Chemical and technological investigations for the valorisation of enological products of a South Tyrol native grape variety: Gewürztraminer

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Abbreviations

3MH: 3-mercaptohexan-1-ol or 3-sulfanylhexan-1-ol

3MHA: 3-mercaptohexyl acetate or 3-sulfanylhexyl acetate

4MMP: 4-methyl 4-mercaptopentan-2-one

Cys: Cysteine

Cys-3MH: 3-S-cysteinyl mercaptohexan-1-ol

Cys-4MMP: 4-S-cysteinyl-4-methylpentan-2-one

GSH: Glutathione

GSH-3MH: 3-S-glutathionyl mercaptohexan-1-ol

GSH-3MHAl: 3-S-glutahionyl mercaptohexan-1-al

GSH-4MMP: 4-S-glutathionyl-4-methylpentan-2-one

GWT: Gewürztraminer

ha: Hectares;

LOD: Limit of detection

SB: Sauvignon Blanc

SECTION 1. AIM OF THE THESIS

Aim of the thesis

The presence of the precursors of thiol compounds in Gewürztraminer juices was reported for the first time at the beginning of the present century, with mean concentrations higher than Sauvignon Blanc's, the benchmark among grape cultivars for the cited and internationally appreciated thiolic aromas. The Gewürztraminer wine aroma is usually perceived by tasters as positively correlated to floral and rose-like aromas and therefore the presence of terpenes, but the complexity of the aroma of this wine, the intriguing presence of citrus-like aromas and the relationships with the typicality perception are not yet totally understood. Trentino, one of the few regions for which the Italian Ministry of Agriculture declared Gewürztraminer as a recommended variety for cultivation, follows the Alto Adige as the largest area for Gewürztraminer grape growing, with a increase importance over the last 10 years. The knowledge related both to thiol formation in wine and the biogenesis of their corresponding precursors in grape and juice, is as for now incomplete, showing the existence of great matrix effects not completely explained. At the same time, it is mandatory for the wine industry to take advantage and maintain elevated quality standards of cultivar*s,* like Gewürztraminer, with an increasing importance on the regional wine scenario and a great commercial appeal.

The aim of this work - indirectly supported by the *Provincia Autonoma di Trento* through the experimental activity carried out at the Technology Transfer Centre of the Edmund Mach Foundation (San Michele all'Adige, TN) - was to deepen the comprehension of the presence and the technological role of the thiol precursors and, at a later time, the sensory significance of the corresponding volatile derivatives in Gewürztraminer produced in the Trentino alpine environment. The objectives looked at the possibility of implementing new technological approaches useful to manage better prefermentative and fermentation phases, in order to increase typicality and quality perception of the aroma of Gewürztraminer wines. Moreover, they looked at the possibility of giving value to vinification by-products.

The thesis - which includes peer-reviewed and published papers together with others *in litteris* - is conceived following possible steps along a hypotethical viticultural and winemaking process, focusing on:

- The clonal variability of precursors in Gewürztraminer grapes, the accumulation during ripening, and the distribution between juices and skins;

- The impact of the main vinification techniques and the role of oxygen in the *de novo* formation of thiolic precursors;

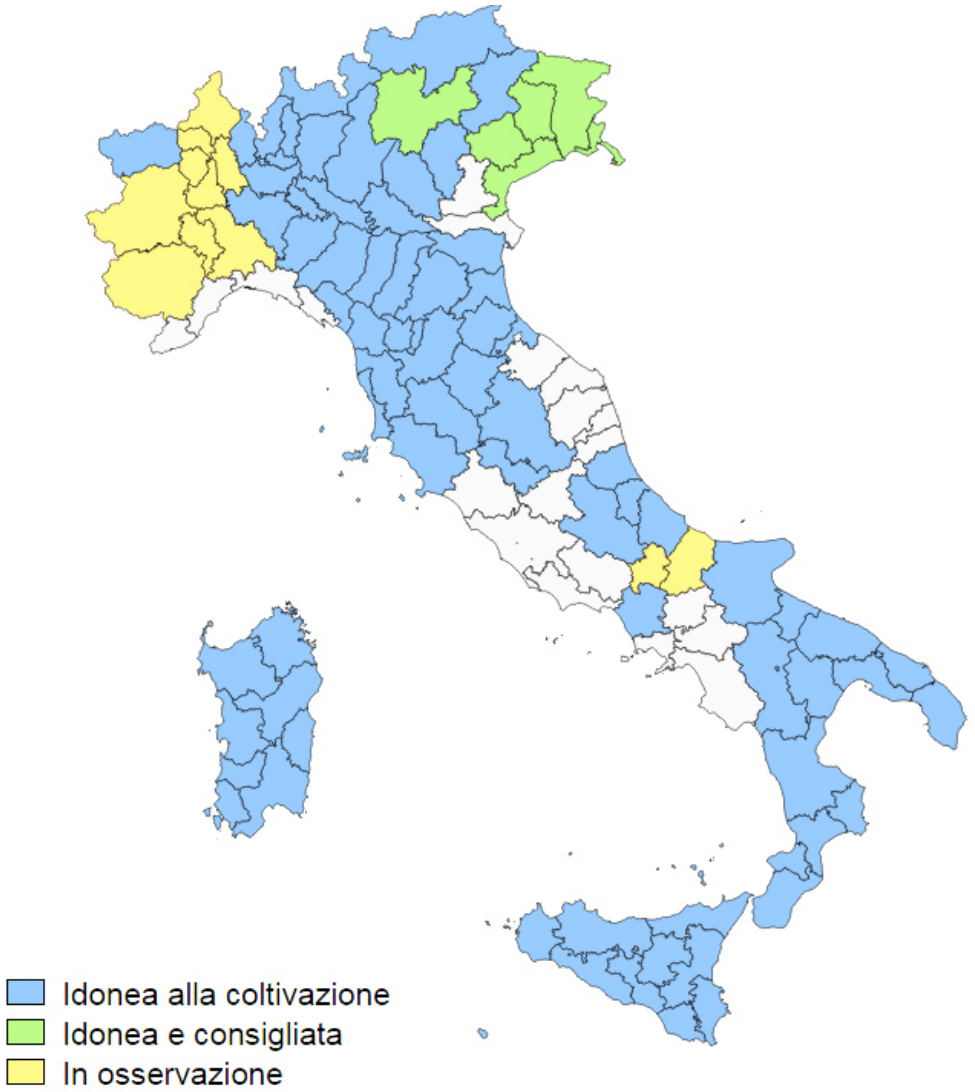
- The impact of recent findings related to grape tannins on thiols, on their precursors and on the aroma of wines processed at lab and semi-industrial scale;
- The sensory evaluation of the impact on typicality and overall appreciation of the thiolic scents in Gewürztraminer wines;
- New strategies to exploit and add value to Gewürztraminer pomace.

To this end, some experiments have been conducted along with international varieties - mainly Sauvignon Banc - characterised by their thiolic features, so allowing to relativise the findings related to Gewürztraminer.

SECTION 2. INTRODUCTION

Trentino is an alpine region situated in North-east Italy close to the border with Austria. The morphology and climatic features of this area are highly dependent on these mountain ranges and impact hugely on the agricultural landscape; the area for vine growing is limited and barely exceeds 10,300 ha, frequently terraced and with high slopes, sometimes over 30%, in these cases making agricultural mechanization almost impossible. Vines are planted out between 200-850 m.a.s.l. or over. All the above features make agricultural practices quite expensive resulting in final products which are not competitively priced. Even so the economic importance of viticulture in the agricultural industry is paramount with almost 8,000 grape growers, covering 40% of the cultivated terrains (Statistical service *Provincia Autonoma di Trento*, Report 2014). Trentino's viticulture is represented by almost 70% of white skin cultivars - where Chardonnay and Pinot Grigio hold a place of honour. A special quality distinction should be made for aromatic varieties like Sauvignon Blanc (SB), Müller Thurgau, Moscato Giallo and Rosa, Riesling and Gewürztraminer (GWT), which in Trentino find a suitable terroir for their development: the thermal excursion, altitude and exposure features of vineyards allow grapes to ripen not only technologically but also aromatically. Most of these varieties are cultivated all around the world but only one of them has its origin in Tyrol, the historical region to which Trentino belonged up to the end of World War I: Gewürztraminer. More precisely, this cultivar is deemed to be native to the small town of Tramin, a few kilometres north of the Trentino border with South Tyrol region, in the Adige valley. Its presence here is reported since the XIIIth century, from where it was then exported firstly to Alsace and to the Rheingau (Huyn 1990); nowadays, it is significantly present in 19 different countries as reported by Fregoni (2010). In Italy there are almost 1,100 ha of GWT, Alto Adige being the most important region for its production. Trentino is the 2nd producer of this variety with almost 360 ha - representing 3.3% of the regional vine planted area - where its cultivation has strongly increased in the last few years, almost doubling its surface. GWT is favoured by the renewal of existing varieties and stimulated by the high remuneration obtained by growers: GWT is the best priced grape variety of Trentino, valued on average 74% more than Chardonnay or 25% more than Pinot Grigio (CCIAA Trento, uve Trentino DOC, 2015 harvest; updated to November the 16th 2016). Figure 3.1 represents the regions recommended (green), suitable (blue) and under surveillance (yellow) for Gewürztraminer wine production as reported by the italian "*Ministero dell'Agricoltura*" (2016).

Fig. 2.1. Map of the Italian regions recommended (green), suitable (blue) or under surveillance (yellow) for Gewürztraminer wine production as reported by "Ministero dell'Agricoltura MIPAA (2016)".



This aromatic variety has been extensively studied for its composition in the past years in particular in the region under study (Versini 1985; Versini *et al.*, 1989 and 1990; Scienza *et al.*, 1990; Stefanini *et al.*, 2000; Malossini *et al.*, 2002). Its aroma is largely defined by terpenes, a family of compounds that resemble citric and floral scents. Among them, geraniol and nerol are characteristic components, present both in their free and glycosylated forms in grape, juice and to a lesser extent, in wine (Mandery 1983; Versini 1985; Marais 1987). Geraniol is always present at the highest concentration and, along with rose oxide (cis-4-methyl-2-(2-methyl-1-propenyl)-

tetrahydropyran), is responsible for the lychee-like scents of Gewürztraminer's wines, that also characterises this fruit (Wu *et al.*, 2009). The aroma complexity is enhanced during the alcoholic fermentation thanks to the partial metabolisation of geraniol - among other terpenes, such as nerol - resulting predominantly in citronellol, due to the liberation of bound aromatic molecules (Versini *et al.*, 1990). The quality perceived by tasters is positively correlated to the content of terpenes in their free form, but the aroma of this cultivar, its complexity and the relationship with the tipicallity perception has unfortunately not yet been fully understood (Rapp, 1990; Versini *et al.*, 1998). Table 3.1 shows a typical composition of aromatic compounds in Geürztraminer wines as reported by Versini *et al.,* in 1990.

Since the beginning of the last decade of the 20th century a new family of compounds has captured the attention in wine research: the initially so-called varietal thiols. These aromatic molecules - at the time already reported in passionfruit (Engel and Tressl, 1991), blackcurrant (Rigaud *et al.*, 1986) and grapefruit (Demole *et al.*, 1982) - resemble the tropical and citric fruit scents, strongly perceived in SB wines. Clearly, due to their aromatic characterization and the worldwide economic importance, research has focused on this cultivar, but they are not exclusive to Sauvignon Blanc and have been reported in a number of varieties (Mateo Vivaracho *et al.*, 2010; Concejero *et al.*, 2014). Knowledge of the biogenesis of the non-volatile precursors of thiols and on the liberation and the *de novo* formation in wines as come a long way, but the main contribuition to the final concentration of such molecules in the final product still cannot be explained. At this point though, industry needs to improve the practical knowledge related to the maximization of the final concentration of thiols in wines of some grape varieties. This knowledge shall include the matrix effects related to the content of precursors in grape and juice and the subsequest formation and/or liberation in wines of the free thiols (Pinu *et al.*, 2014). In GWT, thiols have been reported over the olfactory threshold in only a few studies with small datasets (Tominaga *et al.*, 2000b; Roland *et al.*, 2010b; Concejero *et al.*, 2014), highlighting the potential role played on GWT final aroma. However, as far as we know, there are no previous works on the quality perception of Gewürztraminer wines in relation to the content of thiols. For all these reasons, the next paragraphs will report the state of the art of thiols in the wine industry.

Table 3.1. Composition of Gewürtraminer wines as reported by Versini *et al,*(1990).

Composti		Mosto				Uva	
		Piante non clonali vendemmia 1987		Piante clonali vendemmia 1988		Piante clonali vendemmia 1989	
		A(8)	B(23)	C(4)	D(4)	E(16)	F(6)
f. Lox tr	l	1,0 (0,7)	0,6 (0,7)	0,32 (0,05)	0,44 (0,12)	1,8 (1,08)	0,95 (0,72)
	c	10,3 (6,0)	2,0 (0,7)	29,5 (10,6)	1,7 (0,9)	27,8 (12,6)	3,3 (1,9)
f. Lox cis	l	0,6 (0,9)	0,2 (0,3)	0,4 (0,1)	0,27 (0,09)	0,67 (0,73)	0,68 (1,16)
	c	22,7 (8,1)	2,4 (0,7)	47,8 (5,3)	2,4 (1,1)	25,0 (13,6)	2,9 (2,0)
Linalolo	l	0,4 (0,2)	0,21 (0,26)	0,31 (0,08)	0,14 (0,07)	6,3 (3,8)	0,25 (0,23)
	c	11,0 (6,4)	0,36 (0,17)	21,4 (11,6)	0,48 (0,37)	35,1 (23,1)	2,7 (1,9)
α-Terpin.	l	0,5 (0,3)	0,48 (2,24)	0,12 (0,05)	0,1 (0)	1,4 (1,7)	0,38 (0,56)
	c	30,2 (10,2)	9,3 (4,4)	45,2 (7,8)	7,7 (3,1)	31,6 (31,6)	16,1 (15,6)
p. Lox tr	l	4,6 (1,8)	0,97 (1,25)	2,1 (0,8)	0,52 (0,25)	33,8 (10,2)	5,2 (2,0)
	c	8,7 (5,1)	0,50 (0,22)	12,50 (5,0)	0,77 (0,24)	14,1 (6,9)	2,1 (1,8)
p. Lox cis	l	2,3 (1,5)	0,32 (023)	0,85 (0,37)	0,32 (0,26)	3,6 (1,9)	0,53 (0,35)
	c	7,3 (7,2)	0,39 (0,16)	11,2 (3,9)	0,61 (0,28)	10,5 (8,0)	1,1 (0,8)
Citronel.	l	11,7 (6,7)	0,80 (0,93)	0,85 (0,24)	0,31 (0,20)	54,3 (27,9)	1,3 (1,0)
	c	42,3 (22,3)	0,61 (0,41)	10,1 (7,2)	0,36 (0,22)	31,9 (27,7)	1,2 (1,2)
Nerolo	l	33,3 (23,89)	0,66 (1,7)	3,0 (1,1)	0,32 (0,14)	249,4 (175,7)	5,0 (2,4)
	c	268,4 (130,1)	2,5 (1,39)	97,9 (34,8)	2,4 (1,1)	353,2 (258,7)	5,2 (2,9)
Geraniolo	l	251,7 (130,5)	3,5 (2,5)	44,3 (16,4)	2,1 (1,2)	1301 (725)	43,1 (5,9)
	c	1341 (553)	20,2 (8,4)	802,9 (298,7)	29,5 (10,5)	1391 (905)	36,6 (23,0)
Ac. geran. tr	l	63,0 (33,0)	0,83 (0,67)	20,2 (13,7)	3,1 (1,2)	541,4 (215,8)	9,6 (6,8)
	c	246,7 (161,3)	6,1 (5,7)	254,8 (41,0)	6,3 (2,3)	764,8 (307,4)	33,1 (34,1)
Diendiol (1)	l	175,4 (120,1)	23,5 (9,7)	100,5 (44,6)	34,0 (16,1)	222,6 (132,0)	26,4 (13,2)
	c	233,6 (267,9)	11,5 (7,1)	173,1 (78,6)	6,4 (2,8)	165,0 (75,9)	25,3 (26,2)
Diendiol (2)	l	1,6 (2,5)	0,30 (0,48)	3,0 (1,1)	0,62 (0,17)	3,3 (1,7)	0,63 (1,08)
	c	2,5 (1,1)	0,14 (0,06)	3,4 (1,9)	0,37 (0,09)	3,4 (2,6)	0,45 (0,40)
OH-Citron.	l	50,3 (28,1)	8,3 (3,0)	4,9 (3,7)	1,4 (1,9)	140,0 (62,0)	8,4 (4,1)
	c	49,7 (19,4)	7,5 (3,8)	30,8 (10,0)	0,97 (0,50)	48,8 (22,9)	2,9 (3,1)
8-OH-Lin.tr	l	14,2 (9,5)	2,1 (2,6)	1,2 (1,0)	1,3 (1,1)	6,3 (2,2)	2,7 (2,7)
	c	62,0 (22,5)	19,4 (14,7)	34,9 (7,6)	6,8 (2,8)	65,7 (23,0)	26,4 (18,1)
8-OH-Lin. cis	l	20,2 (12,6)	2,6 (1,8)	5,6 (4,4)	2,3 (1,4)	52,6 (21,5)	12,4 (6,5)
	c	224,1 (66,3)	22,8 (12,8)	305,4 (78,9)	30,0 (2,5)	473,1 (178,6)	135,0 (124,4)
Dichetone norisop.	c	33,0 (7,9)	23,2 (12,4)	29,6 (9,1)	14,9 (6,0)	24,3 (6,7)	27,5 (28,9)
3-Cheto-α-ionolo	c	202,4 (242,1)	96,4 (43,2)	149,5 (27,1)	71,7 (11,9)	178,8 (81,2)	253,8 (249,5)
7-OH-Geran.	l	75,4 (44,9)	4,4 (3,3)	8,2 (4,3)	1,0 (0,9)	459,2 (215,0)	32,8 (14,9)
	c	113,5 (67,1)	6,3 (8,3)	104,2 (42,8)	4,7 (1,9)	127,0 (53,8)	7,3 (5,5)
2-OH-1,8-Cin.	l	1,7 (0,8)	0,92 (0,30)	1,0 (0,2)	0,37 (0,22)	3,3 (1,1)	0,88 (0,45)
	c	14,4 (7,6)	7,0 (2,3)	18,9 (2,8)	4,0 (1,3)	11,5 (4,1)	8,7 (10,4)
p-Menten diolo (1)	c	20,7 (7,7)	9,2 (3,9)	28,4 (9,5)	11,1 (1,1)	16,8 (7,4)	6,8 (4,3)
p-Menten diolo (2)	c	72,2 (18,0)	52,2 (19,8)	80,8 (15,3)	58,5 (8,8)	63,0 (22,6)	58,0 (39,6)
Vanillato metile	c	73,7 (29,2)	42,6 (13,5)	55,1 (21,5)	13,5 (5,3)	72 (19)	38 (21,7)
Alc. benz.	l	41,7 (13,4)	23,9 (13,1)	14,2 (3,0)	11,2 (5,8)	220,2 (47,1)	275,2 (105,1)
	c	428,5 (106,7)	308,2 (120,7)	320,2 (26,6)	255,0 (68,5)	317,7 (138,9)	341,6 (119,1)
Alc. β-fen. et.	l	108,7 (80,1)	105,4 (104,3)	11,6 (5,2)	12,9 (3,8)	199,6 (283,4)	126,2 (62,3)
	c	100.3 (27,3)	76,8 (33,7)	46,0 (7,8)	30,1 (5,9)	82,0 (14,9)	131,5 (75,0)

(*) Concentrazione in µg/l per il mosto e µg/kg per l'uva.
A - C - E = gruppi ad elevato contenuto terpenico; B - D - F = gruppi a basso contenuto terpenico.
(n) = numero di individui costituenti il gruppo.
l = forma libera - c = forma complessata

2.1. Polyfunctional thiols in grapes and wines

Chemically, thiols are a family of compounds whose molecules contain a carbon-bond sulphydryl group (McGorrin, 2011). Many of them - particulary those with low molecular weight - have an extraordinary low perception thresholds, having human olfactory detection limits at a few parts per billion or even less. Often thiols are characterised by repulsive odours resembling garlic, onion, cooked cabbage, boiled potatoes or smoky notes that frequently influence hugely the aroma of vegetable, juices and other fermented beverages. There are however, some thiols with pleasant aromas in their volatile form, typically fruity, reported in nature such as 4-methoxy-2-methyl-2-butanethiol (black currant), 4-mercapto-4-methyl-2-pentanone (4MMP) and 8-mercapto-p-menthane-3-one (grapefruit), 3-mercaptohexan-1-ol (3MH) and 2-methyl-4-propyl-1,3-oxathiane (passion fruit) or ethyl-(methylthio)-propionate (pineapple). In the wine and brewing industry, a large number of compounds from this class have been reported, more frequently with unpleasant or even nauseating scents when their concentrations are above a certain threshold. These compounds are usually linked to the so-called "reduction defect" but the scents vary among compounds and concentration of the molecules. Some of them, at very low concentration, can even positively contribute to the fruity aroma perception of wines.

Recent enological research has focused on a subclass known as polyfunctional thiols, as together with the sulphydryl group, these possess another chemical function (aldehyde, ketone, ester etc.). Polyfunctional wine thiols are characterized by the tropical fruity scents of boxwood, passion fruit, grapefruit or black currant, but at high concentrations - particularly 4MMP – its aroma resembles cat pee (Darriet *et al.*, 1995). For many years the olfactory scents related to these compounds have been associated with certain varieties (particularly SB) whose wines are markedly characterized by these aromas (Lund *et al.*, 2009). However, the aroma does not emerge from tasting grapes, immediately suggesting they could be found in berries as non-aromatic precursor (Darriet, 1993) where the free forms have not yet been found. Small amounts of the free thiols have been reported in some grape juices obtained right after grape veraison (Capone *et al.*, 2011c).

Polyfunctional thiols are found ubiquitously in nature in some plant tissues under their non-volatile form, and are a response to stress (Blum *et al.*, 2007), thus permitting to deepen the comprehension of plant metabolic pathways and suggesting that these compounds are part of the metabolic system of all *Vitis vinifera* varieties (Concejero *et al.*, 2014).

Table 3.2 summarizes some of the main aromatic polyfunctional thiols found in wine, as reported by Dubourdieu and Tominaga (2009). Not all of them play a significant role in wine aroma, due to having a high organoleptic threshold and/or the usual concentration at which they are normally found in wines, if not for some synergistic effects not yet deeply studied. Among all members of this class, 3 of them should be highlighted for their definitive importance in wine aroma: 4MMP, 3MH and its acetate (3MHA). As will be reported in the following paragraphs, all of them are characterized by low organoleptic thresholds and their reported concentrations in wines are reported well above it. The thiols were first discovered in wine by Dubordieu's research group during the last decade of the XX^{th} century (Darreit *et al.*, 1993 and 1995; Tominaga *et al.*, 1996 and 1998a), and precisely 4MMP in particular was the first of them, found in wines from the cultivar Sauvignon Blanc (Darriet *et al.*, 1995), following a previous study by the same group that found it in a fermented model solution, previously enriched with a grape extract of this variety and containing precursors at that time unidentified (Darriet *et al.*, 1993). At concentrations normally found in wines this compound is characterized by a strong scent of boxwood and blackcurrant bud; higher concentrations (above 5 ng/L) can cause a penetrating smell of cat urine (Marais and Swart, 1999) and blackcurrant in red wines (above 16 $ng \cdot L^{-1}$; Rigou *et al.*, 2014). In fact, it has the lowest sensory threshold among wine thiols: 0.1 $ng \cdot L^{-1}$ in water, 0.8 $ng \cdot L^{-1}$ in model solution and 3 $ng \cdot L^{-1}$ in wine (Darriet *et al.*, 1993 and 1995). Its thioether, the 4-methylthio-4-mercapto-2-pentanone, presents a perception threshold around 20,000 times higher both in water (35 $\mu g \cdot L^{-1}$) and in wine (650 $\mu g \cdot L^{-1}$) demonstrating that the thiolic function confers the aromatizing power (Darriet *et al.*, 1993 and 1995).

3MH was first identified by Tominaga and co-workers (1998a) in Sauvignon Blanc wines. Grapefruit and passion fruit hints characterize the aroma of this compound, that has sensory perception thresholds of 17 and 60 $ng \cdot L^{-1}$ in water and model solutions respectively (Tominaga *et al.*, 1998a). The acetate of this alcohol, 3MHA, was firstldiscovered in passion fruit by Engel and Tressl in 1991, but it was not until some years later that it was found in Sauvignon Blanc wines (Tominaga *et al.*, 1996). 3MHA is characterized by a strong fruity aroma of grapefruit, passion fruit, or even boxwood, with a mild hint of what is sometimes referred to as 'Riesling-like'. The perception threshold is lower compared to the corresponding alcohol and it was determined to be 2.3 and 4.2 $ng \cdot L^{-1}$ in water and in hydroalcoholic model solution respectively (Tominaga *et al.*, 1996). At high concentrations, its odour can be unpleasant and green as for all thiol compounds. Contrary to other free thiols, it is not present as a non-volatile precursor in grapes or juices (Tominaga *et al.*, 1996). Rather, its presence in wine is a consequence

of an enzymatic acetylation reaction of 3MH during alcoholic fermentation (Tominaga *et al.*, 1996; Swiegers and Petrorius, 2007).

Other polyfunctional thiols have been reported in wines - mostly Sauvignon Blanc - like 4-mercapto-4-methyl-2-pentanol (4MMPOH, Tominaga *et al.*, 2000a), known for its lemon peel scent, or 3-mercapto-3-methyl-1-butanol, known for its cooked leek scent (Tominaga *et al.*, 1998b). Their importance in wine aroma is limited due to the perception threshold (20 and 1300 $ng \cdot L^{-1}$ in water, and 55 and 1.500 $ng \cdot L^{-1}$ in model solution, respectively) as compared to the humble content in wines (up to a few tens of $ng \cdot L^{-1}$). Other polyfunctional thiols with reported concentration in wines above the sensory threshold have been found in red Bordeaux and sparkling wines, such as 2-furylmethanthiol (0.3 $ng \cdot L^{-1}$) that resembles woody or roasted aromas (Tominaga *et al.*, 2000a and 2003) as well as benzyl mercaptan (0.4 $ng \cdot L^{-1}$), with scents of brewed coffee.

Table 2.2.Main polyfunctional thiols reported in wines, with olfactory descriptors and corresponding odour thresholds, as reported by Dubourdieu and Tominaga (2009).

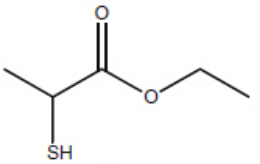
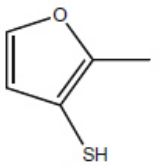
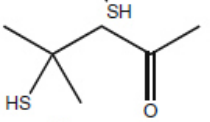
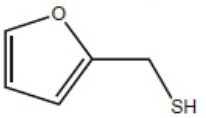
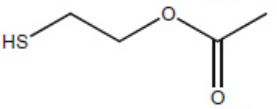
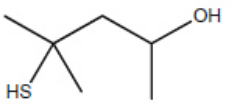
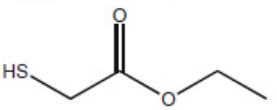
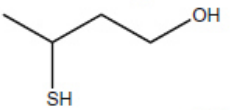
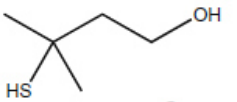
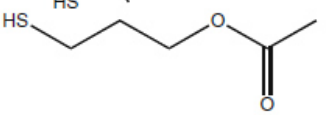
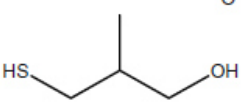
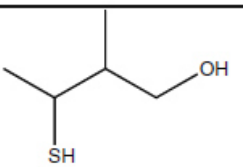
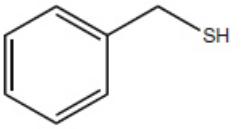
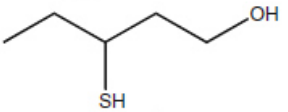
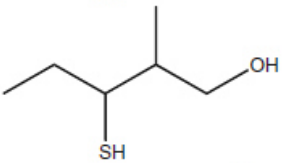
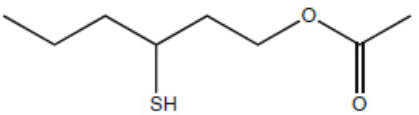
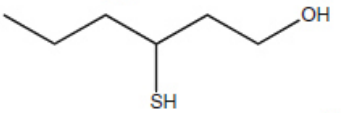
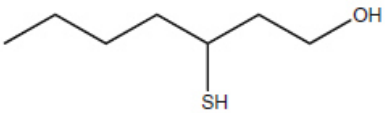
Compound	LRI (BP20)	Descriptor	Formula
I: Ethyl-2-sulfanylpropionate	n.d.	fruity	
II: 2-Methyl-3-furanthiol	1341	meaty	
III: 4-Methyl-4-sulfanylpentan-2-one	1389	box-tree	
IV: 2-Furanmethanethiol	1420	Coffee	
V: 2-Sulfanylethyl acetate	1420	meaty	
VI: 4-Methyl-4-sulfanylpentan-2-ol	1568	Citrus zest	
VII: Ethyl-3-sulfanylpropionate	n.d.	meaty	
VIII: 3-Sulfanylbutan-1-ol	1670	onion, leek	
IX: 3-Methyl-3-sulfanylbutan-1-ol	1677	Cooked leek	
X: Sulfanylpropyl acetate	n.d.	Meaty	
XI: 2-Methyl-3-sulfanylpropan–1-ol	1745	Broth, sweat	

Table 3.2 cont. Main polyfunctional thiols reported in wines, their olfactory descriptors and corresponding odour thresholds, as reported by Dubourdieu and Tominaga (2009).

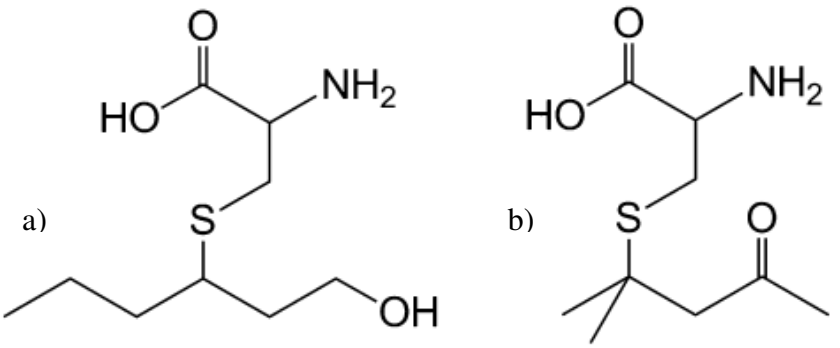
XII: 2-Methyl-3-sulfanylbutan-1-ol	1745	Raw onion	
XIII: Benzenemethanethiol	n.d.	Smoky	
XIV: 3-Sulfanylpentan-1-ol	1772	Grapefruit	
XV: 2-Methyl-3-sulfanylpentan-1-ol	1833	Raw onion	
XVI: 3-Sulfanyl-hexyl acetate	n.d.	Box-tree	
XVII: 3-Sulfanylhexan-1-ol	1857	Passion fruit Grapefruit	
XVIII: 3-Sulfanylheptan-1-ol	1956	Grapefruit	

2.2. The non-aromatic precursors of grape berries

As previously mentioned, the first hypothesis about the presence of non-volatile precursors in juices was proposed since the very beginning, when an unidentified molecule isolated from Sauvignon Blanc grapes, freed 4MMP, firstly in a synthetic medium after alcoholic fermentation (Darriet *et al.*, 1993) and subsequently, in fermented wine from juices of the same variety (Darriet *et al.*, 1995). The exact nature of the precursor however, was not identified until a few years later (Tominaga *et al.*,1998c), when it was first reported that some molecules - linked to L-cysteine by its S atom (fig. 3.2) - released 4MMP, 4MMPOH and 3MH, after both an enzymatic treatment of a Sauvignon Blanc juice extract, and after the alcoholic fermentation by the VL3c yeast strain of a model solution spiked with the same juice extract. This indirectly demonstrated the presence of the non-volatile precursors in that grape variety. These molecules were 4-S-cysteinyl-4-methylpentan-2-one

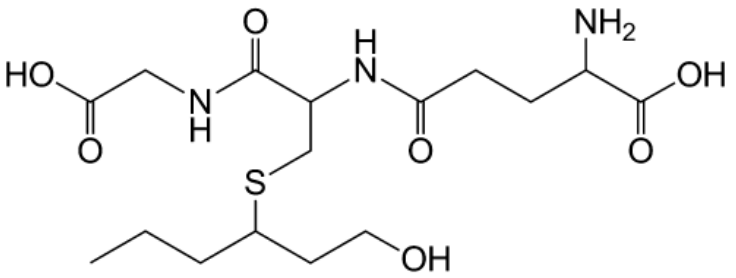
(Cys-4MMP) and 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH). The enzyme used for the first experiment was a *β*-lyase (EC 4.4.1.13) purified from a *Eubacterium limosum* culture, characterized by its high specificity (Larsen, 1985; Larsen and Stevens, 1986).

Figure 2.2. Chemical structure of (a) 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) and (b) 4-S-cysteinyl-4-methylpentan-2-one (Cys-4MMP).



A few years later, the 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH, figure 3.3) was suggested as a new non-volatile thiol precursor. Following the leaching of an aqueous solution of GSH-3MH through a column containing the enzyme γ-glutamyl transpeptidase (EC 2.3.2.2), Peyrot des Gachons and her collaborators (2002a) detected the formation of Cys-3MH and a decrease in the original content of GSH-3MH. In fact, initially, this compound was considered just a precursor of Cys-3MH, later confirmed by several studies (Roland *et al.*, 2010a; Kobayashi *et al.*, 2011), while the ability to release 3MH directly from GSH-3M has only been established recently (Cordente *et al.*, 2015).

Figure 2.3. Chemical structure of 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH).



The last precursor to be found in grape juice has been the 4-S-glutathionyl-4-methylpentan-2-one (GSH-4MMP) suggesting that this compound was involved in the biosynthesis of Cys-4MMP (Fedrizzi *et al.*, 2009), as previously reported for the GSH-3MH (Peyrot des Gachons *et al.*, 2002a).

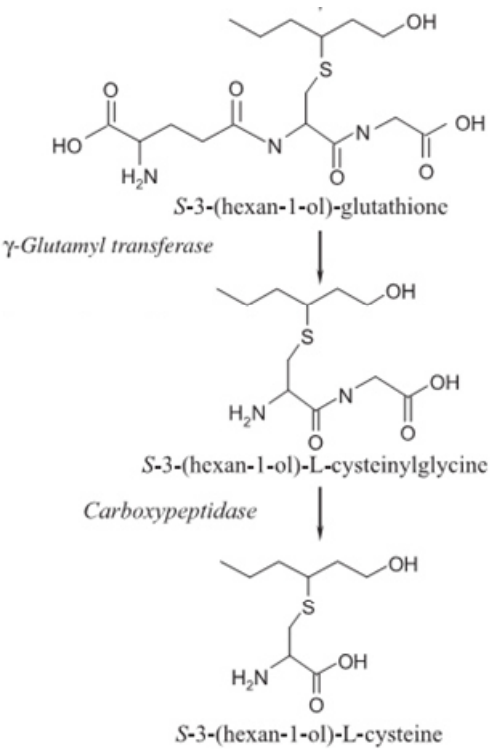
The authors reaffirmed the hypothesis that the glutathionylated molecules present in berries would be the true precursors of free thiols in wines, which in biological systems undergo detoxification reactions resulting in the enzymatic formation of cysteinylated molecules (Blum *et al.,* 2007).

2.3. Biogenesis of the precursors in grape berries

The role of glutathione (GSH) in plants is widely reported as a chelating agent for detoxification of metals and other extraneous compounds (Lallement *et al.*, 2015). The reaction is governed by a transferase present in the cytoplasmic medium, creating an adduct with the foreign molecule via its sulfur atom (Anders and Dekant., 1998; Blum *et al.*, 2007). Once formed, the cells are able to transport the adduct within the vacuoles and catabolize enzymatically into less toxic molecules. The 2 enzymes involved in this reaction are: (i) the *γ*-glutamyltranspeptidase, which cleaves the glutamic acid from the tripeptide forming a glycinecysteinyl adduct (GlyCys-S-), and (ii) a carboxypeptidase that removes the glycine, leaving the sole Cys-S- bond remaining (McIntyre and Curthoys, 1980; Jakoby and Stevens, 1984; Wolf *et al.*, 1996). Figure 3.4 reports the metabolism scheme of GSH-3MH within cells as proposed by Kobayashi and his team in 2011.

The first enzyme of the reaction (γ-glutamyltranspeptidasi) has been thoroughly studied in other plants such *Arabidopsis* (Blum *et al.*, 2007); it is essential in order for the peptide bond cleavage between the cysteine and the glutamate to take place. It has not yet been found in grapes, even though the reaction product (CysGly-3MH and/or CysGly-4MMP) has already been proposed as a potential precursor of free thiols (Dubordieu and Tominaga, 2009). After several failed attempts (Allen *et al.*, 2011; Kobayashi *et al.*, 2011), the CysGly-3MH adduct was finally identified in *Vitis vinifera* grapes (Capone *et al.*, 2011b and 2012; Pinu *et al.*, 2012). Previous works suggested the presence in grape cells of some protease, able to release Cys-3MH inside the cell vacuoles (Cordonnier and Dugal, 1968; Van Rensburg and Pretorius, 2000). The success of this reaction in grape juice was first demonstrated at lab scale when, following the leach of a Sauvignon Blanc juice in a column containing the immobilized enzyme γ-glutamyltranspeptidase, the content of Cys-3MH increased (Peyrot des Gachons *et al.*, 2002a). Furthermore, the content of Cys-S- precursors is higher in skins - where vacuoles are bigger - rather than pulp (Peyrot des Gachons *et al.*, 2002b, Murat *et al.*, 2001a). In addition, the literature often reports a higher content of GSH-3MH compounds than Cys-3MH (Peyrot des Gachons 2002b) supporting the theory that the GSH-S- are the original precursor of the polyfunctional thiols present berries (Fedrizzi *et al.*, 2009).

Figure 2.4. Enzymatic catabolism of 3-S-glutathionyl mercaptohexan-1-ol in the vacuoles as porposed by Kobayashi *et al.*, (2011).



2.4. Biogenesis of the precursors and free thiols in grape juice

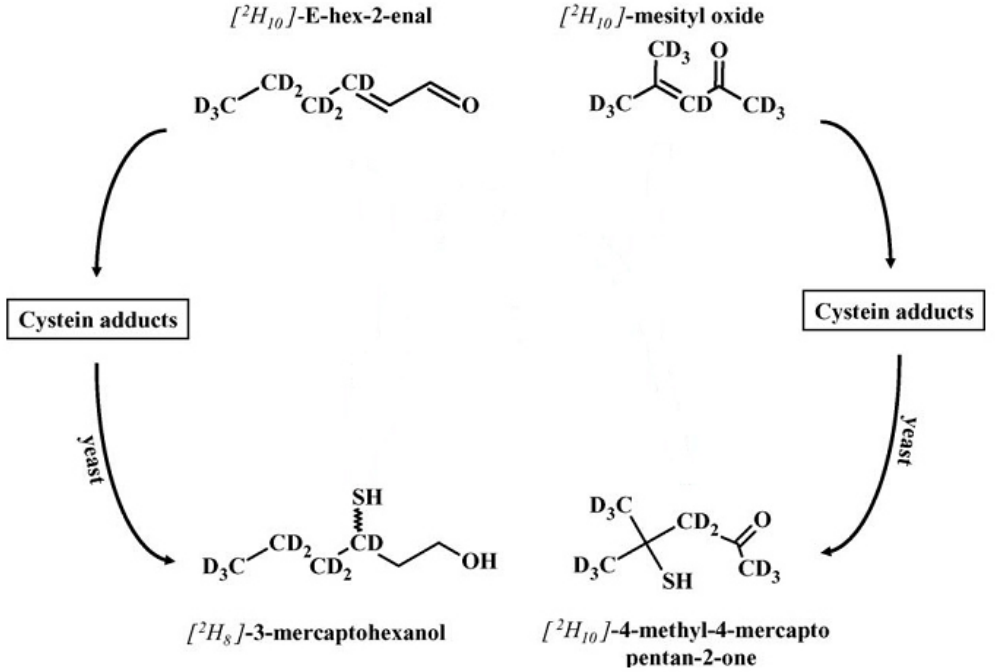
After grape crushing and pressing, the thiolic precursors are partially released into the juice. The liberation of the corresponding thiols during fermentation, as mentioned above, is governed by the β-lyase activity present in the enzymatic pool of some *Saccharomyces cerevisiae* strains (Tominaga *et al.*, 1998c; Masneuf Pomerade *et al.*, 2006) but non-*Saccharomyces* species also proved to be able to release thiols (Anfang *et al.*, 2009; Zott *et al.*, 2011). This reaction is quantitative so the higher the content of precursors and enzyme, the more thiols should be freed up; However, the huge variability found in the liberation ratios - even with the same yeast (Howell *et al.,* 2004) - as well as more intense liberation of polyfunctional thiols in wine compared to synthetic media (Subileau *et al.*, 2008a; Kobayashi *et al*, 2010; Pinu *et al.*, 2012), led to the assumption of the existence of other biogenesis pathways in the formation of both the precursors and the free thiols.

2.4.1. The (E)-2-hexenal pathways

This exogenous *de novo* formation pathway of the precursors of varietal thiols, as well as their impact on the production of volatile thiols, have not yet been fully understood. This process is based on the high reactivity of two sulfur compounds - glutathione and L-cysteine - found in grape juices in significant amounts hailing from berries, but not only. The *(E)*-2-hexenal was simply the first of a number of the targeted molecules studied for the reaction due to its chemical similarity with hexanol, the high reactivity of its aldehyde group and its ubiquitous presence in juice due to being as a main product of the enzymatic degradation of grape lipids (Joslin and Ough, 1978; Cayrel *et al.*, 1983).

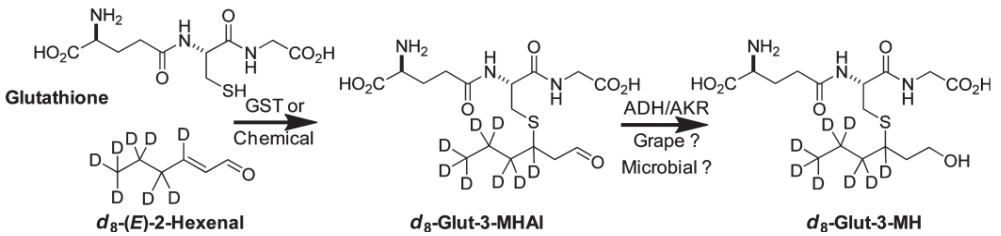
L-cysteine [(2R)-2-amino-3-sulfanyl-propanoic acid] is a well known precursor of sulfur compounds in the food industry, and due to its nucleophilic thiol group is easily oxidized. The reaction of L-cysteine with α and β unsaturated aldehydes usually produces complex mixtures thanks to its N atom; once the S-C bond is established, the aldehyde immediately reacts with the N, forming a Shiff base intermediate that goes on to form diastereomeric mixtures by means of the nature of the substitution. At lab scale, Starkenmann discovered in 2003 the formation in acid medium of Cys-3MH as a minor product in the addition of the L-cysteine S atom across the double bond of the *(E)*-2-hexenal and its subsequest reduction. Moreover, the researcher also obtained the adduct formed between the L-cysteine and the mesityl oxide, previously reported as a precursor of 4MMP (Tominaga *et al.*, 1998a). Mesityl oxide has not yet been found in grape or juice, but its presence in grape is assumed due to its hydrate, being identified in some wines from Japanese varieties (Roland *et al*., 2011). Based on the work of Starkenmann, Schneider and his working group (2006) suggests the plausibility of these routes in the formation of Cys-3MH and the Cys-mesityl oxide adduct, following the demonstrated production in wines of 3MH and 4MMP, coming from grape juices spiked with deuterated [$^2H_8$]-*(E)*-hexen-2-al and [$^2H_{10}$]-mesityl-oxide. The required subsequent reduction would be achieved via the yeast enzymes. Figure 3.6 shows the scheme of the possible pathways for formation of precursors and the liberated functional thiols as proposed by these authors.

Figure 2.5. *De novo* pathways of 3-S-cysteinyl mercaptohexan-1-ol and 4-S-cysteinyl-4-methylpentan-2-one partially modified from those proposed by Schneider *et al.* in 2006.



Glutathione (GSH) is the lowest molecular weight thiol widespread in plants (Pivato *et al.*, 2014), used by plants to interact with the environment due to its high reactivity. It appears in berry tissues after veraison (Adams and Liyanage, 1993), disappearing quickly once extracted from the berry cells (Dubourdieu and Lavigne, 2004; Mattivi *et al*., 2012). The evidence of the *de novo* GSH-3MH formation in grape juice was reported for the first time as a result of the aeration of Sauvignon Blanc and Melon de Bourgogne juices, increasing the mean content of GSH-3MH by 45% and 67% respectively (Roland *et al* 2010b). This was later confirmed by Capone and Jeffery (2011) by the addition of the deuterated molecule [$^2H_8$]-*(E)*-2-hexenal in juice. The biochemistry of the reaction is not clear and it has not yet been elucidated whether it is a direct addition, if it is purely enzymatic, if the enzymes are endogenous or exogenous from spontaneous microflora, or even if the compounds initially react chemically. Nevertheless, it has been demonstrated that the formation of 3-S-glutahionyl mercaptohexan-1-al (GSH-3MHAl; Capone and Jeffery, 2011) occurs in higher concentration than GSH-3MH, and this aldehyde was proposed to be an intermediate in the GSH-3MH formation reaction (fig. 3.6). The *(E)*-2-hexenal concentration plays a decisive role in this reaction, 200 $\mu g \cdot L^{-1}$ having being proposed as the a minimum threshold for the formation to occur (Roland *et al.*, 2010c).

Figure 2.6. 3-S-glutathionyl mercaptohexan-1-ol *de novo* formation pathway from glutathione and *(E)*-2-hexenal, according to Capone and Jeffrey (2011).

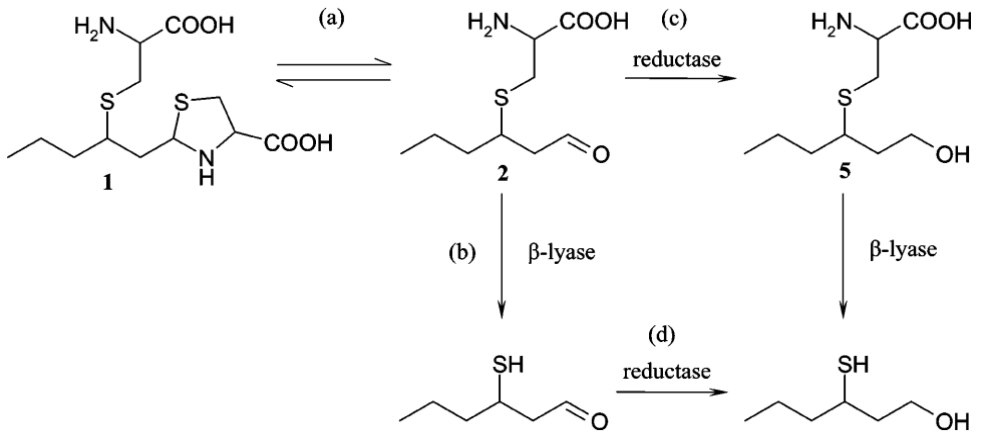


The enzymatic activity in this pathway seems to play an essential role as confirmed by the up to 6-fold lower content of GSH-S- in deproteinized grape juices, while the Cys-S- remained almost unchanged (Capone and Jeffery, 2011). This *de novo* formation pathway includes an intermediate, the GSH-3MHAl, whose formation should be rather fast in the pressed juice or may even occur within the cell vacuoles as a plant detoxification response to certain stresses. These stresses, particularly water stress, result in an increase in the glutathionyl-S-transferase production (Kobayashi *et al.*, 2011), catalysing the aldehyde formation, which is subsequently reduced to the much more stable GSH-3MH.

The previous pathway does not seem to play an important role in the precursor content in juices and its impact on the liberation ratio is very low, especially from the Cys-3MH (Schenider *et al.*, 2006; Subileau *et al.*, 2008a). For this reason, research is still ongoing, looking for other precursors or reactions that could explain the totality of thiols found in wines. In synthetic media, the (2-S-L-cysteinylpenthyl)-1,3-thiazolidine-4-carboxylic acid has been found as a result of the reaction between L-cysteine and *(E)*-2-hexenal (Wakabayashi *et al.*, 2004). This compound, after enzymatic treatment with *β*-lyase, resulted in the formation of 3-MHAl, while after a fermentation with some *S. cerevisiae* strains presenting *β*-lyase activity, 3-MH was detected, probably as a result of the reductase activity of yeasts (fig. 3.7). The Cys-3-hexen-2-al and the GSH-3-hexen-2-al were already proposed as a hypothetical precursor of 3MH by Roland and his collaborators (2011) when, following a 7-day skin-contact, the endogenous content of Cys-3MH and GSH-3MH did not vary in juices but the *(E)*-2-hexenal decreased while 3MH content increased in wine.

The reaction between GSH and *(E)*-2-hexen-1-ol or *(Z)*-2-hexen-1-ol have also been studied for similar biosynthetic pathways as *(E)*-2-hexenal but without encouraging results (Subileau *et al.*, 2008a).

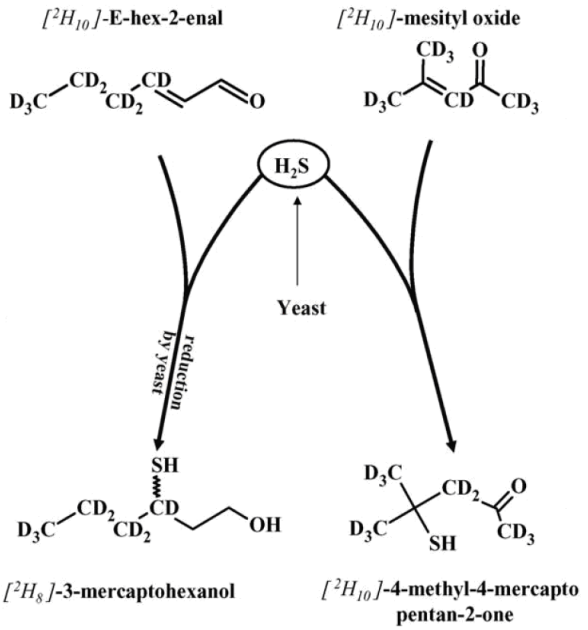
Figure 2.7. Alternative pathway of 3MH formation by reaction between cysteine and *(E)*-Hexen-2-al as proposed by Wakabayashi *et al.,* (2004).



2.4.2. Direct generation of polyfunctional thiols in grape juice and wines from H_2S

At this point, the variability of polyfunctional thiols in wines cannot be explained. The direct addition of H_2S onto *(E)*-2-hexenal is another aim of this research. This reaction has been hypothesized on the basis of the results obtained in onions regarding the enzymatic 1, 4 addition of a molecule of H_2S on the *β* position of the 2-methyl-2-pentenal (generally for all *α, β* unsaturated compounds) and the subsequent reduction, forming 3-mercapto-2-methylpentanol (Widder *et al.*, 2000). The same direct addition, catalyzed by yeasts, was assumed to occur in beer to form 3MH and 4MMP (Vermuelen *et al.*, 2006). As previously mentioned, it has been proven that a higher production of varietal thiols in wines results from a greater content of *(E)*-2-hexenal and mesityl oxide in juices (Schneider *et al.*, 2006). However accordingly to the authors, it is not yet clear whether it is generated by the formation of new L-cysteine precursors or by the direct formation of 3MH and 4MMP. The proposed direct formation pathway implies the addition of H_2S produced in the early stages of fermentation onto the double bond of the aldehyde function of *(E)*-2-hexenal, as shown in figure 3.8. This pathway was confirmed a few years later when Sauvignon Blanc juices spiked with NaSH - that under acidic conditions releases H_2S - resulted in wines richer in 3MH and its acetate (Harsch *et al.*, 2013), also ratifying some of the considerations exposed by Subileau and collaborators (2008a), who proposed the lack of H_2S as the reason of the low release ratios of equivalent 3MH (<1%).

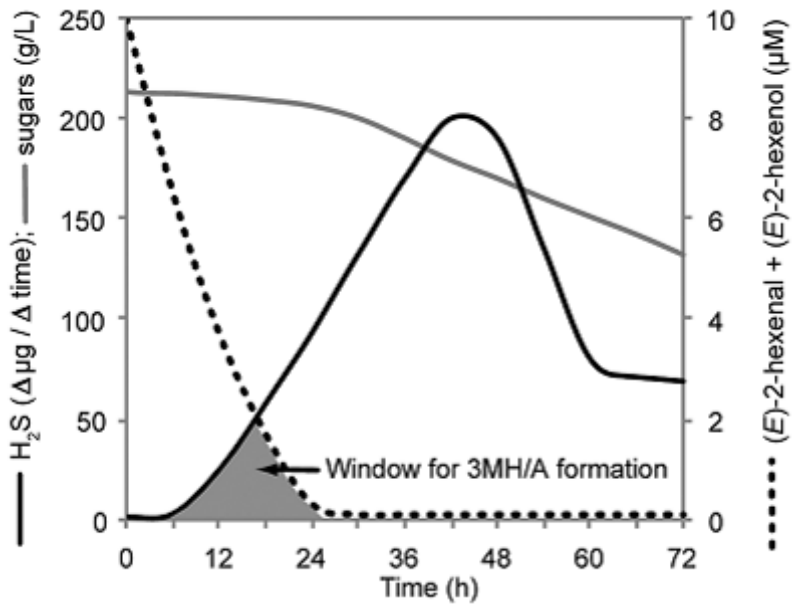
Figure 2.8. Direct addition of H_2S for the production of free thiols by yeasts partially modified from those proposed by Schneider *et al.* in 2006.



(E)-2-hexenol participates in this reaction through its interconversion in *(E)*-2-hexenal by yeasts as was previously ascertained (Joslin and Ough, 1978), confirming it as a new 3MH precursor (Harsch *et al.*, 2013) although at a very low rate if compared to *(E)*-2-hexenal. The balance of interconversion depends on the ratio among *(E)*-2-hexenol and *(E)*-2-hexenal and it is proportional to the amount of equivalent 3MH formed (Harsch *et al.*, 2013). The yeast metabolism of C6 compounds occurs in the early stages of the alcoholic fermentation and the concomitant presence of H_2S in the fermentation medium is necessary for 3MH formation. The kinetics of the synthetic scheme of the kinetics, as proposed by these authors, is represented in figure 3.9.

These pathways would permit a manoeuvring margin in the winemaking procedures, both in the aldehyde production and in the choice of yeast strain in relation to its ability to produce H_2S during sugar catabolism. In any case, this pathway still does not explain why some varieties report more volatile thiols.

Figure 2.9. Theoretical scheme reporting the copresence of H_2S and C6 compounds during alcoholic fermentation (Harsch *et al.*, 2013), highlighting the usefull window for the production of 3MH and its acetate *via* direct addition.



2.4.3. Sulfonic aldehydes pathways

Lately, it has been suggested that the sulfonic aldehydes formed from the reaction between SO_2 and *(E)*-2-hexenal, could be reduced enzymatically by yeasts during alcoholic fermentation, liberating the corresponding thiol (Duhamel *et al.*, 2015). Moreover, S-3-(hexanal)-glutathione and its bisulfite adduct have been found in grape juices, both molecules proposed as new potential polyfunctional thiol precursors (Thibon *et al.,* 2016). However, this pathway has not yet been completely explored.

2.5. Glutathione and cysteine precursors in winemaking

Since the presence of precursors in grapes has been confirmed, several works have reported their contents and those of the corresponding volatile thiols in grape, juice and wine of different varieties, although data was sometimes discordant. This may be partially explained, not only by the variation in geographical origin and environmental features, but also by the variety of sampling and preparation procedures, that can lead to further differences. In any case, the following paragraphs will attempt to report the most recent advances.

2.5.1. Localization of the precursors in leaves and shoots

There is little data in the literature regarding the concentration of thiol precursors in leaves and shoots. The study of the Japanese variety Koshu shows how the content of precursors in the shoots is negligible and in the leaves is almost 20 times that in berries (Kobayashi *et al.*, 2010). To our knowledge there are no other studies regarding these tissues.

2.5.2. Localization of precursors in the berry

As often reported with other precursors and aromatic compound, the contents in seeds is almost negligible (Peyrot des Gachons *et al.*, 2002b; Kobayashi *et al.*, 2010). Within the berry, most thiol precursors are distributed between skin and pulp; the concentration in the skin is greater, but the quantity and distribution depend on the cultivar, the geographical origin and the specific precursor under study (Roland *et al.*, 2011; Concejero *et al.*, 2014). Peyrot des Gachons *et al* (2002b) analysed the relationship among tissues, finding average concentrations of Cys-3MH in the skin about 8 times higher than in the pulp of ripe Sauvignon Blanc grapes. However, in terms of total content the compound was equally distributed among tissues. These results confirmed data previously published, where the average concentration of Cys-3MH was on average 13 times higher in the skins than in the pulp of Cabernet Sauvignon and Merlot grapes (Murat *et al.*, 2001), and are also in agreement with Roland *et al.*, (2011), who reported mean concentrations of GSH-3MH and Cys-3MH up to 4 times higher in the skin of Sauvignon Blanc grapes, with more marked differences for the cysteinylated precursor. However, for Melon B specifically, Roland's study reports a higher content of GSH-3MH in the pulp. For the Koshu variety, concentration values were comparable between skin and pulp for both Cys-3MH and GSH-3MH, with almost double the content in the skin (Kobayashi *et al.*, 2010).

The distribution data between skin and pulp of the 4MMP precursors depend more on the study: Roland *et al.*, (2011) found a concentration 4-5 times higher of the GSH-4MMP in Sauvignon Blanc skins compared to the pulp, finding this precursor only in the skins for the cultivar Melon B. This study did not it find any Cys-4MMP above the limit of detection (LOD). The situation is turned upside down in a study of the cysteinylated precursors of 4MMP and 4MMPOH led by Peyrot des Gachons in 2002(a), where the concentration was almost the same in the skins and pulp, although the total content is greater in the latter.

The content of 3MH precursors are constantly and significantly higher than those of 4MMP (Peyrot des Gachons *et al.*, 2002b; Concejero *et al.*, 2014) with reported concentrations of cysteinylated 3MH precursors in the berry 13 and 20 times higher than those for 4MMP and 4-MMPOH respectively

which. In addition the 4MMP presented lesser variability in their content, whenever detected.

In light of these published studies, 3MH precursors in berries appear to be ubiquitous, even in Chardonnay grapes, a grape variety well-known for its neutral aromatic character (Concejero *et al.*, 2014). The contents of GSH-3MH are higher than those of Cys-3MH and strongly depend on the sampling process and preparation procedure (Roland *et al.*, 2010b; Capone and Jeffery, 2011). As previously mentioned, at the cellular level, the cysteinylated precursors appear to be the products of the degradation of GSH- precursors and their presence is due to the endogenous content, and as a result is relatively less affected by the sample obtaining conditions.

2.5.3. Evolution of precursors during ripening

The concentration of varietal thiols precursors increases with the ripening of grapes, and reaches a maximun right before the technological maturity (Roland *et al.*, 2011; Kobayashi *et al.*, 2010; Capone *et al.*, 2011a), even though the riper the grapes, the higher the final content of thiols in the corresponding wines (Pineau *et al.*, 2011). The accumulation is more pronounced in the last days of grape ripening even though the precursors are present from the onset of veraison. Literature reports that both the accumulation kinetics and the final content are strongly dependent on the variety, on the clone, on the year and on the environmental and agricultural conditions (Roland *et al.*, 2010b; Capone *et al.*, 2010 and 2011a; Kobayashi *et al.* , 2010 and 2012; Pinu *et al.*, 2012). Capone and her co-workers (2011a) monitoring the evolution of the precursors in 5 different clones of SB, found an increase of up to 10 times the total content of the 3MH precursors between the last two sampling points – corresponding to a difference of 14 days – in comparison to marginal increases up to that moment. The increasing concentration was reported in both the isomeric forms - R and S - of the related precursors analysed (GSH-3MH and Cys-3MH). These results corroborated the data published by Kobayashi's research team (2010), which showed an increase in the precursor concentration in Koshu grapes, reaching the maximum content near the technological ripeness. Also the biological cycle of the plant seems to play an important role, in that the precursors in the berry showed sharp daily fluctuations. The plant breathing processes, which occur during carbon fixation or dark phase, augment the precursors content, showing a maximum in the early morning, gradually reducing during the light period, with intraday variations up to 4 times the content (Kobayashi *et al.*, 2012).

The concentration of the 4MMP precursors is less dependent on the ripening stage but highly dependent on the precursor analysed. Roland *et al.*, (2010b)

have found no remarkable increase of GSH-4MMP in Sauvignon Blanc grapes during the final stages of ripeness, and almost a stable mean content of Cys-4MMP within the period under study, although a high variability among the plots investigated.

2.5.4. Concentration of precursors in in grape juices

The distribution between pulp and skin, characteristic for each precursor, means that the extraction process plasys an important role on the final concentration of precursors in juice (Murat *et al.*, 2001a; Peyrot des Gachons *et al.*, 2002b; Maggu *et al.*, 2007; Allen *et al*., 2011). In addition, the different pathways for the *de novo* formation so far proposed, and those that will surely come in the near future, are a source of high intravariety variability. Several studies report the precursor concentration in grape juices, paying special attention to the 3MH precursors due to their higher content and widespread importance in varietal aroma of the wines of many cultivars. Roland *et al.,* (2010a) analyzed the content of Cys-3MH and GSH-3MH in 3 international varieties (SB, Riesling and GWT, n = 35) and found a statistically higher average concentration of the cysteinylated precursor than GSH-3MH, with the ratio Cys-3MH/GSH-3MH clearly differentiated between varieties. The mean concentration of Cys-3MH was significantly higher for every variety if compared to GSH-3MH, and GWT was statistically differentiated from the other varieties, even though it was investigated using a smaller sample size. This data contrasta that reported by Capone *et al.* in 2010 in a study of Australian grape juices of 4 international varieties: SB, Riesling, Chardonnay and Pinot Gris; GSH-3MH concentrations were higher than Cys-3MH in all analyzed samples, averaging a GSH-3MH/Cys-3MH ratio of 14, even reaching 29 in some sample. SB and Pinot Gris showed concentrations greater than Riesling and Chardonnay for both precursors. These concentrations were almost 20 times higher than those determined in the study conducted by Roland and collaborators (2010a) for SB, GWT and Riesling, in which, incidentally, the Cys-3MH was higher in all varieties. Pinu and associates (2012) in a 4 year study of SB juices, found similar concentrations to those reported by Capone *et al.* in 2010: GSH-3MH was always the main precursor, the GSH-3MH/Cys-3MH ratio was always above 5 and the GSH-3MH/CysGly-3MH above 20. Table 3.3 reports the range of published data regarding the concentration in juices of each precursor, broken down by variety.

Table 2.3. Range of concentration of thiol precursors in grape juices split by cultivar as reported in the literature. ([a]Thibon *et al.*, 2008b; [b]Capone *et al* 2010; [c]Roland *et al.*, 2010d; [d]Roland *et al.*, 2010a; [e]Roland *et al* ., 2010d; [f]Thibon *et al.*, 2009; [g]Peyrot des Gachons *et al.*, 2002b; [h]Maggu *et al.*, 2007; [i]Peyrot des Gachons *et al.*, 2000; [j]Subileau *et al.*, 2008b; [k]Allen *et al.*, 2011; [l]Pinu *et al.*, 2012; [m]Mattivi *et al.*, 2012)

Cultivar	Cys-3MH ($\mu g \cdot L^{-1}$)	GSH-3MH ($\mu g \cdot L^{-1}$)	Cys-4MMP ($\mu g \cdot L^{-1}$)	GSH-4MMP ($\mu g \cdot L^{-1}$)
Cabernet Sauvignon	2,1-2,2[a]			
Chardonnay	7-38[b]	111-517[b]		
Gewürztraminer	52,9-65,2[c]	5,6-7,1[c]	0,5-0,8[e]	0,1-0,2[e]
Trebbiano di Lugana	36-363[m]	7,1-173[m]		
Melon B.	0,4-1,05[d]	0,08-0,2[d]	1,77[d]	n.d.[d]
Merlot	1,66-3,5[e]			
Petit Arvine	18-85[k]			
Pinot Grigio	23-27[b]	338-467[b]		
Riesling	10-30,8[c-e]	0,7-275[c-e]	<1,9[e]	0,6-1,8[e]
Sauvignon Blanc	6-15859[a-l]	0,8-642[d,e,k,l]	2,41-8,7[d,e]	0,03-4,3[d,e]
Semillon	3,6-686[a,f]			

2.5.5. Concentration of thiol precursors in wine

There are few studies focusing on the quantification of the precursors in wine. Capone *et al.* (2010) presented data from precursors including wines from 9 to 50 $\mu g \cdot L^{-1}$ for Cys-3MH and 90-480 $\mu g \cdot L^{-1}$ for the GSH-3MH albeit a very small data set (n = 6). Later, the same working group published the data of the precursors contained in Australian white wines (n = 25) of 5 different international varieties (Capone *et al.*, 2011a) ranging from below the LOD to 106 $\mu g \cdot L^{-1}$ for Cys-3MH and from 30 to 503 $\mu g \cdot L^{-1}$ for GSH-3MH, with a strong cultivar variability. The data indicate a 3MH "reserve" in wines that could have not only a considerable technological significance, but also sensorial during the swallowing (Starkenmann *et al.*, 2008).

2.6. Environmental and viticultural influence on the precursors content in berries and polyfunctional thiols concentration in wines

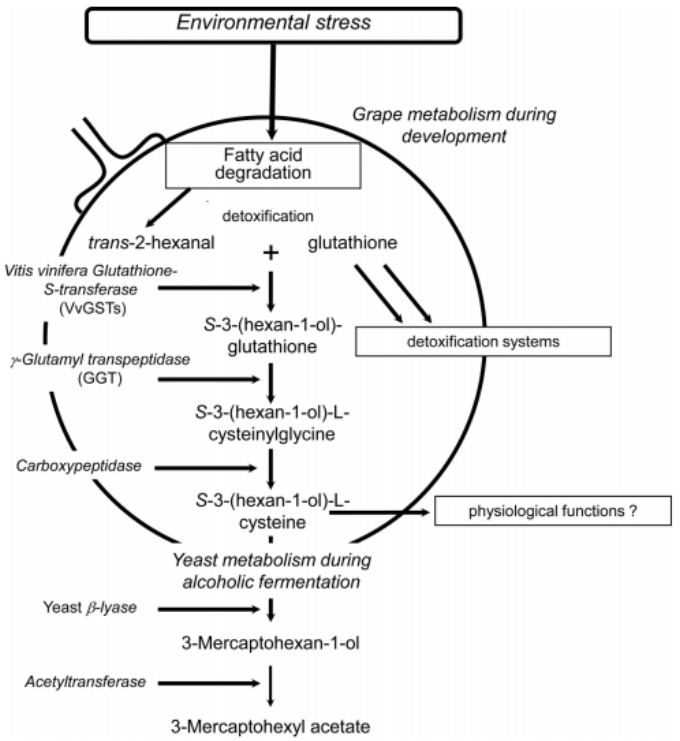
The OIV defines the *terroir* as "the concept that refers to an area in which the collective knowledge of the interactions between the identifiable physical and biological environment and developed wine-growing practices that lead to distinctive characteristics of the products originating in those areas" (resolution OIV/VITI 333/2010). Often, the high variability of the concentration of polyfunctional thiols in wine is justified with this concept; however, it is not yet possible to explain all the mechanisms that determine the biogenesis of the precursors and/or thiols, of which the final content in

wines is attributable to the composition of the juice precursor concentration (Murat *et al.*, 2001). Intravarietal variability of precursors has been widely reported in juice and can vary between different plots of the same wine-growing area up to 7 fold (Kobayashi *et al* ., 2010; Allen *et al.*, 2011; Roland *et al.*, 2010b and 2011a; Johanneau *et al.*, 2012; Pinu *et al.*, 2012).

In biological systems, the accumulation of secondary metabolites is strongly affected by environmental conditions, water and mineral availability, as well as the physico-chemical characteristics of the soil, which may partially explain the interannual variability detected in literature (Pinu *et al.*, 2012). It has been suggested that the plant reaction to moderate stress (radiation, water, cold and/or hot) leads to an accumulation of GSH-3MH, Cys- 3MH and Cys-4MMP, although stress too severe results a decrease (Peyrot des Gachons *et al.*, 2005; Kobayashi *et al.*, 2012). The increase is possibly due to the activation of genes coding for some transferase: ensuing stress, the expressed enzymes degrade fatty acids forming *(E)*-2-hexenal, that the plant uses as a signal for other cells (Blee, 2002). The formation of *(E)*-2-hexenal - a extremely reactive molecule - would activate the whole series of chain reactions leading to the formation of the different 3MH precursors, as explained in the previous paragraphs (Kobayashi *et al.*, 2012). This hypothesis would also explain the lower variability of the 4MMP precursor during maturation. The biosynthesis of the proposed scheme is shown in figure 3.11 as reported by Kobayasi *et al.*, in 2010.

Based on this and on the fragmented data reported, it has been postulated that a deep, chalky soils could be very suitable for the production of Sauvignon Blanc with a high aromatic potential (Peyrot des Gachons *et al.*, 2005). The greater availability of N for the plant can modulate the aromatic potential of Sauvignon Blanc wines, increasing the content of precursors (Peyrot des Gachons *et al.*, 2005). The use of foliar fertilizers during veraison, specifically those containing N, increases the 3MH and 3MHA content in the resulting wines, amplified if in conjunction with S (Lacroux *et al.*, 2008; Dufourcq *et al.*, 2009). In addition, the higher availability of N boosts the vigour of the plant and increases the leaf surface of the foliage in the later stages of maturation, which is positively correlated to the content of 3MH and 3MHA (Šukle *et al.*, 2012). Higher exposure of the bunch to light and UV radiation are other positively related parameters to the content of 3MH and 3MHA (Kobayashi *et al.*, 2010; Šukle *et al.*, 2014), probably because it causes - as speculated by authors - a greater abiotic stress in the cluster which results in increased production of *(E)*-2-hexenal in the berry.

Figure 2.10. Thiol precursor formation whithin *Vitis vinifera* cells as proposed by Kobayashi *et al.* (2010).



In addition, the increase of vigour encountered after high N fertilization increases the risk of a *Botrytis cinerea* attack on berries (Valdés-Gomez *et al.*, 2008). This mold, if present in its "noble" form, raises the content of Cys-3MH and GSH-3MH up to 100 times in the infected grapes (Thibon *et al.*, 2008b, 2009, 2010 and 2011). The plant, through lipoxygenase pathways, sends signals leading to cell death (Rusterucci *et al.*, 1999) with a consequent increase of *(E)*-2-hexenal that, once inside the cell, react with GSH as a detoxification mechanism (Thibon *et al.*, 2011; Kobayashi *et al.*, 2011). The expression of the γ-glutamyltransferase enzyme is also stimulated, increasing the content of Cys-GSH (and probably CysGly-3MH although not confirmed as of yet) in juice, explaining why the resulting wines are richer in varietal thiols (Tominaga *et al.,* 2006; Luisier *et al.*, 2008).

Among the viticultural practices up to now studied, it has been proven that it is better not to hedge vines, meanwhile bunch thinning after veraison enhances the final content in thiols (Šuklje *et al.*, 2013). In fact, it has been demonstrated how an increase in UV-radiation favoured the accumulation of S- precursors in berries (Kobayashi *et al.*, 2011) and the free thiols in wines (Šuklje *et al.*, 2014). Under canopy water nebulisation has been also proposed

as a technique to enhance the precursor content in berries for hot growing seasons (Paciello *et al.,* 2016)

2.7. Harvest and pre-processing treatments on the content of precursors

As previously discussed, the content of varietal thiol precursors increases significantly during the last stages of ripening. Determination of the optimal date of harvest is therefore a fundamental tool for the production of wines highly marked by varietal thiols. The technological variability induced by the harvest operations and grape processing has been well researched by several studies, reporting qualitative and quantitative differences (Capone *et al.*, 2011a; Murat t al., 2001a; Roland *et al.* , 2011c).

2.7.1. Harvest

The importance of harvesting techniques on the final content of the 3MH precursors in juice has always been assumed; in particular, it has been studied how the integrity of the grapes can determine not only quantitative differences but also qualitative, correlated to the endogenous enzymatic activity of the plant (Kobayashi *et al.*, 2010). Results up to now achieved are inconclusive for the mechanical harvest - usually associated with oxidation reactions in pre-processed grapes - although it seems to cause a higher concentration of thiols in wines (Allen *et al.*, 2011; Jouanneau, 2011). The particularities linked to the different experimental designs might partially explain the observed differences. Allen and his colleagues (2011) quantified higher values for GSH-3MH and Cys-3MH in juices obtained from undamaged hand-harvested grapes when compared to the mechanical, although the final content of 3MH and 3MHA was statistically higher in 3 out of the 5 wines resulting from grapes mechanically harvested. The results agreed with other studies that found contents of 3MH + 3MHA 5-10 times higher in wines coming from mechanical harvests (Jouanneau, 2011; Herbst-Johnstone *et al.*, 2012), but contrasted those found in a similar study, where the concentration of the 3MH precursors was statistically higher in juice from mechanical harvesting (Capone and Jeffery, 2011); the particularity of this study arose from the long time necessary for transportation from mechanical harvest (12 hours). The authors suggested how the time would enable further action of endogenous or exogenous enzymes in the production of *de novo* compounds. This hypothesis was reinforced by the lower amount of GSH-3MH and Cys-3MH in the thesis treated with sulfur dioxide (SO_2), which was inversely proportional to a higher dosage of this antioxidant, later confirmed in another study with long term grape storage which obtained similar results (Capone *et al.*, 2012). On the contrary, the SO_2 treatment of grapes determineed richer 3MH and 3MHA wines, as reported by Mahkotkina *et al.,* (2013) in sulfite-rich grapes from mechanical harvest. The research about the contribution of SO_2 to the aroma potential of juices is still

ongoing, not only from the direct formation of thiols (Duhamel *et al.*, 2015; Thibon *et al.,* 2016), but also because of the technological implications, as SO_2 is known both for its antioxydasic activity against grape lipoxygenase and for its strong reactivity with aldehydes formed enzymatically (de Azevedo *et al.*, 2007). Besides, this molecule impacts on the oxidative status of wines, determining the formation of *O*-quinones, known for their capacity to react with 3MH, or form peroxides, leading to a lesser content of free thiols on the resulting wines (Nikolantonaki *et al.,* 2012).

Based on some results discussed in the paragraph above, some authors suggested night harvesting, as the grapes picked early in the morning produced juices and wines richer in precursors and in 3MH and 3MHA respectively (Kobayashi *et al.*, 2012).

2.7.2. Prefermentative skin-contact maceration

The greater concentration of precursors in the skin, as observed for other aromatic compounds, led to the assumption that prefermentative maceration could impact on the content of these molecules in juice, and hence, increase the concentration of polyfunctional thiols in wine after alcoholic fermentation. There are many studies that have dealt with this issue and, as expected, the maceration actually tends to increase the concentration of 3MH precursors in both white and red varieties (Murat *et al.*, 2001a, Peyrot des Gachons *et al.*, 2002b). The main technological variables in the maceration (time and temperature) have a different impact depending on their combination. At room temperature there were reported average increases of Cys-3MH of 62% after 24 hours of skin contact at 20°C for Cabernet Sauvignon and Merlot (Murat *et al.*, 2001a) and double the content of this precursor in Sauvignon Blanc after 18 hours at 18°C (Peyrot des Gachons *et al.*, 2002b). Low skin contact temperatures limit the extraction, especially for the precursors of the 3MH (Peyrot des Gachons *et al.*, 2002b) probably due to the reduced extraction from the solid parts (Peyrot des Gachon *et al.*, 2002b; Maggu *et al.*, 2007; Roland *et al.*, 2011a). This hypothesis is confirmed by the minimal increase observed of Cys-4MMPOH and Cys-4MMP - which are found mainly in the pulp -, if compared to the Cys-3MH, whose content is equally distributed (Peyrot des Gachons *et al.*, 2002b). When the temperature is sufficiently low (<5 ° C) maceration does not lead to significant increase of precursors (Roland *et al.*, 2011), although the final content in varietal thiols is greater in the resulting wines (Roland *et al.*, 2011; Olejar *et al.*, 2015a).

Short maceration time does not result in significant increases at room temperature and does not allow a complete extraction, especially of the 3MH precursors (Peyrot des Gachons *et al.*, 2002b; Maggu *et al.*, 2007). Thus the type of precursor thus also seems to determine the kinetics of the extraction,

where the precursors of 3MH are those which most benefit from the macerative process: in the work of Peyrot des Gachons and co-workers (2002b), the extraction *plateau* of Cys-3MH still wasn't reached after a 7-hour maceration of SB grapes, while the concentration of Cys-4MMP remained stable afterwards.

2.7.3. Pressing

The greater the pressure exerted on the skins, the greater is the precursor concentration on the corresponding juice (Peyrot des Gachons *et al.*, 2002b). This phenomenon results from the differing distribution of precursors between the skin and the pulp and is more or less accentuated by the grape variety and its initial composition (Roland *et al.*, 2011a).

Literature reports concentrations of 3MH precursors in press juice between 1.5 and 7 times higher than the free run (Maggu *et al.*, 2007; Allen *et al.*, 2011), and GSH-3MH significantly increases more than Cys-3MH (Allen *et al.*, 2011). It could be assumed that the oxidative conditions (Roland *et al.*, 2010b) and the reaction time (Capone and Jeffery, 2011) favour the formation of *de novo* GSH-3MH. In a comparison between oxidative and reductive techniques, Mattivi *et al.* (2012) stated that the former had a more marked impact on the formation of GSH-3MH than on Cys-3MH. In contrast, the results reported by Roland *et al.* (2011), showed L-cysteinylated precursors were markedly higher at the end of the pressing cycle. This difference could be explained by the higher content of GSH-3MH found in the pulp of the grapes used for Roland's experiment, which would facilitate its extraction during the first moments of grape processing. To our knowledge, no further data is available in the literature concerning the evolution of 4MMP precursors during pressing.

The greater content of precursors in the pressing fractions is not always related to wines richer in varietal thiols (Patel *et al.*, 2010), especially coming from the highest pressure fractions. The differences on the chemical composition of wines from pressed grapes, result in a depleted protective capacity of the medium towards the free molecules in compared to the free-run wines (Blanchard *et al.*, 2004). The corresponding juices contain both a higher concentration of phenols - able to capture the free thiols (Patel *et al.*, 2010) – and yeast assimilable nitrogen, that modulates the response of yeast in terms of liberating the thiols from their non volatile precursors (Subileau *et al.*, 2008b; Thibon *et al.*, 2008b). Oxidative techniques of pressing result in juices richer in precursors than the reductives', and consequently in wines characterized by higher concentrations of 3MH and 3MHA at the end of alcoholic fermentation (Mattivi *et al.*, 2012); although, during wine evolution,

the concentration of the free compounds increased in the "reductive" samples during wine evolution, overtaking the "oxidative" press wines.

2.8. Release of varietal thiols

In addition to the *de novo* pathways previously described, the liberation from non aromatic precursors occurs during the alcoholic fermentation, following two paths that differ depending on the molecule to which the free thiol is linked. Both need the intervention of microorganisms with an enzyme able to break the peptide bond with 3MH or 4MMP: the *β*–lyase. The activity of this enzyme has been reported in several microorganisms used in the wine world (Tominaga *et al.*, 1998c; Anfang *et al.*, 2009; Zott *et al.*, 2011), and displays a strain-dependent activity (Murat *et al.*, 2001b; Howell *et al.*, 2004; Dubordieu *et al.*, 2006; Subileau *et al.*, 2008a and b; Swiegers *et al.*, 2009), although it is not found in all commercial strains (Masneuf-Pomarède *et al.*, 2006; Roncoroni *et al.*, 2011; Dufour *et al.*, 2013). Genetic research in this area is aimed at better understanding the transport mechanisms within the cell vacuole of the precursors - where the reaction takes place - and to identify the genes that encode the expression of this enzyme (Howell *et al.*, 2005; Thibon *et al.*, 2008b; Swiegers *et al.*, 2009; Holt *et al.*, 2011 and 2012; Harsh and Gardner, 2013). Currently the Opt1p seems to be the main protein responsible for the transportation of glutathionylated precursors in *Saccharomyces cerevisiae* and the generic Gap1p, an aminoacid permease, is involved in the transport of the Cys-3MH (Subileau *et al.*, 2008a; Cordente *et al.*, 2015). The Ecm38p and Irc7p proteins appear to be among the key enzymes in the liberation of 3MH from GSH-3MH and Cys-4MMP (Thibon *et al.*, 2008a; Cordente *et al.*, 2015), but the mechanisms that regulate gene expression, directly or indirectly, resulting in a higher release of varietal thiols, are still to be fully explored.

2.8.1. Yeast liberation pathways

The first route concerns the direct liberation from the cysteinylated precursors of 3MH and 4MMP controlled by the *β*-lyase that cleaves the bond between the aminoacid and the corresponding thiol (Tominaga *et al.*, 1998c). The second path instead provides the release in stages from GSH-3MH and GSH-4MMP, both acting as pro-precursors (Peyrot des Gachons *et al.*, 2002a; Fedrizzi *et al.*, 2009; Grant-Preece *et al.*, 2010). This reaction considers the cleavage of the peptide bond of glutamate as a first step, and is governed by a γ-glutamyl enzyme, resulting in the subsequent formation of the CysGly-S- adduct. The following enzyme - a carboxypeptidase - cleaves the glycine bond, permitting the *β*-lyase to free the corresponding thiol. The reaction was first tested in a model solution (Grant-Preece *et al.*, 2010) and later observed in juice, following the release of 3MH during alcoholic

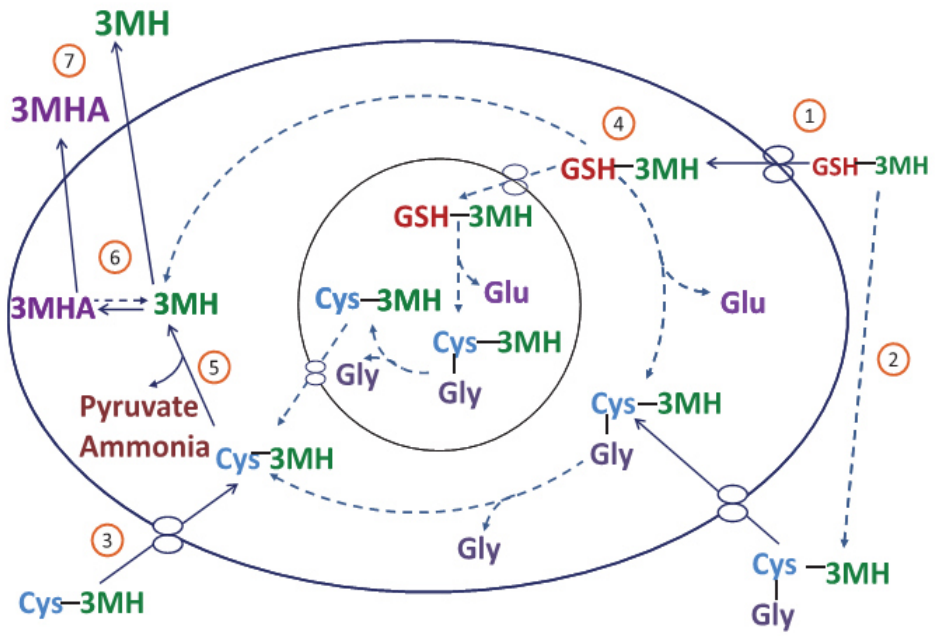
fermentation after the addition of the deuterated precursor d2/d3 of GSH-3MH (Roland *et al.*, 2010a; Capone *et al.*, 2011b). Recent studies have reported that the enzymes involved in the liberation of 3MH and 4MMP, despite belonging to the same category, are not the same (Cordente *et al.*, 2015), confirming previous metabolomic studies that have suggested different biosynthetic pathways between 3MH/3MHA and the 4MMP (Pinu *et al.*, 2014). It remains unclear whether the yeast can lead to the liberation of thiols from the glutathionylated precursors, even if it has been proposed recently (Cordente *et al.,* 2015). The proven pathways described above are summarized in figure 3.12 as proposed by Winter *et al.* in 2011.

The release of thiols during fermentation is highly variable even with the use of the same yeast strain and grape variety (Murat *et al.*, 2001b; Pinu *et al.*, 2012). The free-to-bound ratio in respect to the initial precursor content is very low after the alcoholic fermentation, particularly in synthetic media (Dubordieu *et al.*, 2006; Schneider *et al.*, 2006; Subileau *et al.*, 2008a; Pinu *et al.*, 2012 ; Kobayashi *et al.*, 2010). Often this ratio does not exceed 3-4% although the range is quite wide and can be up to 10-15% (Subileau *et al.*, 2008a; Kobayashi *et al.*, 2010; Roland *et al.*, 2010a; Thibon *et al* ., 2010; Winter *et al.*, 2011). The use of deuterated precursors has allowed the determination of the free-to-bound ratio in regards to each precursor: the Cys-3MH presents values <7% (Subileau *et al.*, 2008a) and GSH-3MH <4-5% (Roland *et al.*, 2010c). For Cys-4MMP, it has been proposed free-to-bound ratios reach up to 14% (Dubordieu *et al.*, 2006) but at that time the presence of GSH-4MMP had not yet been reported in musts (Fedrizzi *et al.*, 2009). The results of the ratios reported and the % of the varietal thiols formed in juices remains still to be clarified as the *β*-lyase activity should theoretically be quantitative, suggesting the presence of additional variables not yet understood, and highlighting how only a small fraction of 3MH/3MHA comes from non-volatile precursors present in juice (Subileau *et al.*, 2008a; Roland *et al.*, 2010c).

GSH-3MH and Cys-3MH contribute almost equally in the production of 3MH and 3MHA (Roland *et al.*, 2010c) although in model solution, the Cys-3MH impact is up to 4 times greater (Kobayashi *et al.*, 2010). Researchers have focused their attention on the relationship between the initial concentration of precursors and the amount of free thiols at the end of alcoholic fermentation. This relationship often shows very low correlation indexes or are not correlated at all (Patel *et al.*, 2010 Roland *et al.*, 2010c; Allen *et al.*, 2011a; Capone *et al.*, 2011a; Pinu *et al.*, 2012). Only Kobayashi and his colleagues (2010) founded a positive correlation between the GSH-3MH and Cys-3MH in juices and the free thiols quantified in wines (r^2= 0.58 and 0.70 respectively) for the cv Koshu, supporting the statement of Murat *et*

al., (2001a) for which juices richer in precursors resulted in wines richer in polyfunctional thiols.

Figure 2.11. Reaction mechanism inside of the yeast cell, as reported by Winter *et al.*, (2011)



During the fermentation, the kinetics of the metabolism of Cys-3MH is faster than GSH-3MH, releasing thiols from the very first day (Kobayashy *et al.* 2010) and achieving the maximum liberation ratio within the first third of the alcoholic fermentation (Dubourdieu *et al.*, 2006). This allows speculations about how the enzyme γ-glutamyltranspeptidase can govern the overall reaction of GSH-3MH metabolism. In the conditions of Kobayashi's experiment (2010), it may be assumed that the oxidation processes of these molecules can greatly impact the final content, but any further metabolisation by *Saccharomyces cerevisiae* cannot be ruled out. This biologic degradation, presumably, follows the same routes and transport carriers as that *(E)*-2-hexenal and 1-hexenol, which yeast reduces into 1-hexanol, much less toxic to cells and less reactive (Kubo *et al.*, 2003). In literature, it has not yet been any data reported on the metabolisation kinetics of the 4MMP precursors.

Temperature affects the release of thiols during alcoholic fermentation, but the available data is highly variable and not conclusive. The final concentration of 3MH, 3MHA and 4MMP seems to be higher at 20°C than at 13°C regardless of the yeast strain (Masneuf-Pomarède *et al.*, 2006). Higher

fermentation temperatures led to the increased production of 4MMP, although the variability between strains is quite high (Howell *et al.*, 2004).

2.8.2. 3-mercaptohexyl acetate formation

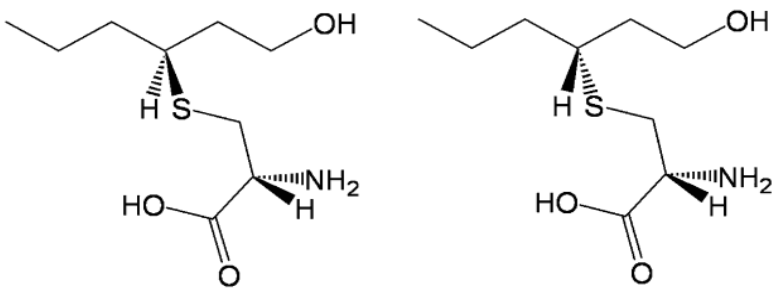
The acetylation of 3MH into 3MHA is governed exclusively by yeast and it is a strain dependent reaction, as demonstrated following the overexpression of ATF1 gene in certain strains of *Saccharomyces cerevisiae* (King *et al.*, 2008). This gene codes for the expression of an alcoholacetyltransferase that induce an increase in the formation of 3MHA (Swiegers and Petrorius, 2007). Many genes encode for a pool of proteins able to cleave the adduct and free the thiol molecule (Thibon *et al.*, 2008b) and it seems that depending on the protein, enzymes have a steroisomeric preference (Wakabayashi *et al.*, 2004; Thibon *et al.*, 2008a). The level of esterification does not depend on the concentration of 3MH in the fermentation medium, showing a highly variable 3MH/3MHA ratio depending on the strain, with ranges varying between 5-25 (King *et al.*, 2008; Swiegers *et al.*, 2009). In any case, the genes coding for the thiol expression are not yet elucidated, even though it seems clear that those involved in nitrogen and sulfur metabolism play an important role (Harsch and Gardner, 2013). In a model solution, despite a lower level of 3MH release, a higher rate of 3MH acetylation deriving form GSH-3MH was found when compared to Cys-3MH's: 2.3% and 1.4% respectively (Winter *et al.*, 2011). This could be because the gene expression of the acetyl transferase - as for the nitrogen catabolite repression genes (NCR) in general - is mediated by the nutrient availability in the fermentation medium (Thibon *et al.*, 2008b; Deed *et al.*, 2011), contributing each precursor with a different weight to that purpose.

2.8.3. Stereoisomeric distribution of precursors and thiols in wine

The concentration of the S enantiomer in the 3MH precursors is significantly greater than the R, with more marked differences for GSH-3MH, even though this distribution is highly variable between varieties (Thibon *et al.*, 2008; Capone *et al.*, 2010, Capone *et al.*, 2011a). In wines, the isomeric ratio of 3MH is maintained, and the S isomer is present in greater concentration than R, regardless of the variety (Capone *et al.*, 2011a), in accordance with what is reported by Tominaga *et al.* (2006) in botrytized wines; however these results contradict the data regarding dry wines, which present a racemic mixture of enantiomers. Differences in the final ratio could be due to the endogenous pool of β-lyase enzymes of yeasts, which that seem to be stereoselective (Wakabayashi *et al.*, 2004). These differences in the final distributions of the enantiomers could impact on final aroma as the S enatiomer of 3MH resembles passion fruit, meanwhile R is more likely to grapefruit, both presenting very similar perception thresholds (Tominaga *et al.*, 2006). The S form of 3MHA is also more abundant in wines, whilst

showing a 4 times lower perception threshold from the R form. As for 3MH, both 3MHA enantiomers have different aromas: R presents scents of passion fruit and S is instead more herbaceous instead, resembling the boxtree aromas of 4MMP (Dubourdieu and Tominaga, 2009). As an example, figure 3.10 represents the chiral structure of 3MH enantiomers.

Figure 2.12. Chiral struture of 3-S-cysteinyl mercaptohexan-1-ol enantiomers, as reported by Thibon *et al.,* (2008b)



(*RS*)-P-3MH (*RR*)-P-3MH

2.8.4. Reaction with quinones and polyphenols

In red wines a decrease of 3MH was observed following oxygenation processes, starting inmediately after the stabilisation of the O_2 concentration (Blanchard *et al.*, 2004), thus indicating the possibility that other molecules could react more easily with oxygen than thiols. The reported decrease of 3MH could be due to its interaction with the resulting oxidised molecules, in particular peroxides deriving from reactions catalysed by metals (Fe and Cu, mainly), as thiols on their own, are not easily oxidised (Jocelyn, 1972; Blanchard *et al.*, 2004; Kreitman *et al.*, 2016a and b). Following this hypotesis, different works have reported a more significant decrease of 3MH in the presence of catechin, epicatechin and caftaric acid following oxygenation treatments (Blanchard *et al.*, 2004, Nikolantonaki *et al.*, 2010 and 2012; Laurie *et al.*, 2012), when compared to the presence of oxygen only, suggesting two possible reaction mechanisms. The first is based on the reaction between thiols and the resulting quinones of the previous phenols, well known highly electrophilic oxydising agents, (Singleton, 1987) via a Michaels addition, as suggested by Chernier *et al.*, (1986); indeed thiols are highly nucleophilic and react rapidly with these molecules. The second is due to the peroxide formation following coupled reactions of *O*-quinones as proposed by Kreitman *et al.*, (2013).

Catechin, epicatechin and caftaric acid have been suggested (Roland *et al.*, 2010c) and then reported (Nikolantonaki *et al.*, 2012) to react with 3MH, mainly in juices due to the faster enzymaticaly mediated formation rate of the

quinones as opposed to chemically. The adduct formation rate depends on the molecule, with the quinone of epicatechin reacting faster than any other, and 3MH is more reactive than 4MMP (Nikolantonaki *et al.,* 2010). In fact, these phenols have been found in higher concentrations in macerated SB along with a reported lower typicality of the wines obtained (Olejar *et al.*, 2015b); moreover, a prefermentation skin-contact phase at low temperature limits enzymatic reactions (Olejar *et al.*, 2015a).

2.8.5. The effect of SO_2 supplementation on juice and wine

SO_2 has been reported to affect a number of mechanisms regarding the *de novo* formation of precursors in juices (Schneider *et al.*, 2006) and free molecules during alcoholic fermentation by sulfonation reactions (Allen *et al.*, 2011; Makhotkina *et al.,* 2013; Duhamel *et al.*, 2015; Thibon *et al.*, 2016), or simply by enhancing the extraction mechanisms from grape cells, helping with the disruption of the cell membranes (Jackson, 2008). In wines, it has been reported that it has a protective action toward 3MH and 4MMP, by avoiding the formation of *O*-quinones, taking them back to their original diphenol (Blanchard *et al.*, 2004; Laurie *et al.,* 2012; Mathotkina *et al.*, 2012) as both compete with the dissolved oxygen. The reaction between quinones and SO_2 is quite fast and, apparently, there is no synergic effect on the antioxidant activity towards other wine preservative, as with glutathione or ascorbic acid (Nikolantonaki *et al.*, 2014). However, SO_2 cannot avoid the formation of every quinone, especially for those coming from epicatechin, whose *O*-quinone can create an adduct with 3MH. The kinetics of the formation of this new adduct is not modified by SO_2, probably due to the higher oxidation rate of epicatechin if compared to the SO_2's (Nikolantonaki *et al.*, 2012).

2.9. Thiols and ageing

The evolution of the concentration in polyfunctional thiols in wines is strongly dependent on the matrix conditions. In particular, oxygen and all the the deriving reactions linked to its presence and its management that play a critical role in the stability of these molecules (Blanchard *et al.,* 2004; Brajkovich *et al*, 2005; Nikolantonaki *et al.,* 2010; Ugliano *et al.,* 2011).

3MH evolution during aging in white wines is differentiated in two phases: first, its concentration clearly increases, followed then by a loss in its content (Herbst-Johnstone *et al*., 2011; Mattivi *et al.*, 2012). The first stage has been suggested to derive from the hydrolysis of 3MHA, which takes place right after or even during the alcoholic fermentation (Herbst-Johnstone *et al*., 2011), or from the 3MH disulfides, already reported in wines (Sarrazin *et al.,* 2010) overlapping the losses caused by oxidations mechanisims (Nikolantonaki *et al.*, 2010), as thiols react easily with oxygen in the

presence of trace amounts of metals (Jocelyn, 1972). The continued presence in wines of the 3MH and 4MMP precursors after the alcoholic fermentation (Capone *et al.*, 2010), made initially envisage that the related thiols could be freed during evolution. However, it seems that at wine pH these forms are stable (Ugliano *et al.,* 2011) and unlikely to do what initially expected, and do not contribute to the aroma formation during aging. The second stage is governed by a net loss, as these compound can undergo oxidations catalysed by metals, direct oxidations from peroxides or linkage with copper or quinones (Jocelyn 1972; Nikolantonaki *et al.*, 2010), but is not particularly affected by room temperature (Makhotkina *et al.*, 2012).

The presence of SO_2 during aging plays a double role as it can react directly with thiols provoking a loss in the aroma potential, or indirectly, competing with them on the reaction with quinones (Nikolantonaki *et al.,* 2010) or limiting the effect of H_2O_2 formed following the oxidation of phenols (Danilewicz *et al.,* 2008). However, wines treated with SO_2 always contain higher concentrations of thiols (Nikolantonaki *et al.*, 2010; Ugliano *et al.*, 2011 and 2013). In the same vein, the impact of other preservatives on the stability of 3MH in wines. The presence of these preservatives permits the maintainance of a higher level of 3MH during ageing, as for GSH in bottles (Brajkovich *et al.,* 2005; Ugliano *et al.,* 2011) or for lees during barrel ageing (Dubourdieu and Lavigne, 2004), where 4MMP were less impacted.

3MHA behave diversly, as this molecule also undergoes - as other esters in wine – acid hydrolysis, resulting in 3MH and acetic acid (Makhotkina and Kilmartin, 2012), characterised by a higher sensory threshold. Herbst-Johnstone *et al*., (2011) have reported a loss of 40% in the 3MHA concentration after 3 months of dark storage of the bottled wine in a dark environment, and 69% after 7 months, following a pseudo-first-order rate, loss later confirmed in wine by other studies (Ugliano *et al.*, 2011; Mattivi *et al.*, 2012). Lower storage temperatures, permit a slowing down of the reaction (Makhotkina *et al.,* 2012). The contribution of oxidation mechanisms in the loss of 3MHA depends on the oxygen dissolved, due to either a direct reaction or the oxidation of 3MH in this way enhancing the hydrolisation rate of 3MH (Herbst-Johnstone *et al*., 2011). However in industrial conditions, where the oxygen concentration is controlled, this path appears to be minimal (Herbst-Johnstone *et al*., 2011).

Copper addition, usually carried out right before bottling to eliminiate unpleasant sulfur off-flavours (Ugliano *et al.,* 2009) due to its reactivity with the sulfur atom (Jocelyn, 1972), causes a sudden drop in thiols (Ugliano *et al.,* 2011), not affecting the rate of 3MH loss afterwards. This effect was previously reported in wines coming from grapes treated with copper fungicides in the vineyard (Hatzidimitriou *et al.,*1996; Darriet *et al.,* 2001),

probably originated at the beginning of the alcoholic fermentation. Indeed, thiols undergo a number of reactions with copper: directly - binding the free thiol, oxidazing to a disulfide or catalysing the formation of quinones. All of them provoke the loss of their aroma potential in wines.

Summarizing, thiols - particularly 3MH and 4MMP - suffer important changes during ageing linked to the content of oxygen in the bottle headspace and its diffusion rate from the sealing (Lopes *et al.,* 2009; Ugliano, 2013), or the presence of metal ions, such as Cu^{2+} or Fe^{3+} (Ugliano *et al.,* 2011), more or less important depending on the chemical characteristics of the medium, the storage conditions and the sealing usued (Blanchard *et al.*, 2004; Lopes *et al.*, 2009; Ugliano 2011 and 2013)

2.10. Concentration of free thiols in wine and their correlation with juice precursors

With all the above mentioned features regarding the precurosor content in juice and thiol liberation during the alcoholic fermentation, numerous works have reported the concentrations of 3MH, 3MHA and 4MMP in wines of several cultivars, with special attention paid to Sauvignon Blanc. As was expected, concentrations vary widely depending on the thiol studied and the paper however, it has been suggested that the concentration is not correlated to the region or sub-region of grapes but to the chemical composition of the juice (Jouanneau *et al.*, 2012). Table 2.4 shows data reported for thiol and variety, where it can be deduced that SB is the most characteristic grape variety for every thiol, with an extraordinary variability that includes terroir and matrix effects.

Table 2.4. Range of free thiol concentration in varietal wines as reported in literature. ([a]Pinu *et al*, 2014; [b]Allen *et al.*, 2011; [c]Dubourdieu *et al.*, 2006; [d]Jouanneau *et al.*, 2012; [e]Lacroux *et al.*, 2008; [f]Masneuf-Pomerade *et al.*, 2006; [g]Mateo-Vivaracho *et al.*, 2010; [h]Mateo-Vivaracho *et al.*, 2007; [i]Murat *et al.*, 2001b; [j]Swiegers *et al.*, 2009; [k]Anfang *et al.*, 2009; [l]Capone *et al.*, 2011a; [m]Herbst-Johnstone *et al.*, 2013; [n]Lund *et al.*, 2009; [o]Plan *et al.*, 2012; [p]Šuklje *et al.*, 2014; [q]Blanchard *et al.*, 2004; [r]Murat *et al.*, 2001a; [s]Schneider *et al.*, 2003; [t]Rapp *et al.*, 1997; [u] Escudero *et al.*, 2004; [v]Fretz *et al.*, 2005. [w]Mateo Vivaracho *et al.*, 2010; [x]Bouchilloux *et al.*, 1998; [y]Guth *et al.*, 1997; [z]Mattivi *et al.*, 2012; [A]Tominaga *et al.*, 2000b; present: Found but not quantified; n.d. not detected or below the *detection limit).*

Cultivar	Concentration range ($ng \cdot L^{-1}$)		
	4MMP	3MH	3MHA
Albariño	n.d.-11[h,g]	n.d.-800[h,g]	n.d.-33[h,g]
Cabernet Franc		4560 [q,x]	present[x]
Cabernet Sauvignon		366-6800[q,r,x]	present[x]
Chardonnay	n.d.-25[g]	10-150[g]	20-45[g]
Gewürztraminer	n.d.-25[y,A]	96-3278[l,A]	1-6[A]
Grenache	n.d.-8[g]	100-500[g]	30-60[g]
Trebbiano di Lugana		50-325[z]	3-30[z]
Macabeo	n.d.-2[g,h,u]	n.d.-114[g,h]	n.d.-28[g,h]
Merlot		n.d.-1000[q-r,x]	present[x]
Moscato	14-97[A]	47-911[l,A]	
Mueller Thurgau	n.d.-1[A]	88-248[t,A]	
Muscadet	n.d.[s]	63-445[s]	n.d.-6[s]
Petit Arvine		212-6112[v]	
Pinot Grigio	n.d.-2[A]	108-1021[l,A]	n.d.-51[A]
Riesling	n.d.-6[A]	172-1060[l, A]	n.d.-5[l,A]
Sauvignon Blanc	n.d.-402[a-j, s]	25,8-18681[a-s]	n.d.-2507[a-s]
Schreube	present[y]		
Semillon	8-40[A]	3911-5969[g,A]	n.d.-101[A]
Sylvaner	n.d.-1[A]	58-145[A]	
Verdejo	n.d.-8[h]	n.d.-931[h]	n.d.-216[h]

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SECTION 3. VITICULTURAL INVESTIGATIONS

3.1. Clonal variability of 3-mercaptohexan-1-ol precursors in Gewürztraminer juice

3.2. Accumulation of cysteine-3-mercaptohexan-1-ol and glutathione-3-mercaptohexan-1-ol during Gewürztraminer ripening in Trentino

3.3. Distribution of the 3-mercaptohexan-1-ol precursors in Gewürztraminer grapes and the effect of the enzymatic maceration on content in juice

SECTION 3.1

Clonal variability of 3-mercaptohexan-1-ol precursors in Gewürztraminer juices

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Aim of the work

In this study we have focused on assesing the content of glutathione-3-mercaptohexan-1-ol and cysteine-3-mercaptohexan-1-ol in juice of 7 Gewürztraminer clones: ISMA-AVIT 904 (904), ISMA-AVIT 906 (906); ISMA-AVIT 916 (916), ISMA-AVIT 918 (918) and ISMA-AVIT 920R (920R), LB14 and 1101. For this aim, 4 plots, with different heights, sun exposures and training systems in a vast alpine area, were sampled and analysed for the 3-mercaptohexan-1-ol precursors to verify the clonal variability.

Introduction

Much has been already written regarding the precursors of 3-mercaptohexan-1-ol - also known as 3-mercaptohexan-1-ol (3MH) - since the discovery of their presence in grapes and juices (Tominaga *et al.*, 1998a; Peyrot des Gachons *et al.,* 2002). These precursors - i.e. 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) and 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) - free 3MH during the alcoholic fermentation (Zott *et al.*, 2011) thanks to endogenous *β*-lyase enzymes present in some yeast strains (Swiegers and Pretorius, 2007; Anfang *et al.*, 2009). Precursor content is positively correlated to a higher concentration of 3MH and its acetate in wines (Murat *et al.*, 2001; Kobayashy *et al.* 2010), although not for every variety (Capone *et al.*, 2011; Pinu *et al.*, 2012). Still, the origin of these precursors in grapes and juices has not completely been explained (Subileau *et al.,* 2008) and various *de novo* formation pathways have been proposed (Wakabayashi *et al.*, 2004; Schneider *et al.*, 2006; Capone & Jeffery, 2011; Harsch *et al.*, 2013). In the meantime, the wine industry requires practical information to better modulate the thiolic aroma of wines, described as tropical, boxtree or grapefruit-like (Tominaga *et al.*, 1996 and 1998b). Several studies have focused their attention on the biological variability of Cys-3MH and GSH-3MH reporting differences between cultivars (Roland *et al.*, 2010; Concejero *et al.*, 2014), growing areas, plots and years (Pinu *et al.*, 2012) and/or training systems (Cerreti *et al*., 2016). Furthermore, a strong matrix effects on the concentration of the free thiols in the resulting wines has been observed (Pinu *et al.*, 2014). To our knowledge, the only clonal variability studied until know is that of Sauvignon Blanc clones in a single vineyard in Australia (Capone *et al.*, 2011). Vines need 2-3 years of development and another 2-3 to reach equilibrium prior to producing grapes suitable for a proper wine quality (Fregoni, 2005), which result in the investment of vine planting being tied-up for several years before revenues are apparent. So, in relation to this long-time investment, it appears fundamental for the wine industry to deepen its knowledge of the clone-related thiolic potential, in particular for some varieties (like

Gewürztraminer) already known by their interesting thiol precursors content (Tominaga *et al.*, 2000; Roland *et al.*, 2010; Román Villegas *et al.*, 2016).

In this work, we studied the performance at industrial ripeness of 7 Gewürztraminer clones cultivates in Trentino (Italy) in 4 plots. They reflect the geological, climatic and agronomical features that can be found for the variety in this Alpine region and provide the biological variability necessary for robust results.

Materials and methods

Gewürztraminer clones and plots

The study has been conducted on 7 clones of Gewürztraminer: ISMA-AVIT 904 (904), ISMA-AVIT 906 (906); ISMA-AVIT 916 (916), ISMA-AVIT 918 (918) and ISMA-AVIT 920R (920R), LB14 and 1101. All clones are already inscribed on the Italian register of grape varieties (G.U. 04/12/1981, 22/12/2001 and 23/07/2011), firstly identified and selected (Malossini *et al.,* 2002; Malossini *et al.*, 2003) at the Fondazione Edmund Mach (San Michele all'Adige, Trentino, Italy) for 904, 906; 916, 918, 920R and 1101, and at the Laimburg experimental Center (Laimburg, South Tyrol, Italy) for LB14. The clonal variability was studied during the 2015 harvest in 4 plots, belonging to the Gewürztraminer growing area in Trentino (fig 1). Table 1 reports the main agronomical and geographical features of the plots under study.

Chemicals

HPLC grade formic acid, methanol and acetonitrile were provided by Sigma-Aldrich (Milan, Italy).

Samples preparation and Ultra High Performance Liquid Chromatography – Mass Spectrometer (UHPLC-MS) essays

10 vines of each clone were randomly sampled, for a total of 5 Kg of grape bunches one day before the date defined for optimal harvest for each growing area by the harvest plan of industrial wineries. Once in the Experimental Winery of the Edmund Mach Foundation, samples were weighed, destemmed (Ares 15, OMAC s.r.l., Corridonia, MC, Italy) and pressed twice (150 atm, 60 seconds) with a R70 hydraulic press (Meccanica Arturo Rossi, Verla di Giovo, Italy). 25 mL of the pressed juice were then infused in 25 mL of methanol and kept at -20°C until precursor analysis.

The analysis of GSH-3MH and Cys-3MH was performed using the method previously reported by Larcher *et al.* (2013b) with an UHPLC Acquity (Waters Corporation, Milford, MA, USA) coupled to a Xevo TQ MS mass

spectrometer (Waters Corporation) injecting 5 µL of sample previously, centrifuged and filtered. The column was a UPLC HSS T3 C18 (18 µm x 2.1 mm x 100 mm, Waters), operating at 40 °C with a flow rate of 0.45 mL·min^{-1} with water (A) and acetonitrile (B) as eluents, both added with 0.1% formic acid. Mass spectrometry analysis was carried out in positive ion mode. Details about the method are reported in the original paper mentioned above.

Fourier transform infrared spectroscopy (FTIR) measurements

30 mL of juice, previously centrifuged (5000 r.p.m., 5 min) and filtered (25 mm×0.45 µm cellulose acetate syringe cartridge; Alltech, Deerfield, IL, USA), were analysed for °Brix, pH, titratable acidity, tartaric acid, malic acid and potassium with a WineScan^{TM} FT 120 Type 77310 (Foss Electric A/S Hillerød, Denmark), calibrated with the official methods (Organisation Internationale de la Vigne et du Vin 2013).

Statistical analysis

Statistical analysis was performed with Statistica 9.0 software (StatSoft Inc., Tusla, OK, USA), applying the procedures each time declared.

Results

Plot effect

As expected, plot origin has affected the basic composition of juices (table 2) and the total precursors concentration (fig. 2). In particular, a large variability has been found regarding pH and malic acid. These parameters, typically correlated negatively in juice, and seem to have suffered more than any other from the extremely hot 2015 summer in Trentino. The increase of the respiration processes of the vines may has led to malic acid degradation, resulting in a higher pH. In fact, the malic acid concentration is meanly 1-2 g·L^{-1} lesser than what is typical for the region at technological ripeness (2.37±0.46 g·L^{-1}, in association with a higher sugar accumulation, 22.2±1.0 °Brix; N= 25; unpublished data). Differences of technological ripeness between plots, even if statistically higher in grapes from Marchi and Padaro (table 2), are not very marked (maximun mean difference: 0.6 °Brix) so the mean effect of the grape maturity on the final concentration could be assumed to be limited (Kobayashi *et al.*, 2010; Capone *et al.*, 2011). At this regard, the concentration of GSH-3MH, the most impacted precursor by plant environment and ripeness (Capone & Jeffery 2011), is the highest in the Padaro plot, but not in Marchi, being this plot undistinguishable from Filippi, the poorest plot. Cys-3MH presents its highest concentration in Sarche juices, differentiated from all the other plots, along with the lowest sugar concentration and the highest yeast assimilable nitrogen (YAN). Vine

nutrition status regarding nitrogen has previously been positively correlated to the final concentration of precursors (Peyrot des Gachons *et al.*, 2005; Lacroux *et al.*, 2008, Dufourcq *et al.*, 2009) and at our conditions, the highest YAN (Padaro and Sarche) corresponded to the highest total precursors concentration.

Regarding the vine training system, it has been hypothesized to have an effect on the accumulation of thiol preccurosors, as reported by Cerreti *et al.*, (2016) in relation to Grechetto. In our case we cannot make any consideration as both training systems under analysis did not coincide in the same plot and for this type of analysis, the number of plots *per* system cannot be considered representative. The same consideration can be made for altitude and sun exposition of the canopy, reported in table 1 just to describe the geographical and agronomical variability.

Clone effect

Along with the large variability observed between plots, the analysis of variance (Anova main effects; Fisher LSD test, $p<0.05$) has shown statistical differences between clones for all the juice parameters (table 3). Regarding the total precursors concentration ($nmol \cdot L^{-1}$), 920R is the richest clone, statistically differentiated from 916 by over a 30%, with the rest of the clones positioned midway between the two. 920R - along with 916 - presents the highest GSH-3MH concentration (fig. 3), but not for Cys-3MH where 906 was the richest, statistically differentiated from 916, 920R and LB14 (fig. 4). Mean concentrations *per* clone ranged 72-103 $\mu g \cdot L^{-1}$ and 15-21 $\mu g \cdot L^{-1}$ for GSH-3MH and Cys-3MH respectively. These values are in accordance with reported literature (Capone *et al.*, 2010), and in particular with regard to this *cultivar* (Roland *et al.*, 2010). GSH-3MH is the main 3MH precursor in all clones and plots, representing meanly the 73% of total precursors, differing with the results of Roland *et al.* (2010a) but in agreement with the majority of works which have stated GSH-3MH as the main precursor both in grape and juices (Capone *et al.*, 2010; Allen *et al.*, 2011; Roland *et al.*, 2011; Pinu *et al.*, 2012), also for Gewürztraminer (Concejero *et al.*, 2014). The differences reported in the literature underline the importance of technological and matrix features in the concentration of these precursors in juices (Peyrot des Gachons *et al.*, 2005; Kobayashi *et al.*, 2012; Pinu *et al.*, 2012), also observed by our research group while studying pomace concentration from the same variety and area under investigation (Roman Villegas *et al.*, 2016; Roman Villegas *et al.*, 2017 *in litteris*).

Conclusions

The present work reports for the first time in Gewürztraminer, the evidence of clonal variability regarding 3MH precursors in juice, with differences up to 30%. The variability of the agronomic and environmental conditions of the plots where clones were grown supports and strengthens the presence of actual clonal differences, even if measured in only one vintage year. Moreover, a larger variability in terms of total precursors has been observed between plots, where the richer doubles the poorest.

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Table 1. Georeferencing and main geographical and viticultural features of plots.

Code	Plot	Municipality	Latitude	Longitude	Altitude (m.a.s.l.)	Training system	Row direction
1	Marchi	Faedo	46.19957	11.14379	313	Pergola	SW-NE
2	Filippi	San Michele	46.19733	11.14089	268	Pergola	N-S
3	Sarche	Sarche	46.04419	10.95207	250	Guyot	N-S
4	Padaro	Avio	45.93199	10.87115	353	Guyot	W-E

Table 2. Mean composition of clonal juice of Gewürztraminer displayed *per* plots. (GSH-3MH: 3-S-glutathionyl mercaptohexan-1-ol ; Cys-3MH: cysteinyl mercaptohexan-1-ol; ∑Prec: sum of GSH-3MH and Cys-3MH). Different letters correspond to statistically different values (Fisher's LSD test, $p<0.05$).

Variable	Filippi			Marchi			Padaro			Sarche		
	Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.	
GSH-3MH ($\mu g \cdot L^{-1}$)	54	9	c	68	20	c	129	20	a	100	11	b
Cys-3MH ($\mu g \cdot L^{-1}$)	14	2	b	15	4	b	17	2	b	24	4	a
ΣPrec ($nmol \cdot L^{-1}$)	196	30	b	233	60	b	394	45	a	354	43	a
GSH/Cys 3MH (mol:mol)	2.04	0.16	b	2.51	0.68	b	4.07	0.91	a	2.28	0.32	b
SST (°Brix)	21.52	0.73	ab	21.78	0.51	a	21.86	0.35	a	21.24	0.33	b
pH	3.54	0.08	b	3.43	0.09	c	3.68	0.09	a	3.27	0.03	d
Titratable acidity ($g \cdot L^{-1}$)	3.33	0.46	b	3.84	0.35	a	2.71	0.20	c	3.23	0.14	b
Tartaric acid (g·L-1)	6.89	0.23	a	6.74	0.16	a	6.71	0.25	a	6.39	0.28	b
Malic acid ($g \cdot L^{-1}$)	1.16	0.22	b	1.48	0.23	a	0.90	0.10	c	0.63	0.08	d
Potassium (mg·L-1)	2.02	0.12	ab	1.92	0.08	b	2.13	0.15	a	1.42	0.07	c
YAN ($mg \cdot L^{-1}$)	114	17	b	83	17	c	135	16	a	145	15	a

Table 3. Compositional characterisation of juices of 7 Gewürztraminer clones. (GSH-3MH: 3-S-glutathionyl mercaptohexan-1-ol ; Cys-3MH: cysteinyl mercaptohexan-1-ol; ∑Prec: sum of GSH-3MH and Cys-3MH; TSS: Total Soluble Solids; YAN = yeast assimilable nitrogen; parameters with the same letter are not significantly different in Fisher's LSD test, $p<0.05$).

Parameter	1101			904			906			916			918			920R			Lb14		
	Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.		Mean (n=4)	Std. Dev.	
GSH-3MH ($\mu g \cdot L^{-1}$)	84	33	ab	85	25	ab	97	20	a	72	46	b	86	41	ab	103	47	a	85	32	ab
Cys-3MH ($\mu g \cdot L^{-1}$)	18	3	ab	19	8	ab	21	4	a	15	4	b	17	8	ab	16	3	b	16	4	b
ΣPrec ($nmol \cdot L^{-1}$)	289	89	ab	295	91	ab	332	58	a	247	129	b	289	123	ab	327	118	a	282	92	ab
GSH/Cys 3MH (mol:mol)	2.44	0.82	b	2.54	0.56	b	2.53	0.54	b	2.38	1.00	b	2.81	1.23	ab	3.54	1.70	a	2.84	0.89	ab
TSS (°Brix)	21.88	0.42	ab	21.11	0.29	c	21.31	0.59	c	21.55	0.52	bc	21.48	0.21	bc	22.35	0.50	a	21.55	0.34	bc
pH	3.54	0.23	a	3.48	0.15	ab	3.47	0.18	ab	3.41	0.18	b	3.46	0.20	ab	3.53	0.19	a	3.47	0.17	ab
Titratable acidity ($g \cdot L^{-1}$)	3.05	0.50	b	3.23	0.22	b	3.28	0.51	ab	3.65	0.72	a	3.30	0.66	ab	3.05	0.42	b	3.40	0.48	ab
Tartaric acid (g·L-1)	6.78	0.30	ab	6.58	0.28	bc	6.44	0.37	c	6.91	0.25	a	6.67	0.23	abc	6.69	0.24	abc	6.72	0.31	abc
Malic acid ($g \cdot L^{-1}$)	0.91	0.28	b	0.90	0.27	b	1.07	0.41	ab	1.23	0.42	a	1.09	0.46	ab	0.95	0.31	b	1.16	0.48	a
Potassium (mg·L-1)	1.95	0.39	ab	1.84	0.30	ab	1.84	0.36	ab	1.80	0.22	b	1.83	0.34	ab	1.96	0.34	a	1.87	0.34	ab
YAN ($mg \cdot L^{-1}$)	122	35	abc	102	34	c	111	26	bc	127	17	ab	118	25	abc	122	45	abc	133	26	a

Figure 1. Geographical distribution of the plots under study: 1 = Faedo; 2 = San Michele all'Adige; 3 = Sarche and 4 = Avio.

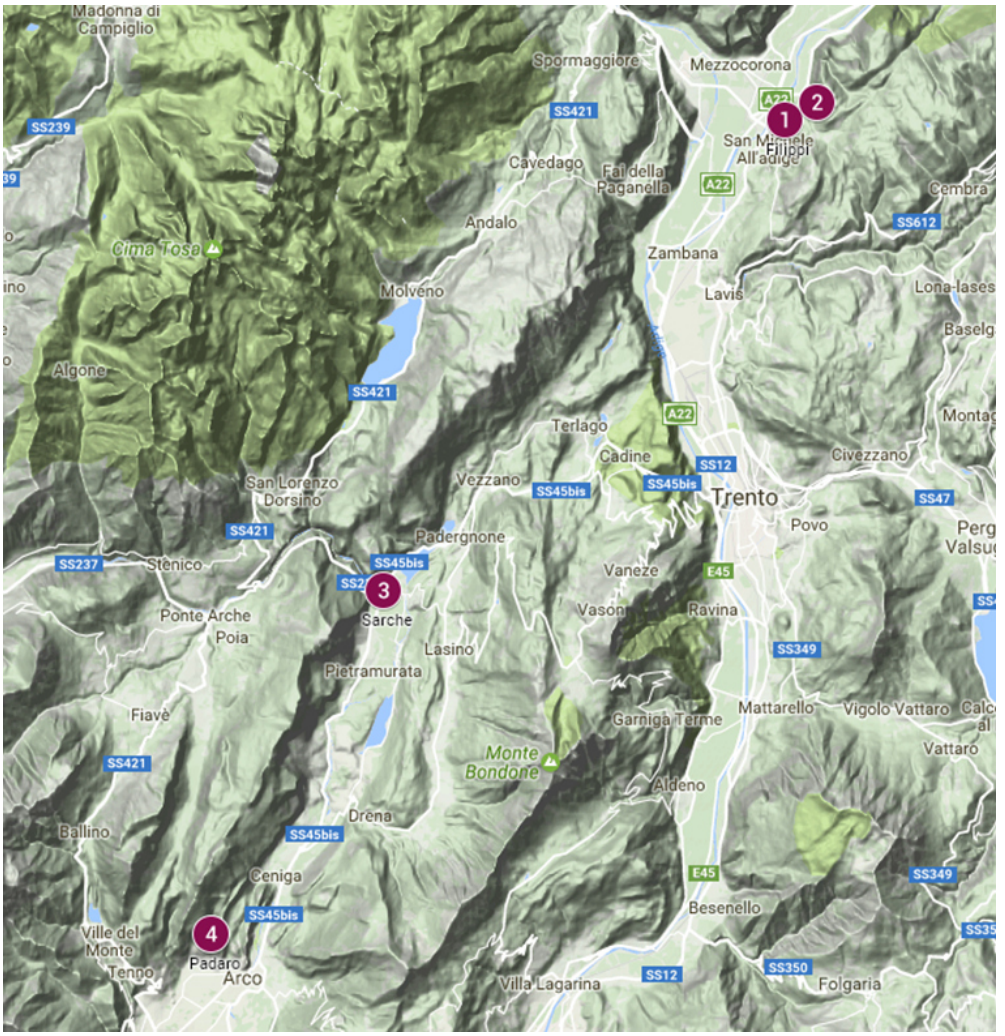


Figure 2. Distribution of the sum of precursors (3-S-glutathionyl mercaptohexan-1-ol + 3-S-cysteinyl mercaptohexan-1-ol) measured in clonal juices (n=7) in relation to the 4 plots under investigation (Box plots with the same letter do not differ significantly in Fisher's LSD test, $p<0.05$).

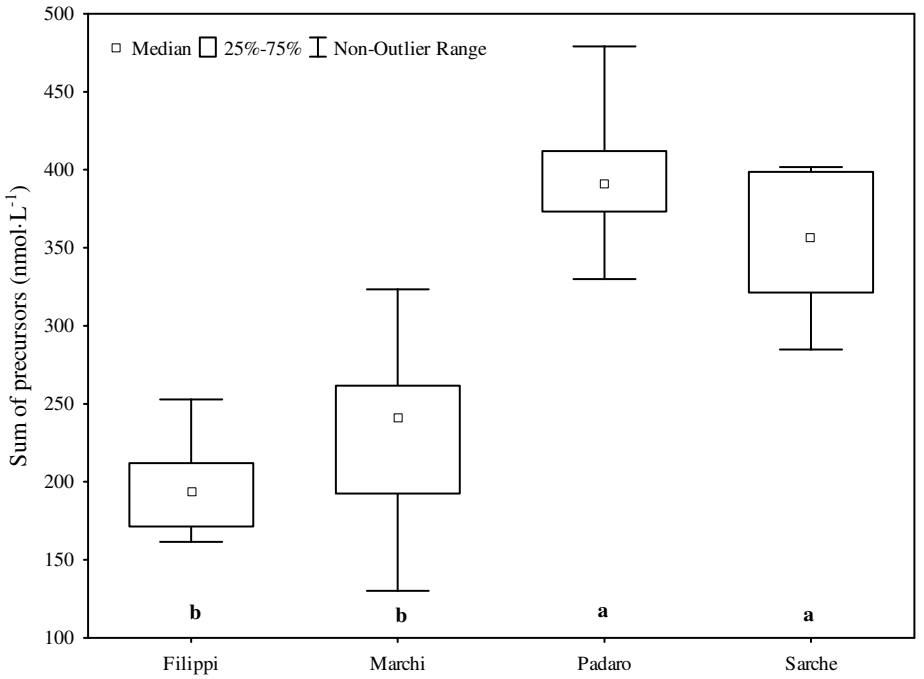


Figure 3. 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) distribution measured in clones grown in 4 plots (Mean value and standard deviation; histograms with the same letter do not differ significantly in Fisher's LSD test, $p<0.05$).

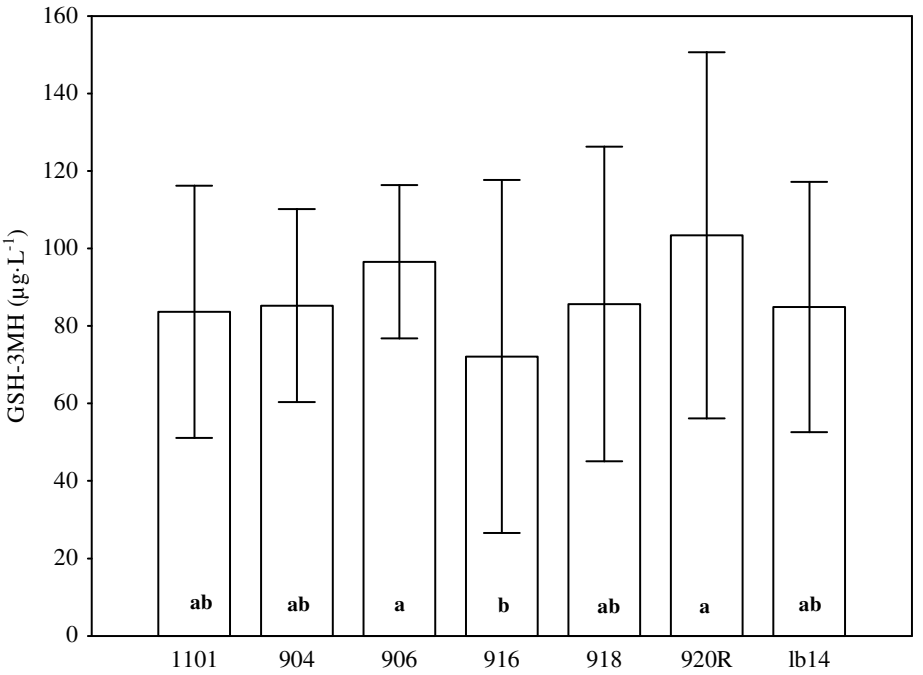
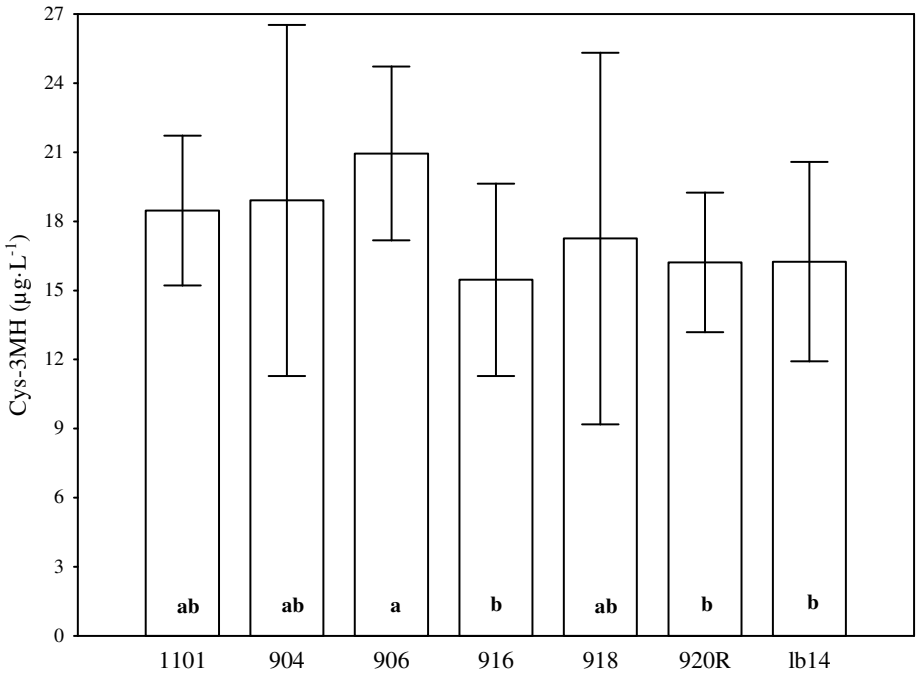


Figure 4. 3-S-cysteinyl mercaptohexan-1-ol (GSH-3MH) distribution measured in clones grown in 4 plots (Mean value and standard deviation; histograms with the same letter do not differ significantly in Fisher's LSD test, $p<0.05$).



SECTION 3.2

Accumulation of cysteine-3-mercaptohexan-1-ol and glutathione-3-mercaptohexan-1-ol during Gewürztraminer ripening in Trentino

Aim of the work

The accumulation of 3-mercaptohexan-1-ol (3MH) precursors during ripening of a few varieties of *Vitis vinifera* has been previously reported (Roland *et al.,* 2010a; Kobayashi *et al.,* 2010; Capone *et al.,* 2011; Cerreti *et al.,* 2016) however the concentration at harvest is highly dependent, not only on grape ripeness (Pineau *et al.,* 2011) but also on the vine and grape environment (Peyrot des Gachons *et al.*, 2005; Lacroux *et al.*, 2008; Kobayashi *et al.*, 2010; Roland *et al.*, 2011; Pinu *et al.,* 2012). Thus the aim of this project was to characterise the accumulation of 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH), 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH), 4-S-glutathionyl-4-methylpentan-2-one (GSH-4MMP) and 4-S-gcysteinyl-4-methylpentan-2-one (Cys-4MMP) in Gewürztraminer juice during grape ripening in 6 vineyards sampling weekly during the last month before harvest. Vineyards were located in a vast area in Trentino (Italy), an alpine region characterised by high differences in terms of orography, soils and microclimatic conditions.

Materials and methods

Chemicals

HPLC grade formic acid, methanol and acetonitrile were provided by Sigma-Aldrich (Milan, Italy).

Samples preparation and Ultra High Performance Liquid Chromatography – Mass Spectrometer (UHPLC-MS) essays

Six plots in different areas, valleys and heights a.s.l. in the Gewürztraminer Trentino DOC production area (fig. 1) were studied at 5 moments during the final month preceding the 2014 harvest (-28, -21, -14, -7 days and harvest). Each sample was composed of 5 Kg of grapes, randomly picked in the vineyard. Once in the experimental winery of the Edmund Mach Foundation, samples were weighed, destemmed (Ares 15, OMAC s.r.l., Corridonia, MC, Italy) and pressed twice (150 atm, 60 seconds) with a R70 hydraulic press (Meccanica Arturo Rossi, Verla di Giovo, TN, Italy), recording the volume obtained and the weight of the marcs. 25 mL of the must obtained was then added to a 25 mL methanol and kept at -20°C until precursor analysis.

The analysis of precursors was performed using a method previously reported (Larcher *et al.,* 2013) with an UHPLC Acquity (Waters Corporation, Milford, MA, USA) coupled to a Xevo TQ MS mass spectrometer (Waters Corporation) injecting 5 µL of previously centrifuged and filtered sample. The column was a UPLC HSS T3 C18 (18 µm x 2.1 mm x 100 mm, Waters), operating at 40 °C with a flow rate of 0.45 mL·min-1 with water (A) and

acetonitrile (B) as eluents, both added with 0.1% formic acid. Mass spectrometry analysis was carried out in positive ion mode. Details about the method are reported in the original paper mentioned above.

Fourier transform infrared spectroscopy (FTIR) measurements

30 mL of juice, previously centrifuged (5000 r.p.m., 5 min) and filtered (25 mm×0.45 μm cellulose acetate syringe cartridge; Alltech, Deerfield, IL, USA), were analysed for °Brix, pH, titratable acidity, tartaric acid, malic acid and potassium with a WineScanTM FT 120 Type 77310 (Foss Electric A/S Hillerød, Denmark), calibrated with the official methods (Organisation Internationale de la Vigne et du Vin 2013).

Statistical analysis

Statistical analysis was performed with Statistica 9.0 software (StatSoft Inc., Tusla, OK, USA), applying the procedures each time declared.

Results

The mean pressing yield (w/w) obtained among plots varied between 64% and 70%. These values are consistent with the ripeness degree of grape samples and similar to those achievable using a correct industrial pressing approach; moreover they testify the apprpriateness of the performance of the lab pressing system. By date of sampling, the first point (-28) showed the lower extraction yield (61%), while the maximum was reached in points -14 and -7 with a 73% and 72% respectively.

The 2014 vintage in Trentino has been strongly characterized by low temperatures and substantial rainfall, that have resulted in a slow ripening, particularily in the last weeks preceding harvest (table 1); and even blocked for some plots, probably due to either dilution phenomena or the inability of the plant to mature further. Moreover, the risk of *Botrytis cinerea* attack and possible gray mold rot appearance led to an early harvest, with grape averaging 4-5 °Brix less than mean values observed in the last 6 years (average data at harvest of the period 2010-2016, excluding 2014; data not shown) for technological ripeness in the same plots.

Table 1 also reports the concentration of the 3MH and 4MMP precursors in juice. Depending on the plot studied, two types of evolution can be observed for GSH-3MH: the first, in Roveré della Luna and in Faedo, where the concentration is maximum in the -7 sample and the second in the other vineyards, where the concentration of precursors mantained a slight but constant increase - more or less accentuated - during the entire maturation period under examination. Cys-3MH did not show a clear pattern and the

concentration in the samples studied seems more aleatory. The mean accumulation trend versus time is shown in figure 2, and confirms the increasing trend previously reported for other cultivars - like Sauvignon Blanc (Roland *et al.,* 2010a; Capone *et al.,* 2011), Koshu (Kobayashi *et al.*, 2012) and Grechetto (Cerreti *et al.,* 2016) - although the sampling point at harvest is statistically indistinguishable from points -14 and -7 as regards the GSH-3MH and Cys-3MH concentration respectively (Anova main effects: plot and harvest dates; Fisher's LSD test, $p < 0.05$).

The concentration of total precursors we found in juice at harvest is consistent with that found for Gewürztraminer by Roland *et al.* (2010b), but not the distribution between the two precursors since these authors reported a higher concentration of Cys-3MH. In this regard, more papers reporting other varieties, showed instead a higher concentration of glutathionylated precursors in juice (Capone *et al.,* 2010; Pinu *et al.,* 2012). The prevalence of GSH-3MH has been also observed by us for GWT (see section 4.3). On a molar base, Cys-3MH is always lower than GSH-3MH with the exception of the -28 point at Calliano where it is almost equal. The molar ratio GSH-3MH/Cys-3MH (fig. 3) is on average initially increasing till point -14 where it reacehes a maximum. These data suggest the consistency of the observation that the unripe grapes can maintain a higher GSH-3MH/Cys-3MH ratio (Kobayashi *et al.,* 2011). In fact, it can be appreciated a decreasing trend near the harvest point. (fig 3). The reason of this evolution could be related to several factors: the enzymatic biogenesis and degradation of these precursors, where Cys-3MH derives from GSH-3MH (Kobayashi *et al.,* 2010), and the higher lipoxygenase activity reported in unripe grapes (Zamora *et al.,* 1985), that produces *(E)*-1-hexenal, a precursor of GSH-3MH (Schneider *et al.,* 2006; Capone and Jeffery, 2011).

We did not find either GSH-4MMP or Cys-4MMP in concentrations over the detection limit (0.5 $\mu g \cdot L^{-1}$) for any sample (table 1). These compounds have been already reported in Gewürztraminer (Roland *et al.,* 2010d), but the concentrations found were very low and did not exceed 0,8 $\mu g \cdot L^{-1}$ and 0,2 $\mu g \cdot L^{-1}$ respectively, very close to their detection limit.

Conclusions

The incremental trend during ripening of 3MH precursors, mainly in the GSH form, has been observed - to the best knowledge for the first time - also in Gewürztraminer, confirming previous findings for other varieties.

The concentration of 4MMP precursors was confirmed to be quite low in Gewürztraminer never exceeding the detection limit.

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Table 1. Plot description and evolution of the chemical composition of Gewürztraminer juices (GSH-3MH: glutathione-3-mercaptohexan-1-ol; Cys-3MH: cysteine-3-mercaptohexan-1-ol; GSH-4MMP: glutathione-4-mercapto-4methilpentan-2-one; Cys-4MMP: cysteine-4-mercapto-4methilpentan-2-one (Cys-3MH)YAN: Yeast asssimilable nitrogen).

Municipality	Plot	Height (m.a.s.l.)	Longitude	Latitude	Sampling date	Days to harvest	GSH-3MH ($ug \cdot L^{-1}$)	Cys-3MH ($ug \cdot L^{-1}$)	GSH-4MMP ($ug \cdot L^{-1}$)	Cys-4MMP ($ug \cdot L^{-1}$)	TSS (°Brix)	pH	Titratable acidity ($g \cdot L^{-1}$)	Tartaric acid ($g \cdot L^{-1}$)	Malic acid ($g \cdot L^{-1}$)	Potassium (mg/L)	YAN (mg/l)
Calliano	Castel Pietra	205	45.92363	11.09427	12/08/2014	28	12.1	7.3	<0,5	<0,5	14.58	2.87	12.5	7.05	8.01	1409	172
					19/08/2014	21	13.9	4.6	<0,5	<0,5	15.14	2.91	11.5	6.64	7.06	1237	177
					26/08/2014	14	57.7	5.5	<0,5	<0,5	16.26	2.99	9.8	6.51	5.29	1474	168
					03/09/2014	7	45.9	5.1	<0,5	<0,5	17.25	3.03	9.1	6.55	4.79	1385	169
					10/09/2014	Harvest	74.6	10.1	<0,5	<0,5	18.28	3.18	7.2	6.43	3.19	1675	126
Cembra	Crosara	525	46.15884	11.21442	26/08/2014	28	18.3	4.8	<0,5	<0,5	14.29	2.86	14.6	7.76	9.11	1517	212
					03/09/2014	21	38.7	6.0	<0,5	<0,5	17.91	3.09	10.6	7.68	5.95	1746	220
					10/09/2014	14	48.2	5.3	<0,5	<0,5	18.00	3.19	8.4	6.95	4.36	1841	155
					18/09/2014	7	53.1	5.3	<0,5	<0,5	18.64	3.24	8.2	6.71	3.88	1716	93
					24/09/2014	Harvest	63.4	20.0	<0,5	<0,5	21.10	3.34	7.8	6.97	4.06	1862	210
Faedo	Centofinestre	118	46.19239	11.14257	12/08/2014	28	12.7	5.0	<0,5	<0,5	15.01	2.79	16.1	7.92	10.71	1788	169
					19/08/2014	21	17.1	5.9	<0,5	<0,5	16.86	2.91	11.8	7.21	6.75	1273	116
					26/08/2014	14	36.7	6.8	<0,5	<0,5	18.01	3.11	10.0	6.73	5.89	1603	162
					03/09/2014	7	45.1	5.4	<0,5	<0,5	18.81	3.15	10.0	6.66	5.87	1662	92
					10/09/2014	Harvest	36.6	5.5	<0,5	<0,5	17.58	3.20	8.2	6.48	4.51	1846	138
Roveré	Winkeli	225	46.24721	11.16304	12/08/2014	28	18.6	6.3	<0,5	<0,5	17.95	3.15	10.7	7.49	7.15	2091	294
					19/08/2014	21	37.6	3.9	<0,5	<0,5	16.89	3.12	11.2	7.40	6.86	1623	294
					26/08/2014	14	72.3	30.5	<0,5	<0,5	17.70	3.23	9.2	6.75	5.68	1890	240
					03/09/2014	7	109.0	14.6	<0,5	<0,5	19.74	3.42	9.0	6.55	5.40	2180	236
					10/09/2014	Harvest	63.9	11.7	<0,5	<0,5	19.63	3.48	6.8	6.20	3.67	2319	188
Tenno	Vandrino	444	45.91976	10.83969	12/08/2014	28	21.3	9.6	<0,5	<0,5	13.43	2.71	18.6	8.65	12.06	1447	183
					19/08/2014	21	16.1	2.5	<0,5	<0,5	14.75	2.83	14.8	7.94	9.21	1365	208
					26/08/2014	14	31.9	2.3	<0,5	<0,5	14.90	2.83	14.4	7.80	8.71	1433	179
					03/09/2014	7	48.1	9.9	<0,5	<0,5	15.94	3.01	12.6	7.64	7.51	1606	241
					10/09/2014	Harvest	56.0	18.5	<0,5	<0,5	18.09	3.11	10.7	7.43	5.60	1967	189
Vezzano	Ciago	472	46.08535	11.00419	12/08/2014	28	31.3	13.5	<0,5	<0,5	11.04	2.72	23.1	8.35	16.89	1230	288
					19/08/2014	21	15.7	5.0	<0,5	<0,5	12.22	2.82	19.1	7.72	13.96	1197	319
					26/08/2014	14	41.9	6.5	<0,5	<0,5	14.96	2.95	13.9	7.07	9.42	1546	279
					03/09/2014	7	32.7	5.7	<0,5	<0,5	15.04	2.95	13.3	7.15	8.48	1437	209
					10/09/2014	Harvest	179.8	34.0	<0,5	<0,5	17.76	3.16	10.5	6.96	6.21	1954	218

Figure 1. Plot location: 1 = Calliano; 2 = Cembra; 3 = Faedo; 4 = Roveré della luna; 5 = Vezzano; 6 = Tenno.

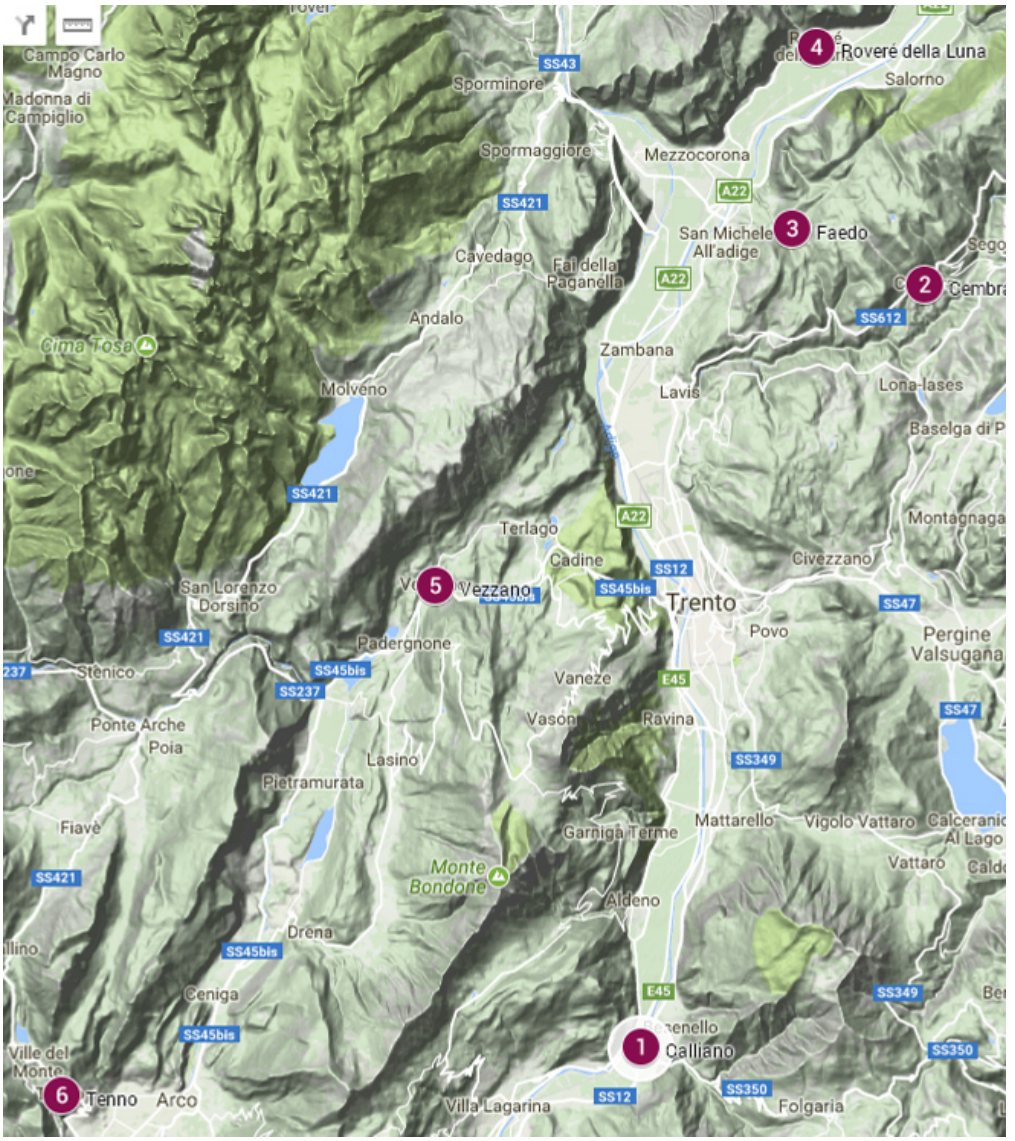


Figure 2. Concentration of 3-mercaptohexan-1-ol precursors in juice from Gewürztraminer grapes processed at increasing ripeness in the last month before technological harvest (Mean value, N.=6, and standard deviation of 6 vineyards; histograms with the same letter do not differ significantly in Fisher's LSD test, $p<0.05$).

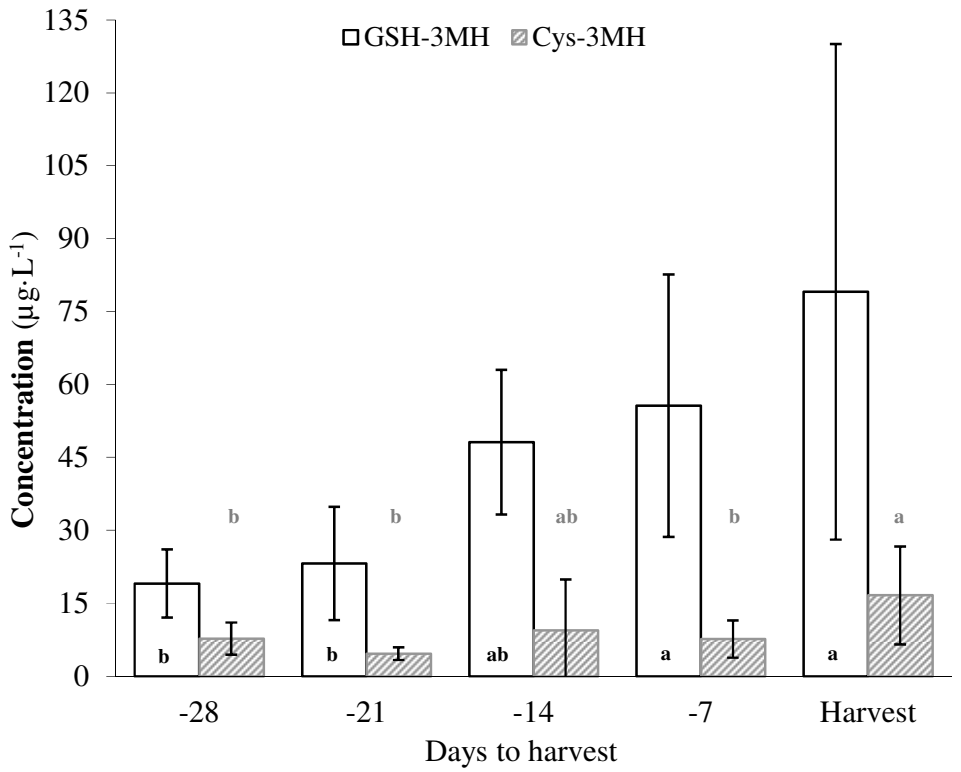
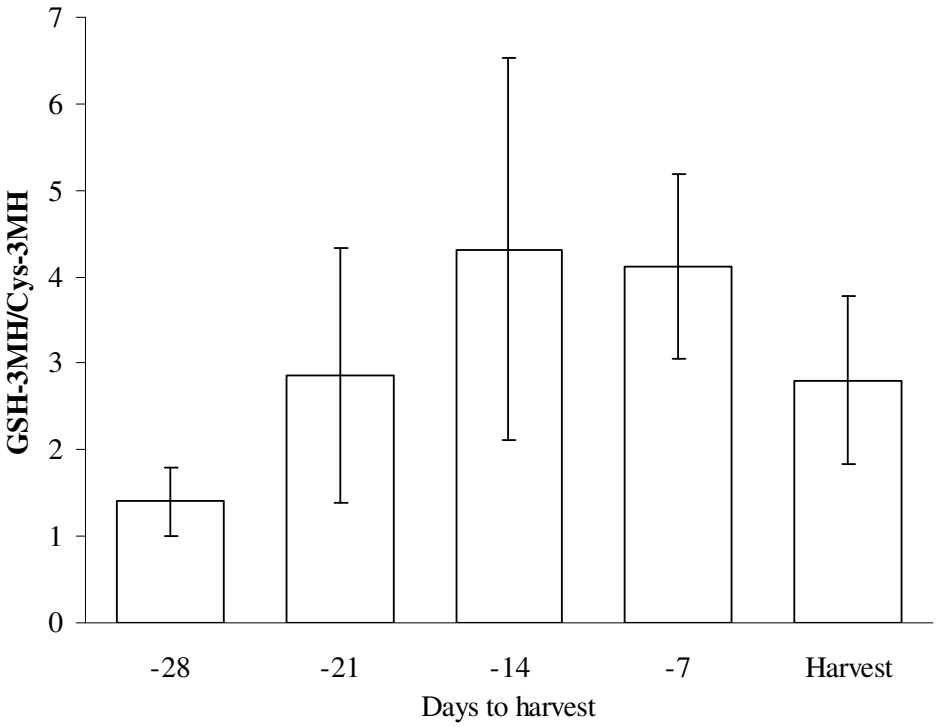


Figure 3. Glutathione-3-mercaptohexan-1-ol (GSH-3MH)/cysteine-3-mercaptohexan-1-ol (Cys-3MH) mean molar ratio versus time to harvest (mean value and standard deviation of 6 vineyards; histograms with the same letter do not differ significantly in Fisher's LSD test, $p<0.05$).



SECTION 3.3

Distribution of the 3-mercaptohexan-1-ol precursors in Gewürztraminer grapes and the effect of the enzymatic maceration on juice content

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Abstract

We quantified the concentration and distribution between pomace and juice of the *S*–glutathionyl and *S*–cysteinyl precursors of 3-mercaptohexan-1-ol (GSH–3MH and Cys–3MH respectively) in 12 Gewürztraminer (GWT) obtained after a semi–industrial pressing. We have compared results with those of 13 Sauvignon Blanc (SB) grapes, processed using the same protocol. Samples, belonging to the whole Trentino growing area (N–E Italy), were collected the same day for each variety during the 2013 harvest. Mean values comparison showed GWT pomace significantly richer in precursors, meanwhile juice situation was flipped over, with an aromatic potential of SB 3 times higher than GWT's. 97% of the total GWT precursors was sited in pomace.

On the basis of these results, during the 2014 harvest we verified at semi-industrial scale the impact of prefermentativa skin cotact on the transfer of 3-mercaptohexan-1-ol precursors from pomace to juice; moreover, the effect of different pectic enzymes used during skin-contact was investigated. At the conditions applied, prefermentativae skin-contact significantly doubled the precursorsors level in juice, while the increase due to the enzyme treatment was not significant.

Introduction

Varietal thiols have a remarkably impact on the aroma of many different fruits and plants - like passion fruit (Engel and Tressl, 1991), blackcurrant (Rigaud *et al.,* 1986) and grapefruit (Demole *et al.,* 1982) – due to their extremely low odour threshold (Darriet *et al*., 1993; Tominaga *et al.,* 1996 and 1998). In wines, these compounds are responsible for the main tropical fruity scents, highlighting 4-methyl-4-mercaptopentan-2-one (4MMP) and 3–mercaptohexyl acetate (3MHA) for box–tree scents (Darriet *et al.,* 1993; Tominaga *et al.,* 1996 & 1998), and 3–mercaptohexan–1–ol (3MH) for passion fruit and grapefruit aromas (Tominaga *et al.,* 1998). Even if the origin of the final content in wine has not yet been completely elucidated, it's clear the liberation during alcoholic fermentation from non–volatile precursors present in grapes (Tominaga *et al.,* 1996; Swiegers and Pretorius, 2007). Some authors have found a positive correlation between the initial concentration of 3MH precursors in juice and the final concentration of the corresponding free derivatives in wine (Murat *et al.,* 2001) but both the biosynthetic origin and the *de novo* formation pathways of the free molecules do not permit this to be conclusive, indicating a strong role of the initial matrix composition and of the fermentation media (Pinu *et al.,* 2012; Larcher *et al.,* 2013).

3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) and 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) are the most important 3MH precursors present in grape (Schneider *et al.,* 2006; Capone and Jefferey, 2011; Roland *et al.,* 2010a). Their occurrence has been deeply studied in Sauvignon Blanc grapes and juice because of the distinctive aroma and economic importance of this cultivar. Distribution analysis of the precursors found that Cys–3MH content is equally distributed between skin and pulp (Peyrot des Gachons *et al.,* 2002) or slightly higher in skins (Murat *et al.,* 2001), however the total concentration of both Cys–3MH and GSH–3MH is higher in skins (Roland *et al.,* 2011). In any case the ratio between GSH–3MH and Cys–3MH in juice is cultivar and site dependent, with a significant higher concentration of GSH–3MH compared to Cys–3MH in juice (Roland *et al.,* 2010c).

Precursor and free thiol concentration in Gewürztraminer juice and wine is barely reported, usually with a small sample size (Dubordieu and Tominaga, 2009; Roland *et al.,* 2010b and c; Concejero *et al.,* 2014), and pomace of this international variety have been stated to posses a high thiolic potential (Román Villegas *et al.,* 2016). Gewürztraminer wines are typically and positively characterized by the powerful rose-like floral scents related to a rather high concentration in varietal compounds belonging to the terpene family – in particular geraniol, nerol, citronellol and rose-oxide –, and by vinylphenols, in particular 4-vinylphenol not exceeding a few hundreds $\mu g \cdot L^{-}$

[1] (Versini, 1985; Marais, 1987; Grando *et al*., 1993; Versini *et al.,* 1999; Koslitz *et al.,* 2008). Recently it has been observed how an augmentation of the grapefruit-like fruity aroma, in response to a higher concentration in thiol precursors, lead to increase in the overall appreciation and aroma typicality of these wines (Roman *et al.,* 2017 *in litteris*). Therefore, the aims of this work are firstly to deepen both the characterization and distribution of the thiol precursors in Gewürztraminer grape in order to exploit the varietal aroma potentiality, and then to investigate the effect of pre-fermentation techniques aimed to increase aroma complexity, always using Sauvignon Blanc as a benchmark.

Materials and methods

Precursors distribution essay

The distribution analysis was carried out with 12 grape batches (10 Kg apiece) of Gewürztraminer (GWT) and 13 of Sauvignon Blanc (SB) harvested on the same day *per* variety in different plots in Trentino (Italy) during 2013. After crushing–destemming (Ares 15, OMAC s.r.l., Corridonia, MC, Italy), must samples were pressed three times (5 min x 3.5 bar; 20L Hydropress, Spiedel GmbH., Ofterdingen, Germany) at the E. Mach Foundation experimental winery (San Michele all'Adige, Italy), recording for each sample the weight of pomace and juice.

Prefermentation skin contact essay

60 Kg of GWT grape, picked from 3 plots during 2014 harvest, were crushed–destemmed and pressed (40L Hydropress, Speidel; Fig. 1). After sampling, the juice was split in 4 aliquots named Control, Maceration (Mac), Enzyme A (EA) and Enzyme B (EB). Mac, EA and EB were added to the pomace, maintaining the original juice/pomace ratio (w/w). The EA and EB fractions were then supplemented with maceration enzymes (Zymopec PXL 09, Perdomini IOC, San Martino Buon Albergo, VR, Italy and Trenolin Bukett, Erbslöh, Geisenheim, Germany respectively). After 24 hours of skin contact at 12°C, Mac, EA and EB were pressed until the achievement of a final extraction rate of 70% (w/w).

Juice and pomace sample preparation

For every juice sample, 25 mL were infused into 25 mL of methanol (≥99,8%, Sigma–Aldrich, Ukraine) whereas pomace samples were prepared grinding 100 g of them with methanol (80 mL) and both samples stored at –20°C until analysis (Larcher *et al.*, 2013; Roman Villegas *et al.*, 2016). Samples were then centrifuged (4.500 r.p.m., 5 min; IEC CL31 Multispeed centrifuge, Thermo Electron Industries S.A.S. Z.I., France) and an aliquot of

2 mL of the supernatant was spiked with both d_3–Cys–3MH and d_3–GSH–3MH (35 µg·L^{-1}) as internal standards. Before the UHPLC–MS injection, samples were filtered through 0.22 µm filters (Millex–GV, Millipore, Carrigtwohill, Ireland).

LC-MS/MS analysis

An LC–MS/MS approach was used for the analysis of the thiol precursors content using an UHPLC Acquity coupled with a Xevo TQ MS mass spectrometer (Waters Corporation, Milford, MA, USA). Sample (5 µL) was injected at 0.45 mL/min onto an Acquity UPLC HSS T3 C18 column (1.8 µm film thickness, 2.1 mm × 100 mm; Waters) operating at 40°C. The features of the method are fully reported in a previous work (Larcher *et al.,* 2013).

Fourier transform infrared spectroscopy (FTIR) measurements and statistical analysis

Juice samples (50 mL) were filtered through a cellulose acetate syringe cartridge (25 mm × 0.45 µm; Alltech, Deerfield, IL, USA) after centrifugation (4.500 r.p.m., 5 min) into a vial for FTIR analysis with FOSS WineScanTM FT 120 Type 77310 (Foss Electric A/S Hillerød, Denmark). The instrument was calibrated for °Brix, pH, total acidity, tartaric acid, malic acid, YAN, and potassium using official methods (Organisation Internationale de la Vigne et du Vin, 2013).

Statistical analysis and figures were executed with STATISTICA v. 9.0 (StatSoft Inc., Tulsa, OK, USA).

Results

Data distribution split by cultivar for GSH–3MH and Cys–3MH are represented in the box plots of figures 2a and b, respectively for juice and pomace. With regards to juice, the Unequal HSD test of Tukey differentiates statistically ($p<0.01$) GWT and SB according to the mean concentration of GSH–3MH (37±19 and 116±37 µg·Kg^{-1} respectively) and Cys–3MH (15±6 and 43±19 µg·Kg^{-1}). In pomace, the mean values of Cys–3MH are also statistically different between varieties (1701±743 and 714±234 µg·Kg^{-1} for GWT and SB respectively), but not those of GSH–3MH (1083±476 and 1463±743 µg·Kg^{-1}). The juice concentration dataset is in accordance to previous works reported both for SB (Capone *et al.*, 2010; Roland *et al.*, 2010c; Pinu *et al.*, 2012) and GWT (Roland *et al.,* 2010c); pomace instead seems to be quite high if compared to what was reported previously in SB skins (Peyrot des Gachons *et al.,* 2002; Murat *et al.,* 2001; Kobayashi *et al.*, 2010; Roland *et al.,* 2011). These latter works were focused on the precursor distribution among skin and pulp, using a lab approach with the removal of

the exocarp from frozen berries that differs significantly from the semi–industrial stamp that characterized the separation procedure used by us. The grapes manipulation during prefermentative winemaking steps can lead to a *de novo* formation of precursors (Maggu *et al.,* 2007; Allen *et al.,* 2011; Roland *et al*., 2011), nonetheless values are similar for both varieties to those reported in our previous work (Román Villegas *et al.,* 2016) where samples came from the same grape growing area and were obtained with a similar procedure. Differences on the content could be also ascribed to the huge variability previously reported not only among plots or years (Pinu *et al.,* 2012), but also on the ripeness stage (Kobayashi *et al.,* 2010; Capone *et al.,* 2011a) or the harvest moment within the day (Kobayashi *et al.,* 2012). In this regard, technological ripeness was comparable among varieties (GWT: 22.16±0.87 °Brix; SB: 21.56±1.42 °Brix) even if it is traditionally known that the potentiality for sugar accumulation in GWT grapes grown in Trentino is greater than in SB. This situation leads us to hypothesize a possible effect of the not complete ripeness of GWT samples impacting the secondary metabolites accumulation.

The pressing yield –which mean values were virtually the same for GWT and SB: 65.1% and 64.7% w/w respectively– has permitted the calculation of the berry total content in precursors, meant as the molar sum of GSH–3MH and Cys–3MH, and the relative distribution in pomace and juice. The calculated parameter for the entire berry was slightly but significantly higher (Unequal HSD Tukey test, $p<0.01$) in GWT compared to SB (3.70±1.40 $\mu mol \cdot Kg^{-1}$ and 2.67±0.89 $\mu mol \cdot Kg^{-1}$ respectively) and the content was mainly located in pomace: 97% in the case of GWT and 88% in SB. Predominance of precursors in this berry fraction agrees with data previously reported (Murat *et al.,* 2001; Roland *et al.,* 2011; Kobayashi *et al.,* 2010) and precisely with SB values, where Peyrot des Gachons *et al.* (2002) has found in skins 8 times the concentration of that found in the respective pulps.

However, on molar base each precursor contributes differently to the berry composition depending on the cultivar studied. Meanly, SB grapes are equally constituted by GSH–3MH and Cys–3MH (53 and 47% respectively), these values deriving from a rather similar distribution among precursors in pomace (54 and 46%) and juice (60 and 40%) that confirms previous works with this cultivar (Peyrot des Gachons *et al.,* 2002). GWT is instead characterized by a higher percentage of cysteinylated precursors, meanly the 73% of total, in accordance with previous observations of Roland *et al.* (2010c), with the same precursor distribution in pomace – respectively 26 and 74% for GSH-3MH and Cys-3MH - but not in juice; the GSH-3MH was higher (58%) than Cys-3MH (42%) in juice, confirming the results found for SB. The molar predominance of cysteinylated precursors in GWT here

observed here is in disagreement with our previous works, where GSH–3MH levels in GWT pomace were largely more important than Cys–3MH (Román Villegas *et al.,* 2016). Differences between precursors could be partially explained by the plant environment since, within the cells, Cys–3MH results from the GSH–3MH enzymatic degradation (Kobayashy *et al.,* 2011) whereas GSH–3MH is more dependent on technological and matrix features for the *de novo* formation in juices (Capone and Jeffery 2011).

For the whole dataset, there is no correlation between the total molar content of precursors in juice and pomace. Plotting them separately by cultivar (fig. 3), significance is still absent, however in SB it appears clear a positive trend correlating the the richer pomace with the higher the aromatic potential of the corresponding juice; meanwhile, GWT's trend seems to drift and no correlation has been found.

Prefermentative skin contact experiment

Results obtained in the above experiment carried out in 2013, showing the high percentage of precursors contained in GWT pomace, pointed out the importance of the extraction processes on the aromatic potential of this cultivar, leading us during the following harvest to explore the consequences of technological approaches potentially enhancing of the precursors extraction. Crushed– destemmed grapes of this second trial showed among batches almost the same initial pressing juice ratio (57.8%, 60.3% and 59.4% w/w respectively for A, B and C batches). The chemical composition of juices reported in table 1 is consistent with the cold and rainy growing season that characterised 2014. That situation forced a premature harvest of unripe grapes due to the high risk of a generalised appearance of *Botrytis cinerea* infection, specifically harmful for aromatic varieties. The concentration of GSH–3MH of the original unmacerated control juices was 40.2, 68.1 and 61.1 $\mu g \cdot L^{-1}$ for A, B and C juices respectively and 13.2, 31.6 and 28.6 $\mu g \cdot L^{-1}$ for Cys–3MH, confirming values published by Roland *et al.,* (2010b) for GWT juices. It's interesting to observe that the technology ripeness of 2013 and 2014 was remarkably different (about 3-4 °Brix lesser in the latter) thus potentially penalising the accumulation of thiols in grapes of 2014 (Kobayashy *et al*., 2012; Roland *et al.,* 2010a). Nevertheless the thiolic concentration was similar for GWT juices in both years. This situation could be partially explained by a different vine N nutritional status (Peyrot des Gachons *et al.,* 2005; Lacroux *et al.,* 2008; Dufourq *et al.,* 2009; Šuklje *et al.,* 2013), the presence of *Botrytis cinerea* (Wakabayashi *et al.,* 2004; Thibon *et al.,* 2009 and 2010) and/or the higher lipoxygenase activity reported in unripe grapes (Zamora *et al.,* 1985) that increases the production of *(E)*–1–hexenal, known as GSH–3MH precursor (Schneider *et al.,* 2006; Roland *et al.,* 2010a, Capone and Jeffery, 2011).

No SO_2 was added to avoid the inhibitory activity on the enzymes added to EA and EB samples. This choice could favour the *de novo* formation of 3MH precursors (Schneider *et al.,* 2006; Capone *et al.,* 2011b; Duhamel *et al.,* 2015) governed by grape enzymes. Moreover, lesser concentrations of precursors in juice treated with SO_2 have been reported (Capone and Jeffery, 2011). These authors highlighted the important effect of time on the enzymatic formation of new GSH-3MH and Cys-3MH in juices. In our case, after 24 hours of skin contact, concentrations remained almost unaltered for every single batch and precursor (GSH–3MH: 40.3, 67.7 and 67.9 $\mu g \cdot L^{-1}$; Cys–3MH: 13.8, 32.7 and 30.7 $\mu g \cdot L^{-1}$ for A, B and C respectively). The desire to mimic an industrial process along with the maceration time chosen imposed a low temperature (12°C) in order to avoid spontaneous fermentations – favoured by lack of SO_2 – and the relative enzymic pool (Schneider *et al.,* 2006). Low temperature however certainly not only slowed down the appearance of new adducts (Capone *et al.,* 2012), but also limited extraction phenomena. All the above reported features justified the relatively long maceration time applied in this semi-industrial scale experiment (Peyrot des Gachon *et al.,* 2002; Maggu *et al.,* 2007; Roland *et al.,* 2011).

All maceration protocols significantly augmented the concentration of both precursors compared to the control juice (fig. 4), almost doubling their concentrations at the conditions studied and perfectly matching with other maceration experiments (Murat *et al.,* 2001; Peyrot des Gachons *et al.,* 2002), even with different experimental conditions. There were instead no significant differences in the concentration of precursors between the macerated thesis, but meanly those supplemented with enzymes slightly augmented concentrations if compared to the non-supplemented macerated: +18% and +10% in EB and EA respectively, for GSH-3MHA and 19% and 6% for Cys-3MH. This lack of significance could be explained either by the long skin contact period that could have flattened differences or, most likely, by the final extraction ratio, set in our experiment at 70%. Pectolitic treatment is known to enhance the pressing performances, extracting faster and more readily juice and skin compounds, thus acheivng higher pressing rates (Nicolini *et al.,* 1996). The fractions obtained at higher operating pressures have been previously demonstrated to be richer in precursors (Peyrot des Gachons *et al.,* 2002; Roland *et al.,* 2011).

Conclusions

The results showed Gewürztraminer pomace to be richer in 3MH precursors compared to Sauvignon Blanc's. These differences inverted in juice obtained according to the experimental conditions, highlighting the importance of prefermentation steps on Gewürztraminer potential aroma, whose precursors are mainly located in the pomace. The ratio among precursors and

comparison with reported data confirm the dependence on technological parameters of the final concentration on GSH-3MH.

The long skin contact maceration time applied almost doubled the final concentration of both GSH-3MH and Cys-3MH in Gewürtraminer juices, but enzymatic treatments did not determine any significant difference when compared to the non-enzymed macerated samples although, at the conditions of the experiment, the mean concentrations of the enzymed samples were slightly higher.

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Table 1. Chemical composition of juices used in the skin contact experiment (TSS: Total soluble solids; YAN: yeast assimilable nitrogen).

Plot	TSS (°Brix)	pH	Titratable acidity ($g \cdot L^{-1}$)	Tartaric acid ($g \cdot L^{-1}$)	Malic acid ($g \cdot L^{-1}$)	Potassium ($g \cdot L^{-1}$)	YAN ($g \cdot L^{-1}$)
A	20.0	3.21	8.0	6.64	4.11	1855	201
B	18.0	3.17	7.8	5.71	3.51	1525	158
C	20.4	3.38	8.8	6.75	4.98	2183	264

Table 2. Chemical composition of control and treated juices (GSH-3MH: 3-S-glutathionyl mercaptohexan-1-ol; Cys-3MH: 3-S-cysteinyl mercaptohexan-1-ol; TSS: Total soluble solids; YAN: yeast assimilable nitrogen).

Plot	Treatment	GSH-3MH ($\mu g \cdot L^{-1}$)	Cys-3MH ($\mu g \cdot L^{-1}$)	TSS (°Brix)	pH	Titratable acidity ($g \cdot L^{-1}$)	Tartaric acid ($g \cdot L^{-1}$)	Malic acid ($g \cdot L^{-1}$)	Potassium ($mg \cdot L^{-1}$)	YAN ($mg \cdot L^{-1}$)
A	Control	40.3	13.8	20.0	3.21	7.9	6.48	4.14	1816	208
	Mac	84.2	37.7	20.0	3.38	6.7	4.52	4.31	1933	241
	EA	119.2	56.7	20.0	3.39	6.7	4.54	4.15	1993	237
	EB	84.5	38.4	20.1	3.40	6.7	4.23	4.63	1940	251
B	Control	67.7	32.7	17.9	3.19	7.7	5.61	3.44	1499	163
	Mac	118.9	50.8	17.8	3.26	6.1	3.93	4.02	1484	180
	EA	132.5	57.5	18.1	3.26	6.1	3.93	4.05	1458	185
	EB	140.8	56.8	18.0	3.25	6.1	3.89	4.15	1495	176
C	Control	67.9	30.7	20.4	3.39	8.7	6.62	4.96	2151	270
	Mac	91.0	41.6	20.3	3.51	7.3	4.80	5.34	2202	295
	EA	95.3	40.4	20.4	3.52	7.3	4.81	5.27	2219	296
	EB	97.5	43.5	20.5	3.52	7.3	4.64	5.56	2211	298

Figure 1. Flowchart of the prefermentation skin-contact essay.

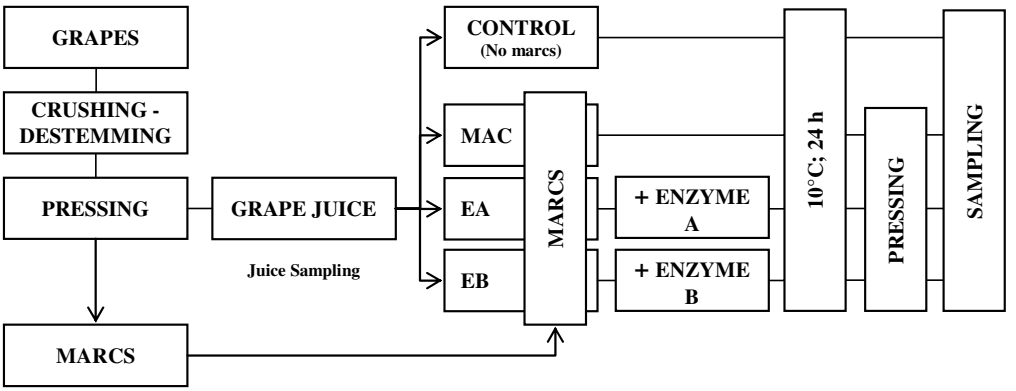


Figure 2a. Concentration distribution of 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) and 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) in Gewürztraminer (GWT, n=12) and Sauvignon Blanc (SB, n=13) juices.

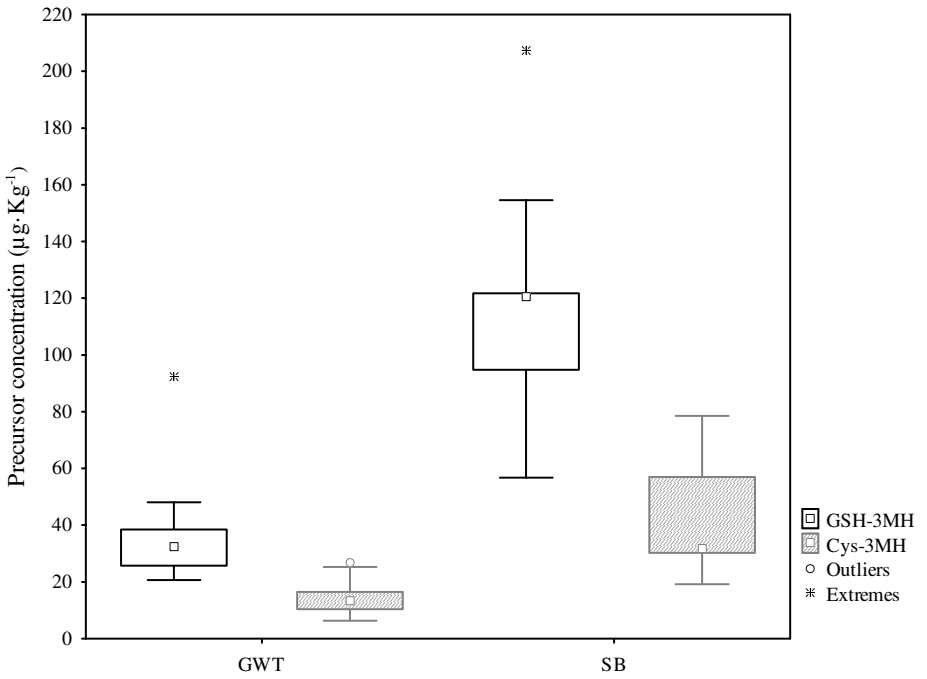


Figure 2b. Concentration distribution of 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) and 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) in Gewürztraminer (GWT, n=12) and Sauvignon Blanc (SB, n=13) pomace.

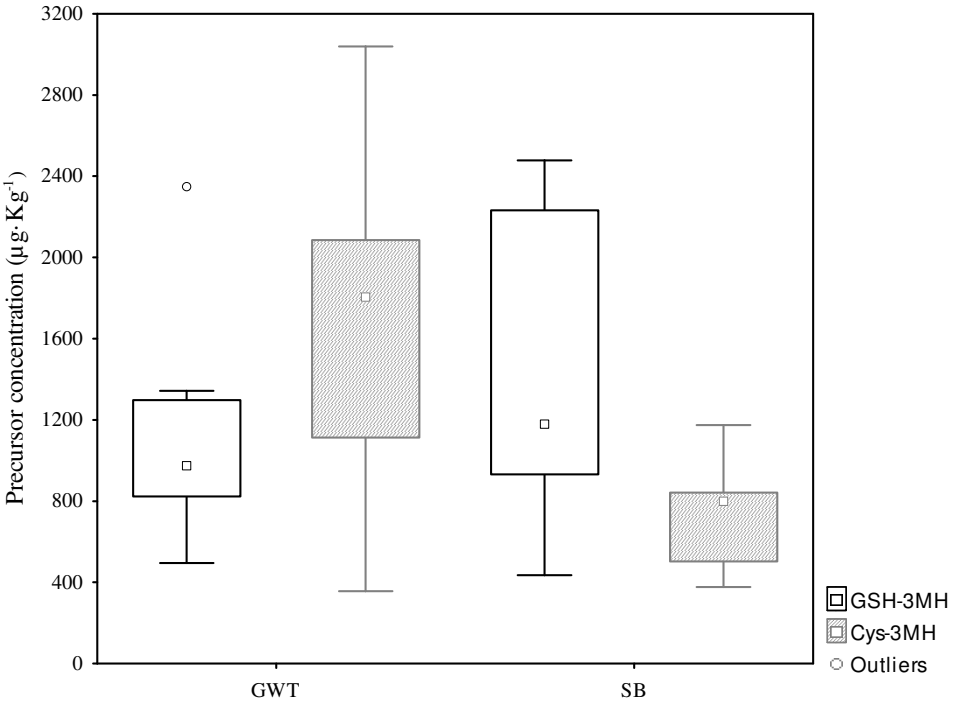


Fig. 3. Correlation of the concentration of the sum of total precursors (3-S-glutathionyl mercaptohexan-1-ol + 3-S-cysteinyl mercaptohexan-1-ol) between pomace and juice split by cultivar.

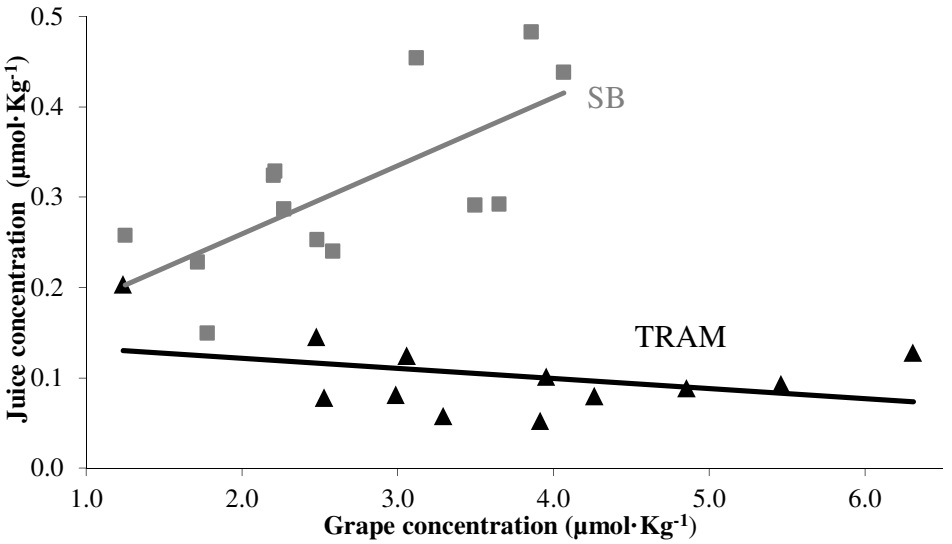
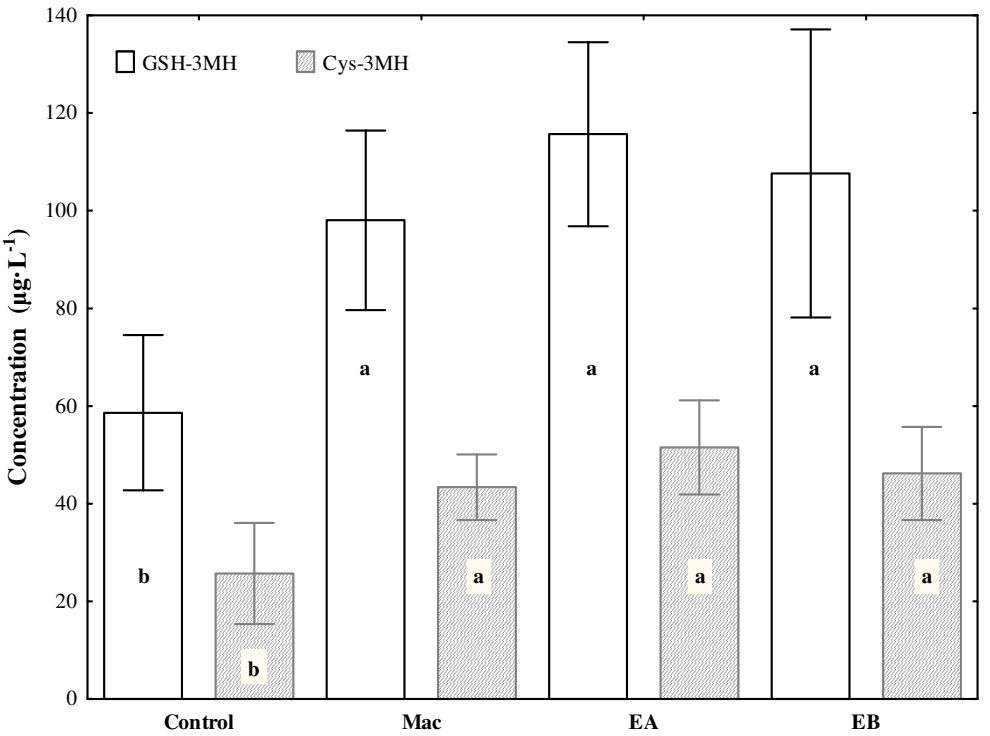


Fig. 4. Distribution of 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) and 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) split by treatment. Mac: skin-contat maceration; EA and EB: skin-contat maceration plus enzymes A and B respectively. Different letters represent values statistically differentiated (Anova main effects: batch and treatment; Fisher LSD Test, $p<0.05$).



SECTION 4. INVESTIGATIONS CONCERNING PREFERMENTATION STEPS

4.1. Influence of oxygen availability during skin-contact maceration on the formation of precursors of 3-mercaptohexan-1-ol in Müller-Thurgau and Sauvignon Blanc grapes

4.2. Extraction of thiol precursors during Gewürztraminer industrial pressing

4.3. Clarifying agents and 3-sulphanylhexanol precursors in grape juices

SECTION 4.1

Influence of oxygen availability during skin-contact maceration on the formation of precursors of 3-mercaptohexan-1-ol in Müller-Thurgau and Sauvignon Blanc grapes

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Aim of the work

Prefermentative maceration is frequently used in the winemaking protocols for the extraction of aroma compounds located in grape skins, in particular for aromatic varieties like Gewürztraminer. In the case of S-glutathionylated and S-cysteinylated precursors of 3-mercaptohexan-1-ol, the effect of this technological option has been already reported but the extent of the oxygen availability during this process and its influence on the *de novo* formation of precursors has not yet been studied. To this aim, 32 Sauvignon Blanc - already known for its high content in precursors in grapes - and 19 Müller Thurgau samples, were macerated under oxidative and reductive conditions at lab scale, and the resulting juices were analysed for composition in 3-mercaptohexan-1-ol precursors.

Influence of oxygen availability during skin-contact maceration on the formation of precursors of 3-mercaptohexan-1-ol in Müller-Thurgau and Sauvignon Blanc grapes

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Abstract

Background and Aims: Grape maceration plays an important role in the formation of precursors of 3-mercaptohexan-1-ol (3MH), 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) and 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH), but its contribution is still not well understood. The aim of this paper was to study the effect of oxygen deprivation on the concentration of these 3MH precursors during skin-contact maceration in two grape cultivars.

Methods and Results: Müller-Thurgau ($n = 19$) and Sauvignon Blanc (32) grapes from Trentino (Italy) were hand harvested and processed under the following conditions: reductive (air protected; 80 mg/kg sulfur dioxide, 80 mg/kg L-ascorbic acid, and 200 mg/kg dimethyl dicarbonate) and oxidative (no addition of adjuvants and air contact). After maceration, GSH-3MH and Cys-3MH were analysed by liquid chromatography-mass spectrometry and isotopic dilution.

Conclusions: Both GSH-3MH and Cys-3MH were identified for the first time in Müller-Thurgau juices. Oxidative maceration increased the GSH-3MH concentration in 16 out of 19 Müller-Thurgau and in 23 out of 32 Sauvignon Blanc juices, while Cys-3MH was higher in 13 and 20 juices, respectively, of the two cultivars. Parametrical and non-parametrical statistical tests confirmed that oxidative maceration of Müller-Thurgau increased GSH-3MH concentration significantly ($P < 0.01$).

Significance of the Study: This work highlights the complexity of the mechanisms involved in the potential *de novo* formation of thiol precursors.

Keywords: *3-S-cysteinyl mercaptohexan-1-ol, 3-S-glutathionyl mercaptohexan-1-ol, maceration skin contact, thiol*

Introduction

Some thiols (Figure 1) are an important class of aroma compounds in the grapes and wine from some grape cultivars (Roland et al. 2011). They have an extremely low sensory threshold (Tominaga et al. 1998) and impart strong tropical and citrus sensory notes to wines. Many efforts have been made (Tominaga et al. 1998) to understand the biogenesis of these molecules and how to increase their concentration in grapes and wine.

Their occurrence appears to be related to non-volatile *S*-glutathionylated and *S*-cysteinylated precursors (Tominaga and Dubourdieu 2000, Peyrot des Gachons et al. 2002, Fedrizzi et al. 2009). Their biogenesis is thought to be initiated by the activity of lipoxygenase cleaving fatty acids (Podolyan et al. 2010) and *S*-glutathionyl transferase catalysing the conjugation of reduced glutathione (GSH) with (*E*)-2-hexenal (Kobayashi et al. 2011). Such a mechanism has been hypothesised in other plants and organisms (Wunschmann et al. 2010, Starkenmann and Niclass 2011, Fedrizzi et al. 2012), even though it is still not clear how this mechanism blends in with the other pathways in which GSH is involved (Rennenberg 1982, Alscher 1989, Dixon et al. 2002).

The wine industry not only needs increased knowledge of the *in vivo* biosynthesis (Kobayashi et al. 2011) of these precursors (Figure 2), which are only poorly converted into their free forms (Roland et al. 2011), but also the connection between bound and free forms (Roncoroni et al. 2011, Winter et al. 2011) and on how grape and wine technology can impact these precursors (Maggu et al. 2007, Patel et al. 2010, Allen et al. 2011, Capone and Jeffery 2011).

Allen et al. (2011) and Capone and Jeffery (2011) suggest that machine harvesting, where extended oxidative skin contact occurs, or a higher pressure during grape crushing, could influence the *de novo* synthesis of the thiol precursors. These reports, however, do not clarify the effect of extended skin contact on S-cysteinyl conjugates and the influence of machine and hand harvesting.

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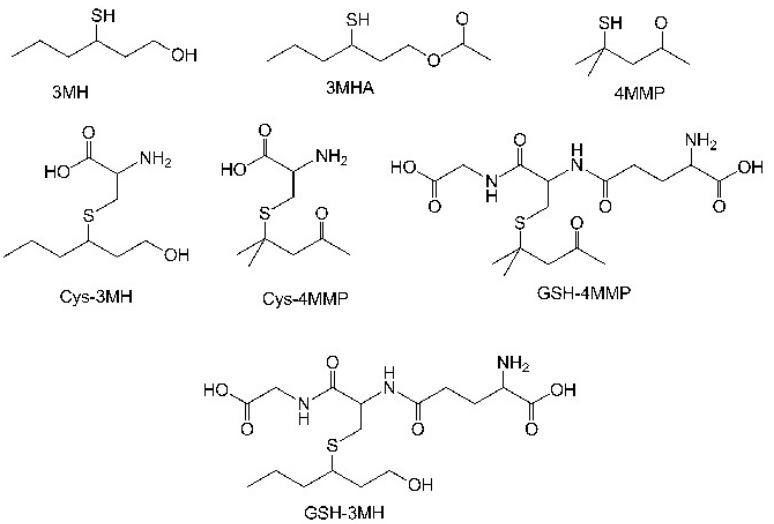

Figure 1. Chemical structures of varietal thiols and their precursors in *Vitis vinifera* L. grapes and wine.

It is acknowledged that maceration influences the liberation of and potentially the *de novo* formation of thiol precursors. Nonetheless, it is still unclear to what extent maceration itself or oxygen availability during maceration influence the occurrence of these thiols and their precursors. In this paper, we have studied carefully the effect of oxygen deprivation during crushing and maceration on the concentration of the 3-mercaptohexan-1-ol (3MH) precursors. To allow the most reliable and reproducible conditions, the comparison was made on a laboratory scale.

Materials and methods

Chemicals

Ultrapure water was produced with an Arium Pro UV DI Ultrapure Water System (Sartorius, Göttingen, Germany). Liquid chromatography-mass spectrometry grade formic acid (FA) and acetonitrile (ACN), ≥ 97% potassium metabisulfite, ≥ 98% L-ascorbic acid, ≥ 98% reduced GSH and anhydrous ≥ 99% dimethyl dicarbonate were supplied by Sigma-Aldrich (Milan, Italy). Buchem B.V. (Apeldoorn, the Netherlands) supplied d_3-(R/S)-3-*S*-cysteinylhexan-1-ol (d_3-Cys-3MH) and d_3-(R/S)-glutathionylhexan-1-ol (d_3-GSH-3MH), along with unlabeled 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) and 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH).

Sample preparation

Good reproducibility and accuracy in the processing of samples were achieved in a controlled, micro-winery environment. During the 2012 harvest, 19 Müller-Thurgau and 32 Sauvignon Blanc grape samples were taken at sugar maturity (Table 1) according to the Trentino Controlled Designation of Origin production protocols. The grapes (about 6 kg, < 20°C) were manually processed to remove any rotten berries and to cut the bunches with scissors into three-berry bunches. Each sample was then divided homogeneously into two subsamples of roughly 2 kg that were separately transferred into 40 × 40 cm polyethylene plastic bags commercially available for vacuum packing of food. The first sample was subjected to reductive skin-contact maceration (RD), while the second was submitted to an oxidative skin-contact maceration (OX). In the RD treatment, sulfur dioxide (160 mg potassium metabisulfite), L-ascorbic acid (160 mg) and dimethyldicarbonate (400 mg) were added to the grape bunches which were sealed in plastic bags after air was removed with the commercial vacuum system VacMaster VP215 (Ary Inc., Kansas City, MO, USA). The sealing was automatically performed by the device during the low-pressure step (air evacuation for about 30 s and bag sealing at a pressure ≤ 0.02 MPa). Within a few minutes, the RD grapes were manually crushed in the sealed bags and held under skin-contact maceration for 24 h at 20°C in the sealed bags. The OX grapes were crushed in open bags without the addition of any adjuvants and macerated at the same temperature and for the same time as the RD treatment. At the end of the maceration, 5 mL of must was collected from each sample. The must in the sealed bags was sampled by piercing them with a 10-mL syringe with a stainless steel needle, while no particular attention was required to collect the oxidatively prepared samples. The must sample was then rapidly infused in 20 mL methanol that was previously chilled at −20°C and spiked with the internal standards d_3-GSH-3MH and d_3-Cys-3MH, both at 35 µg/L. The samples were then stored at −20°C until analysis. Each pair of RD and OX samples, positioned in a random order but always close each other, was analysed within a month as a single batch. The position of the OX and RD samples was systematically inverted along the analytical sequence, in order to have one half of the RD samples analysed before and one half after the OX samples.

Fourier transform infrared spectroscopy (FTIR) measurements

After maceration, the sample (30 mL) was filtered through a cellulose acetate syringe cartridge (25 mm × 0.45 µm; Alltech, Deerfield, IL, USA) and transferred to a 50-mL vial for FTIR analysis. The instrument was calibrated for °Brix, pH, total acidity, tartaric acid, malic acid and potassium using official methods (Oganisation Internationale de la Vigne et du Vin 2013).

High performance liquid chromatography – mass spectrometry (HPLC-MS) experiments

HPLC-MS conditions. Samples were analysed using an Acquity UPLC system (Waters Corporation, Milford, MA, USA), equipped with a Xevo TQ MS mass spectrometer (Waters Corporation). The column was an Acquity UPLC HSS T3 C18 column, 1.8 µm film thickness, 2.1 mm × 100 mm (Waters Corporation), operated at 40°C. Experimental conditions were

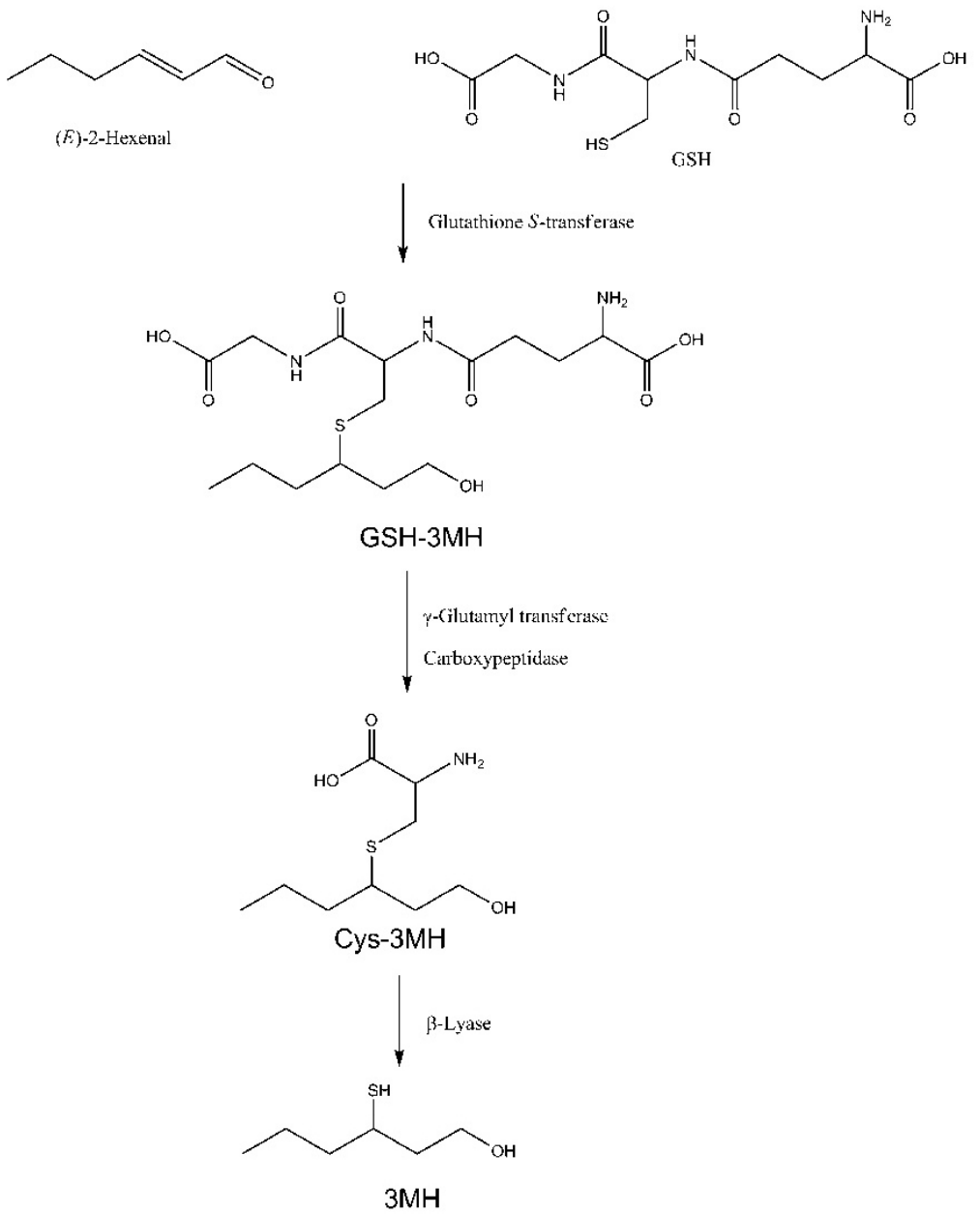

Figure 2. Biosynthetic pathway for the formation of *S*-glutathionylated precursor (GSH-3MH) and the *S*-cysteinylated precursor (Cys-3MH) of 3-mercaptohexan-1-ol (3MH) in the grapevine. 3-Mercaptohexan-1-ol is produced during the alcoholic fermentation (adapted from Kobayashi et al. 2011).

based upon those of Mattivi et al. (2012). The eluents were A (water + 0.1% FA) and B (ACN + 0.1% FA) at 0.45 mL/min. The gradient for solvent B was: 5% for 2 min, to 100% in 5 min, 1 min at 100%, then to 5% in 0.01 min and at 5% per 2 min for column reconditioning. The sample was passed through a 0.22 µm polyvinylidene difluoride syringe filter (Millex-GV; Millipore, Tullagreen, Ireland) and injected (5 µL) using the 'partial-loop with needle overfill' function.

All MS experiments were performed in positive ion mode (capillary voltage, 2.5 kV). Nitrogen (1000 L/h) and argon (0.20 mL/min) were the desolvation and collision gas, respectively. Source and desolvation temperature was set at 150 and 500°C, respectively. Cone voltage potential and collision energy for the product ion experiments were: 16 V and 10 eV for Cys-3MH and 26 V and 12 eV for GSH-3MH, respectively.

Method validation. Linearity was established at five calibration levels, ranging between 0.1 and 30 µg/L, and with three replicated injections. Performance characteristics (evaluated as

Table 1. Chemical composition of Müller-Thurgau (MT) and Sauvignon Blanc (SB) grapes harvested in Trentino (Italy) in 2012.

	Median	Minimum	Maximum	Lower quartile	Upper quartile
Müller-Thurgau (n = 19)					
°Brix	19.27	15.68	20.53	18.36	19.66
pH	3.42	3.18	3.55	3.32	3.46
Total acidity (g/L)	4.50	3.90	6.20	4.20	5.30
Tartaric acid (g/L)	3.19	2.69	3.90	2.90	3.41
Malic acid (g/L)	3.79	2.48	5.08	3.41	4.50
K (mg/L)	1707	922	1981	1476	1830
Assimilable N (mg/L)	128	15	222	73	192
Sauvignon Blanc (n = 32)					
°Brix	21.67	17.85	24.21	20.93	22.45
pH	3.19	3.08	3.54	3.14	3.30
Total acidity (g/L)	6.85	3.20	9.60	5.05	7.25
Tartaric acid (g/L)	3.60	2.84	4.07	3.06	3.78
Malic acid (g/L)	5.25	2.66	7.86	4.32	5.68
K (mg/L)	1402	1193	2163	1305	1584
Assimilable N (mg/L)	97	32	290	79	137

R^2) for the deuterated thiol precursors were always higher than 0.98.

Data analysis

Box plots were prepared and statistical testing undertaken with STATISTICA v. 8.0 (StatSoft Inc., Tusla, OK, USA).

Results and discussion

Juice composition and reduced GSH concentration

The analysis of the Müller-Thurgau and Sauvignon Blanc juices (Table 1) highlights the large variability in composition because of the wide range of altitude (250–750 m above sea level) and of sun exposure in this Alpine region. With Müller-Thurgau grapes, total soluble solids (TSS) was generally low and in agreement with those found in the literature for this cultivar (Falcetti et al. 1990, Nicolini et al. 1999), although juices were obtained by procedures different to that of the current study. Within-sample variability for TSS was about 4.8°Brix in Müller-Thurgau, and about 6.4°Brix for Sauvignon Blanc. Yeast assimilable nitrogen was negatively correlated with °Brix both in Müller-Thurgau ($P < 0.016$) and Sauvignon Blanc ($P < 0.001$).

As expected, GSH concentration (Figure 3) was significantly affected ($P < 0.001$) by the juice treatment, with a concentration practically negligible in the juices obtained via the oxidative protocol (OX) for Müller-Thurgau and below 10 mg/L for Sauvignon Blanc. The protected environment (RD) provided a GSH concentration between 1.64 and 28.7 mg/L and 4.44–45.4 mg/L in the juice of Müller-Thurgau and Sauvignon Blanc, respectively. Such a result confirms the effectiveness of the oxygen deprivation in the RD treatment.

3-S-cysteinyl and 3-S-glutathionyl 3MH

The concentration of the 3MH precursors was evaluated in Müller-Thurgau and Sauvignon Blanc juices after RD and OX treatment (Table 2). A more oxidative environment is expected in machine harvesting, compared with that of hand harvesting, but the literature reports both a higher (Capone and Jeffery 2011) and a lower (Allen et al. 2011) concentration of precursors with machine harvesting. Controlled addition of oxygen

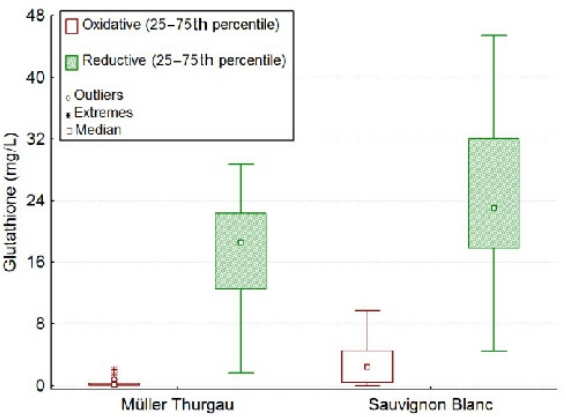

Figure 3. Distribution of glutathione content in Müller-Thurgau and Sauvignon Blanc juices after oxidative and reductive skin-contact maceration.

into micro-fermentations has been reported to increase the concentration of GSH-3MH (Roland et al. 2010a). The experiments appear to suggest a mono-directional pathway (*de novo* conjugation of GSH with the (*E*)-2-hexenal released from degradation of fatty acids), even if they are still insufficient to give a wider explanation of these phenomena. The recently published work of Harsch et al. (2013) sheds new light on the actual effect that the (*E*)-2-hexenal addition could have on the formation of free 3MH.

Figure 4 shows the 3MH precursor concentration in the RD- and OX-treated juices. The concentration range (µg/L for median, min and max, respectively) for Müller-Thurgau/OX was: GSH-3MH = 114, 48, 290; Cys-3MH = 28, 11, 107, and for Müller-Thurgau/RD, GSH-3MH = 80, 26, 267; Cys-3MH = 23, 10, 101. This evidence for Müller-Thurgau (Rhein Riesling × Madeleine Royale) matches the results of Roland et al. (2010a) where these precursors were found in the parent Riesling with a concentration similar to that of Sauvignon Blanc grape juices. The concentration of the precursors of 3MH found

Table 2. Concentration of the precursors of 3-mercaptohexan-1-ol in Müller-Thurgau and Sauvignon Blanc juices prepared according to oxidative/reductive maceration protocols.

Müller-Thurgau #	GSH-3MH (µg/L)		Cys-3MH (µg/L)		Sauvignon Blanc #	GSH-3MH (µg/L)		Cys-3MH (µg/L)	
	Oxidative maceration	Reductive maceration	Oxidative maceration	Reductive maceration		Oxidative maceration	Reductive maceration	Oxidative maceration	Reductive maceration
1	57.2	66.3	12.7	22.6	1	250	268	71.8	80.4
2	47.8	56.7	12.3	15.6	2	172	164	55.5	67.4
3	116	98.1	28.7	29.0	3	225	199	63.8	71.9
4	146	60.5	37.2	20.7	4	179	170	71.1	72.3
5	86.3	71.9	15.4	15.3	5	277	240	81.1	78.5
6	166	123	33.7	36.7	6	230	222	80.4	73.9
7	52.1	43.1	15.1	14.1	7	161	135	63.2	50.9
8	144	132	39.8	37.4	8	168	158	79.5	59.3
9	63.5	52.8	18.0	17.6	9	168	153	77.1	82.9
10	114	70.4	39.3	22.5	10	151	123	72.8	68.1
11	61.2	25.6	11.2	10.4	11	204	177	81.6	71.0
12	87.0	52.0	27.9	16.8	12	192	146	95.2	96.6
13	99.2	121	24.5	27.0	13	146	141	74.9	67.1
14	127	80.2	24.4	24.0	14	173	139	67.5	54.2
15	121	93.5	27.8	25.5	15	228	193	69.9	66.8
16	134	131	28.1	37.6	16	164	142	93.7	84.9
17	128	126	29.2	22.9	17	214	204	84.0	77.9
18	290	267	107	101	18	205	217	77.1	77.7
19	106	91.9	30.2	24.2	19	550	207	250	109
					20	389	246	129	157
					21	577	422	310	272
					22	449	302	145	94.2
					23	330	568	149	273
					24	143	225	29.6	81.8
					25	236	347	149	142
					26	328	118	130	28.3
					27	152	267	83.3	137
					28	154	265	103	142
					29	243	144	97.9	66.2
					30	217	175	119	88.2
					31	244	258	106	117
					32	197	207	138	93.7

Cys-3MH, 3-S-cysteinyl mercaptohexan-1-ol; GSH-3MH, 3-S-glutathionyl mercaptohexan-1-ol.

in Müller-Thurgau appeared to be lower than that measured in Gewürztraminer and Melon B (Roland et al. 2010a,b). This is probably the first time thiol precursor concentration has been measured in Müller-Thurgau.

With Sauvignon Blanc, the GSH-3MH precursor concentration range (µg/L for median, min and max, respectively) was OX = 209, 143, 577 and RD = 201, 118, 568. These values are comparable to those previously reported (Allen et al. 2011, Capone and Jeffery 2011). The Cys-3MH precursor concentration range (µg/L for median, min and max, respectively) was OX = 82, 30, 310 and RD = 79, 28, 273. These values are comparable to those of Capone and Jeffery (2011) but about ten times higher than those of Allen et al. (2011). The mean precursor concentration ratio Cys-3MH/GSH-3MH (the precursor concentration ratio) found for oxidative and reductive conditions was 0.25 and 0.30 for Müller-Thurgau, and 0.43 and 0.44 for Sauvignon Blanc, respectively. Sauvignon Blanc values in both treatments were about ten times lower than that found by Roland et al. (2010b).

The precursor concentration ratio for Sauvignon Blanc and Müller-Thurgau was statistically different according to Tukey's honestly significant different test (HSDT) for unequal number of samples ($P < 0.01$) in the OX and RD treatments. This evidence is in agreement with that already reported for other cultivars (Roland et al. 2010b), where a significant difference was found between Sauvignon Blanc, Gewürztraminer and Riesling.

Tukey's HSDT ($P < 0.05$) indicated that the OX and RD treatments significantly influenced the precursor concentration ratio only with Müller-Thurgau grapes. By comparison with the RD treatment, the OX treatment increased the GSH-3MH and Cys-3MH concentration in 16 and 13 samples, respectively, of the 19 Müller-Thurgau juice samples and in 23 and 20 samples, respectively, of the 32 Sauvignon Blanc juice samples. Table 3 reports, for each grape cultivar, the effect of the OX and RD treatments on juice composition when assessed using parametrical (Tukey's HSDT) and non-parametrical (Wilcoxon matched-pairs test) statistical tests. No significant difference was found for Cys-3MH. A significant difference was found for GSH-3MH when using the non-parametrical approach, with mean values higher for the OX treatment.

Although most samples showed a higher concentration of GSH-3MH with the OX (84% in Müller-Thurgau and 72% in Sauvignon Blanc), some did not. Similar behaviour was observed for Cys-3MH, with the proportion equal to 68% in Müller-Thurgau and 63% in Sauvignon Blanc. No correlation was observed for any of the trials between GSH and either of the thiol precursors, suggesting a more complicated pathway potentially involving other species (Nikolantonaki and Waterhouse 2012). These results are in partial agreement with those reported by Roland et al. (2010a) where a consistent increment for GSH-3MH was observed under oxidative conditions, however, that study took into account only one sample per cultivar.

Even though no quantitative connection between oxidative maceration and machine harvesting has been reported, it is generally recognised that during machine harvesting and transportation an extended oxidative skin contact occurs, often for several hours. The results obtained in our work and the proportion measured appear similar to those found by Allen et al. (2011).

Overall, the statistical findings for the effect of the RD and OX treatments in the Müller-Thurgau and Sauvignon Blanc grape samples strongly suggest that grape cultivar has an influence on these thiol precursors greater than that of oxygen availability during maceration. No correlation between juice composition and the thiol precursors was found for both the reductive and oxidative treatments.

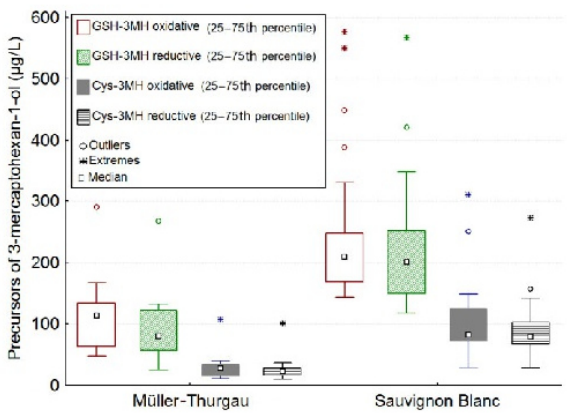

Figure 4. Distribution of the *S*-glutathionylated precursor (GSH-3MH) and the *S*-cysteinylated precursor (Cys-3MH) of 3-mercaptohexan-1-ol in Müller-Thurgau and Sauvignon Blanc juices after oxidative and reductive skin-contact maceration.

Conclusions

This work shows that important thiol precursors in Müller-Thurgau grape juice can occur at a concentration similar to that found in Sauvignon Blanc grape juice. Oxidative maceration

Table 3. Effect of oxidative/reductive maceration protocols on the final concentration of the precursors of 3-mercaptohexan-1-ol in Müller-Thurgau and Sauvignon Blanc juices.

Precursor concentration (µg/L)	Oxidative maceration	Reductive maceration	Wilcoxon matched-pairs test		Tukey HSD test	
	Mean	Mean	*P*-level	Significance	*P*-level	Significance
Müller-Thurgau ($n = 19$)						
GSH-3MH	113	92.9	0.002	**	0.260	n.s.
Cys-3MH	29.6	27.4	0.198	n.s.	0.738	n.s.
Sauvignon Blanc ($n = 32$)						
GSH-3MH	241	217	0.050	*	0.346	n.s.
Cys-3MH	103.1	96.9	0.270	n.s.	0.654	n.s.

*$P < 0.05$; **$P < 0.01$. Cys-3MH, 3-S-cysteinyl mercaptohexan-1-ol; GSH-3MH, 3-S-3-glutathionyl mercaptohexan-1-ol; n.s., not significant.

increased the GSH-3MH content, particularly in Müller-Thurgau, but the concentration of Cys-3MH did not show a clear increase. About a quarter and a third of the juice samples, however, showed a decreasing trend for GSH-3MH and Cys-3MH, respectively. These results confirm that no generalisation can be made on the contribution of grape harvesting and maceration on the *de novo* synthesis of these molecules.

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Conclusions

In addition to the first evidence of the presence of GSH- and Cys-3MH in Müller Thurgau, we have seen that, an oxidative maceration statistically enhances the final concentration of GSH-3MH compared to a reductive maceration, in both Sauvignon Blanc and Müller-Thurgau, meanwhile Cys-3MH did not clearly increase. Even if the oxidative and reductive treatments could be statistically differentiated, GSH-3MH concentration decreased under oxidative conditions in a quarter of the total juice samples and also, in a third of them for Cys-3MH. So, we conclude that a generalisation cannot be easily done regarding the extent of the mechanisms of maceration and *de novo* synthesis on the final concentration of precursors, being this extent highly dependent on the matrix features.

SECTION 4.2

Extraction of thiol precursors during Gewürztraminer industrial pressing

Aim of the work

As stated in section 3.3, Gewürztraminer (GWT) pomace is rich in precursors of 3-mercaptohexan-1-ol (3MH), even more than Sauvignon Blanc's (SB), however this situation does not automatically result in a corresponding richness in juice. The extraction of 3MH precursors during industrial pressing has been already been studied for Sauvignon Blanc (Maggu *et al.,* 2007; Roland *et al.,* 2010; Allen *et al.,* 2011) Melon B. (Roland *et al.,* 2010) and Trebbiano di Lugana (Mattivi *et al.,* 2012), and it is reported that higher pressures result in juices richer in 3MH precursors. The juice fractioning at pressing is routine in the wine industry, aiming for valorising wine through the optimisation of the chemical composition of different fractions. In the case of GWT, the latter fractions, richer in varietal compounds, are also tipically characterised by very elevated pH - high even in free run – and phenols, making juice fractioning still more necessary.

The objective of this section is acquiring records of Gewürztraminer at industrial-scale to compare the appropriateness of the semi-industrial pressing systems reported in previous paragraphs, regarding the concentration and distribution of 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) and 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH).

Materials and methods

Chemicals

HPLC grade formic acid, methanol and acetonitrile were provided by Sigma-Aldrich (Milan, Italy).

Pressing system and sample preparation

Three lots of GWT grapes (A, B and C) were pressed after short prefermentative maceration with industrial pressing systems: Bucher (Bucher Vaslin, Chalonnes-sur-Loire, France), Wilmess (Willmes GmbH, Lorsch, Germany) and Velo (VLS technologies, Zenone degli Ezzelini, Italy). The pressing cycles used by wineries are detailed in table 1. For every fraction, corresponding to a precise working pressure and pomace stir, juice was sampled several times (0.5 L each) and mixed. 25 mL of the mixture was then infused into 25 mL of methanol and kept at -20°C until analysis.

Ultra High Performance Liquid Chromatography –Mass Spectrometer (UHPLC-MS) essays

The analysis of precursors was performed using a method previously reported (Larcher *et al.,* 2013) with an UHPLC Acquity (Waters Corporation, Milford, MA, USA) coupled to a Xevo TQ MS mass spectrometer (Waters

Corporation) injecting 5 µL of sample previously, centrifuged and filtered. The column was a UPLC HSS T3 C18 (18 µm x 2.1 mm x 100 mm, Waters), operating at 40 °C with a flow rate of 0.45 $mL \cdot min^{-1}$ with water (A) and acetonitrile (B) as eluents, both added with 0.1% formic acid. Mass spectrometry analysis was carried out in positive ion mode. details about the method are reported in the original paper above mentioned.

Fourier transform infrared spectroscopy (FTIR) measurements

30 mL of juice, previously centrifuged (5000 r.p.m., 5 min) and filtered (25 mm×0.45 µm cellulose acetate syringe cartridge; Alltech, Deerfield, IL, USA), were analysed for °Brix, pH, titratable acidity, tartaric acid, malic acid and potassium with a WineScanTM FT 120 Type 77310 (Foss Electric A/S Hillerød, Denmark), calibrated with the official methods (Organisation Internationale de la Vigne et du Vin 2013).

Results

The limited number of batches under study along with the elevated number of variables affecting the whole process, do not permit to make univocal conclusions. Figures 1 to 3 report the evolution of the main quality control parameters during the pressing cycles for the Bucher, Willmes and Velo presses respectively. Results show typical values of all parameters for Gewürztraminer during the 2014 harvest, especially cold and rainy. The parameters follow the well-known trend for industrial grape pressing cycles, with an augmentation of pH, potassium, malic acid and yeast assimilable nitrogen (YAN), a slight decrease in titrateble acidity and a nearly unaltered concentration of total soluble solids.

The trend of concentration of each precursor (table 1) also matches with previous studies (Maggu *et al*., 2007), where it has been reported a firm augmentation in the final steps of pressing, corresponding to the higher pressures applied. The concentration in the resulting juices obtained at the lower pressures (and with every pressing system) perfectly matches with the results reported for Gewürztraminer juices in previous sections. At high pressures - indicatively set in 800 mb - the concentrations augmented considerably, but the volume of juice obtained from this pressure, represents approximately 10% or less of the total. The distribution between precursors is also in agreement with previous sections, where GSH-3MH was found to be the main precursor in the juice of every pressing system (fig 4).

Conclusions

The industrial pressing records confirm the appropriateness of semi-industrial and lab pressing system regarding the representativeness on the thiol precursors content in juices. As expected the higher the pressures, the higher

the concentration in juice of thiol precursors. This fractions could be exploited in terms of aroma through specific vinification protocols aimed to maximize the final concentration of 3MH and its acetate in wines, simultaneously, minimizing already known problems of pressed juices (higher pH and polyphenols, green scents etc.). GSH-3MH has been also confirmed as the main precursor in Gewürztraminer juice.

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Figure 1. Evolution of the main quality parameters of juice during the Bucher pressing cycle.

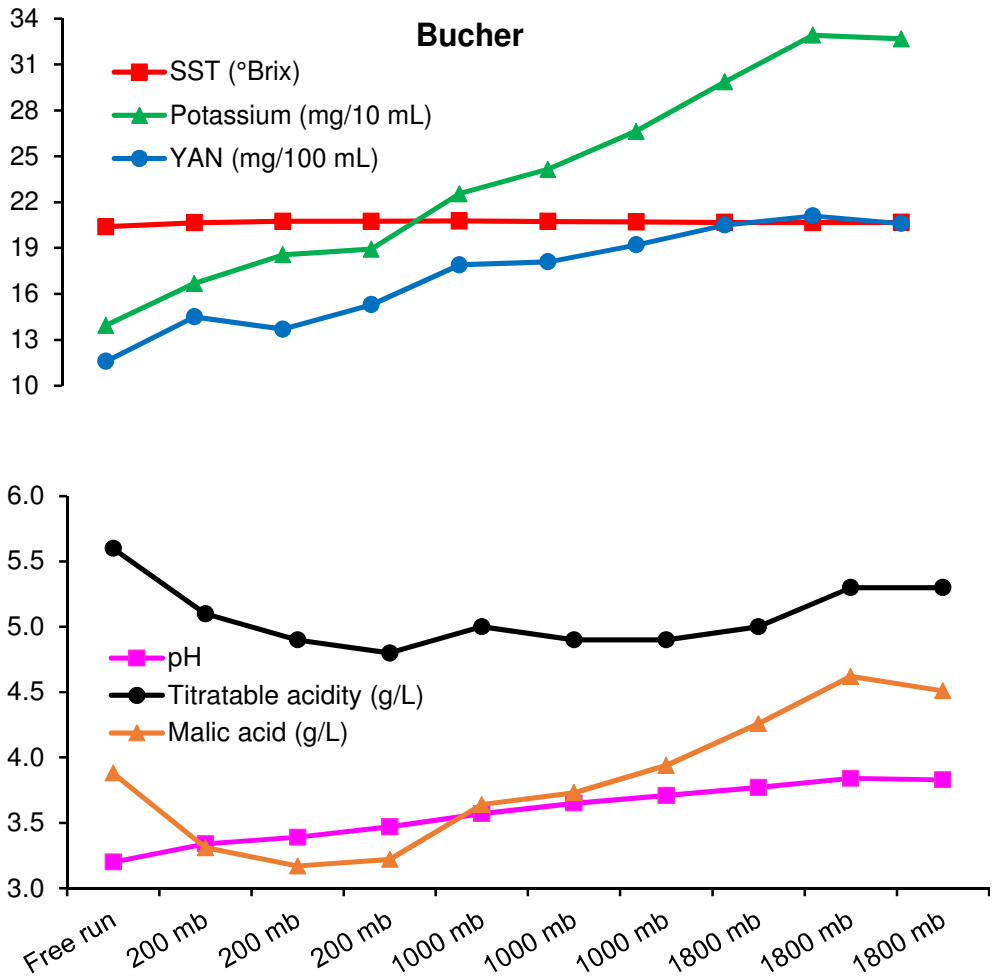

Figure 2. Evolution of the main quality parameters of juice during the Willmes pressing cycle.

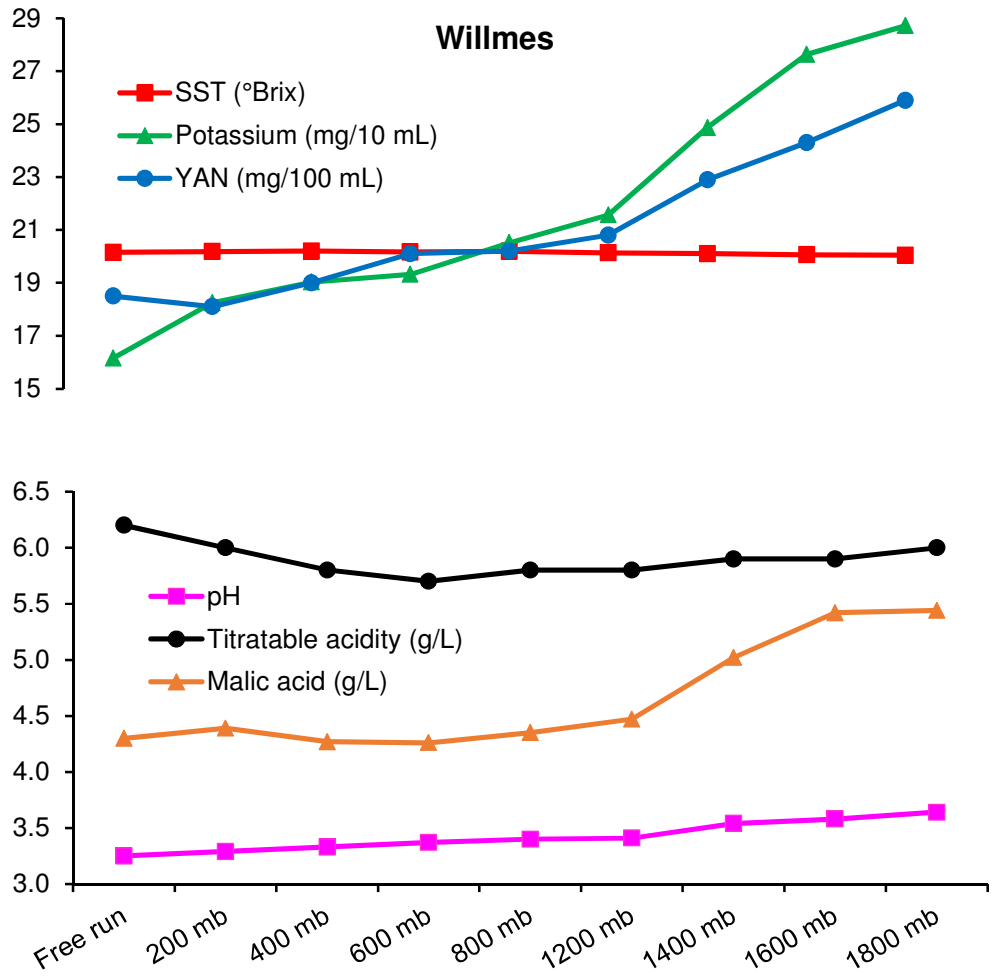

Figure 3. Evolution of the main quality parameters of juice during the Velo pressing cycle.

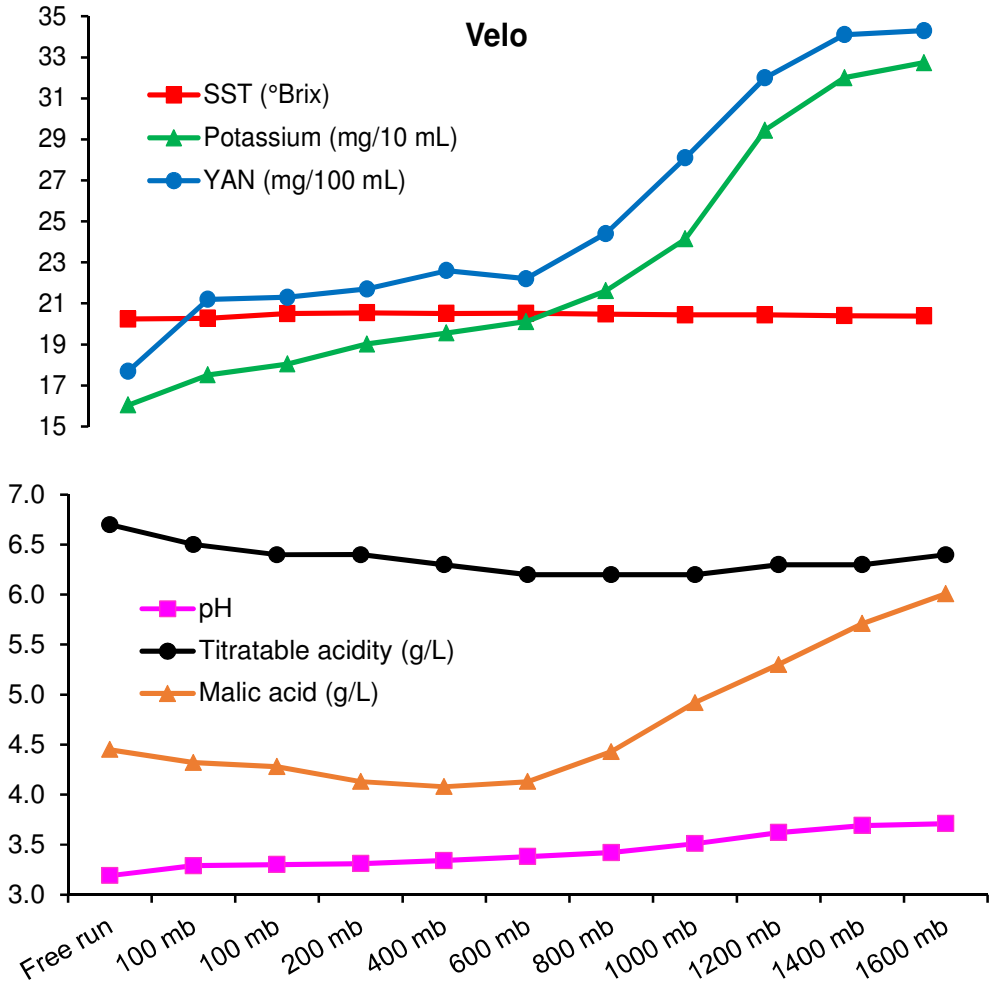

Figure 4. Evolution of precursors with pressure regarding the Bucher, Willmes and Velo pressing systems.

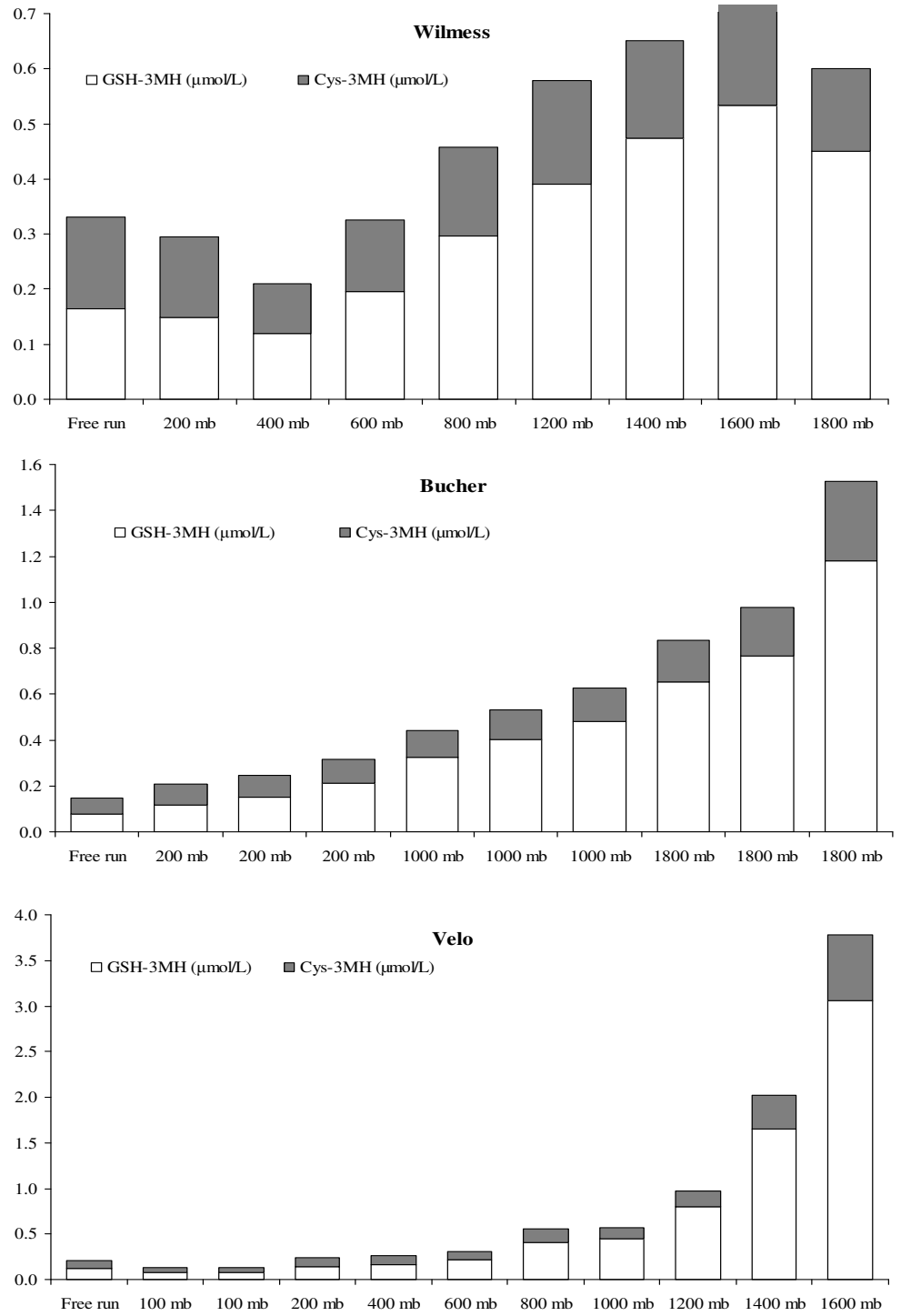

Table 1. Chemical composition of the juice corresponding to the different fractions of the pressing cycles under study (GSH-3MH: 3-S-glutathionyl mercaptohexan-1-ol; Cys-3MH: 3-S-cysteinyl mercaptohexan-1-ol; TSS: Total soluble solids; YAN: yeast assimilable nitrogen).

Batch	Pressing system	Working pressure	Pressure (bar)	GSH-3MH ($\mu g \cdot L^{-1}$)	Cys-3MH ($\mu g \cdot L^{-1}$)	TSS (°Brix)	pH	Titratable acidity ($g \cdot L^{-1}$)	Tartaric acid ($g \cdot L^{-1}$)	Malic acid ($g \cdot L^{-1}$)	Potassium ($mg \cdot L^{-1}$)	YAN ($mg \cdot L^{-1}$)	Potassium (mg/10 mL)
A	Velo	Free run	0	47	20	20.24	3.19	6.7	4.01	4.45	1605	177	16.05
		100 mb	0.1	30	14	20.28	3.29	6.5	4.22	4.32	1752	212	17.52
		100 mb	0.1	30	12	20.5	3.3	6.4	4.12	4.28	1805	213	18.05
		200 mb	0.2	59	20	20.54	3.31	6.4	4.2	4.13	1902	217	19.02
		400 mb	0.4	67	21	20.51	3.34	6.3	4.25	4.08	1956	226	19.56
		600 mb	0.6	87	21	20.52	3.38	6.2	4.3	4.13	2011	222	20.11
		800 mb	0.8	166	33	20.48	3.42	6.2	4.18	4.43	2162	244	21.62
		1000 mb	1	183	27	20.44	3.51	6.2	4.08	4.92	2414	281	24.14
		1200 mb	1.2	323	40	20.44	3.62	6.3	4	5.3	2945	320	29.45
		1400 mb	1.4	672	83	20.4	3.69	6.3	3.8	5.71	3201	341	32.01
		1600 mb	1.6	1245	159	20.39	3.71	6.4	3.75	6.01	3274	343	32.74
B	Willmes	Free run	0	67	37	20.15	3.25	6.2	4.27	4.3	1616	185	16.16
		200 mb	0.2	60	32	20.18	3.29	6	4.14	4.39	1825	181	18.25
		400 mb	0.4	49	20	20.2	3.33	5.8	4.2	4.27	1903	190	19.03
		600 mb	0.6	79	29	20.16	3.37	5.7	4.11	4.26	1932	201	19.32
		800 mb	0.8	121	35	20.19	3.4	5.8	4.06	4.35	2052	202	20.52
		1200 mb	1.2	159	42	20.13	3.41	5.8	4.05	4.47	2157	208	21.57
		1400 mb	1.4	193	39	20.1	3.54	5.9	4.02	5.02	2487	229	24.87
		1600 mb	1.6	217	43	20.06	3.58	5.9	3.89	5.42	2763	243	27.63
		1800 mb	1.8	183	33	20.04	3.64	6	4.01	5.44	2872	259	28.72

Table 1 cont. Chemical composition of the juice corresponding to the different fractions of the pressing cycles under study (GSH-3MH: 3-S-glutathionyl mercaptohexan-1-ol; Cys-3MH: 3-S-cysteinyl mercaptohexan-1-ol; TSS: Total soluble solids; YAN: yeast assimilable nitrogen).

Batch	Pressing system	Working pressure	Pressure (bar)	GSH-3MH ($\mu g \cdot L^{-1}$)	Cys-3MH ($\mu g \cdot L^{-1}$)	TSS (°Brix)	pH	Titratable acidity ($g \cdot L^{-1}$)	Tartaric acid ($g \cdot L^{-1}$)	Malic acid ($g \cdot L^{-1}$)	Potassium ($mg \cdot L^{-1}$)	YAN ($mg \cdot L^{-1}$)
C	Bucher	Free run	0	31	15	20.38	3.2	5.6	3.5	3.88	1395	116
		200 mb	0.2	48	19	20.64	3.34	5.1	3.73	3.31	1668	145
		200 mb	0.2	61	21	20.74	3.39	4.9	3.77	3.17	1855	137
		200 mb	0.2	86	24	20.74	3.47	4.8	3.85	3.22	1892	153
		1000 mb	1	132	26	20.76	3.57	5	3.9	3.64	2256	179
		1000 mb	1	164	29	20.73	3.65	4.9	3.95	3.73	2414	181
		1000 mb	1	195	33	20.7	3.71	4.9	4.07	3.94	2663	192
		1800 mb	1.8	265	40	20.67	3.77	5	3.98	4.26	2986	205
		1800 mb	1.8	312	46	20.66	3.84	5.3	4.06	4.62	3291	211
		1800 mb	1.8	481	76	20.67	3.83	5.3	4.1	4.51	3267	206

SECTION 4.3

Clarifying agents and 3-sulphanylhexanol precursors in grape juices

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Aim of the work

Clarifying agents are extensively used during the pre-fermentation steps of white wine vinification. Treatments with these adjuvants are done to deplete specific compounds such as proteins (bentonite), low molecular weight phenols (PVPP) or, non-specifically, to reduce colour and various undesired compounds (charcoal). Gewürztraminer grapes are characterised not only by a high content in hydroxicinnamic derivatives and proteins, but also by their slightly red coloured skin, so these treatments are usually carried out in juice. Reactivity and efficacy of these treatments often depends on the original composition of grape juices so as to ensure a high variability that validates results, 19 grape clear juices from red and white varieties, spiked with 3-sulphanylhexan-1-ol precursors, were treated with bentonite, PVPP and charcoal. Following this, the devatted juices were sampled and analysed for their concentration in precursors, and compared with the untreated devatted control juices.

SHORT COMMUNICATION

CLARIFYING AGENTS AND 3-SULFANYLHEXANOL PRECURSORS IN GRAPE JUICE

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ABSTRACT

We evaluated the impact of a number of clarifying agents on the concentration of S-3-(hexan-1-ol)-L-cysteine (Cys-3SH) and S-3-(hexan-1-ol)-L-glutathione (GSH-3SH). 19 clear grape juices were spiked with a grape skin tannin rich in Cys-3SH and GSH-3SH. Juices were then treated with Na-bentonite, PVPP or charcoal (1 g/L) and cold settled. The concentration of precursors was measured and compared to the corresponding untreated control juices in the devatted samples. Cys-3SH and GSH-3SH were analysed using UHPLC-MS/MS and accuracy was guaranteed with deuterated internal standards. Only charcoal caused a statistically significant depletion of both precursors, quantitatively limited even at the highest dose adopted. Technologically, the clarifiers used in juice affected the thiol precursors in a marginal manner.

Keywords: bentonite, charcoal, grape juice, polyvinylpolypyrrolidone, varietal thiols

1. INTRODUCTION

S-3-(hexan-1-ol)-l-cysteine (Cys-3SH) and S-3-(hexan-1-ol)-l-glutathione (GSH-3SH) are precursors, present in grapes and/or formed in juice (TOMINAGA and DUBOURDIEU, 2000; PEYROT DES GACHONS *et al.*, 2002; SCHNEIDER *et al.*, 2006; FEDRIZZI *et al.*, 2009; ROLAND *et al.*, 2011a), of 3-sulfanylhexanol (3SH), responsible - together with its acetate - for the tropical and grapefruit-like fruity notes produced during fermentation by some yeast strains having lyase activity (RONCORONI *et al.*, 2011; WINTER *et al.*, 2011). The grape variety, as well as the processing conditions of grape, pomace and juice, are very important in saving/producing a high level of precursors (ROLAND *et al.*, 2011a; CERRETI *et al.*, 2015; ROMÁN VILLEGAS *et al.* 2016). Technologically speaking, for example, 3SH precursors increase with longer skin-contact and stronger pressing conditions (MATTIVI *et al.*, 2012) and GSH-3SH in particular increases when oxidative pre-fermentative maceration is adopted (LARCHER *et al.*, 2013a). Nevertheless, the effects of the main clarifying agents on the content of the precursors cited are little known to date. For this reason, the aim of the experiment reported in this paper was to investigate whether certain common clarifiers used in juice can modify the concentration of Cys-3SH and GSH-3SH.

2. MATERIALS AND METHODS

2.1. Juice preparation

Nineteen lots of must (20 L) were produced from sound white and red grapes coming from different varieties and plots in Trentino (Northern Italy), selected in order to include a wide compositional variability. Grape lots (300 Kg) were destemmed, crushed and pressed (3.5 bar; press mod. UP600, Willmes, Lorsch, Germany) on a semi-industrial scale at the Edmund Mach Foundation experimental winery (San Michele all'Adige, Italy). To ensure a high concentration of thiol precursors, only the juice fraction over 65% w/v yield was used in the experiment (MAGGU *et al.*, 2007; ALLEN *et al.*, 2011; ROLLAND *et al.*, 2011b). Moreover, the juices were indirectly enriched randomly with 500-1000 mg/L of grape skin tannin containing 224.2 mg/kg GSH-3SH and 25.5 mg/kg Cys-3SH, quantified according to LARCHER *et al.*, (2013b), and supplemented with a volume of 15-25% of Sauvignon Blanc juice, a variety well-known for its richness in thiol precursors (CAPONE *et al.*, 2010; LARCHER *et al.*, 2013a). After sulfiting (20 mg/L SO_2), all juices were cold settled (< 20 nephelometric turbidity units), well beyond normal winemaking practice, in order to minimise the effect of solids suspended in the turbid juice. Clear juices were then devatted, divided into 4 fractions of 5 L each and supplemented with activated Na bentonite (Pentagel, 1 g/L; Perdomini-IOC S.p.A., S. Martino Buon Albergo, Italy), charcoal (Eno Anticromos, 1 g/L; Dal Cin S.p.A., Concorezzo, Italy) or polyvinylpolypyrrolidone (PVPP V, 1 g/L; Perdomini-IOC) in comparison with the unspiked fraction respectively. After treatment, all samples were cold settled again for 48h at 4°C.

2.2. Sampling

The settled juice was sampled (25 mL), supplemented with methanol (25 mL, -20°C) and stored at -20°C until analysis. The methanol solution was spiked with d_2-GSH-3SH and d_2-Cys-3SH as labelled internal standards and filtered through a 0.22 μm filter (Millex-GV, Millipore, Ireland) before analysis.

2.3. Chemical analysis

The juice composition was analysed using a WineScan FT 120 Type 77310 (Foss, Hillerød, Denmark), accurately aligned according to the official methods (OIV 2012).
An UPLC Acquity system coupled with a Xevo TQ MS mass spectrometer (Waters Corporation, Milford, USA) was used for LC-MS/MS quantification of thiol precursors. A 5 µL sample was injected into an Acquity UPLC HSS T3 C18 column (1.8 µm film thickness, 2.1 mm × 100 mm; Waters) set with a flow rate of 0.45 mL/min and a temperature of 40°C. MS isotopic dilution analysis was performed in positive ion mode (capillary voltage, 2.5 kV), using argon (0.20 mL/min) and nitrogen (1,000 L/h) as collision and desolvation gas respectively. Other characteristics of the method are specified in LARCHER *et al.* (2013a).

2.4. Statistical analysis

Anova (main effects: juice, clarifier) and Tukey's HSD test were carried out using STATISTICA v. 8.0 (StatSoft Inc., Tulsa, OK).

3. RESULTS AND CONCLUSIONS

The clarifiers were chosen because they are extensively used in winemaking during prefermentation manipulation of white grape must, due to their depletion features in relation to specific classes of compounds (bentonite vs. proteins; PVPP vs. polyphenols) or to their high but non-selective adsorption capacity (charcoal). To our knowledge, there are no reports that specifically link fining agents and thiol precursor content, while their depletion capacity has been previously reported in relation to free and bound primary aromas (MOIO *et al.*, 2004) and other odour active compounds in juice (LAMBRI *et al.*, 2010).
The juices were chemically characterised by their base composition (mean ± st. dev.; min - max) for total soluble solids (21.2±2.0 °Brix; 18.6-26.0), pH (3.23±0.11; 3.01-3.45), titratable acidity (6.63±1.23 g/L; 4.70-10.00), tartaric acid (5.76±0.69 g/L; 4.93-7.62), malic acid (3.25±0.76 g/L; 1.93-4.68), potassium (1348±158 mg/L; 1086-1618). These data highlight the considerable compositional variability used to ensure the robustness of the results, since the grape cultivar and ripeness not only affect the precursor content (KOBAYASHI *et al.*, 2010; CERRETI *et al.*, 2015) but also influence either the composition (Pirie and Mullins, 1977; POCOCK *et al.*, 2000) or the haze (MESQUITA *et al.*, 2001) of the most usual target molecules for these clarifiers and hence the clarifying activity.
The ranges obtained for GSH-3SH (min-max: 240 - 564 µg/L) and Cys-3SH (36,5 - 244 µg/L) in the control juices match the literature (PEÑA GALLEGO *et al.*, 2012; LARCHER *et al.*, 2013a;). Comparison of the results of the corresponding control juices and treated samples showed that bentonite and PVPP had a limited and not statistically significant effect (Table 1) on the concentration of GSH-3SH and Cys-3SH. On the contrary, charcoal treatment significantly reduced ($p<0.05$) the two thiol precursors, however this reduction was limited, being roughly 20% for GSH-3SH and 10% for Cys-3SH.
The significance of these results is not limited to winemaking, but could also be of interest for the grape juice industry, where there is the possibility of using hybrid varieties, resistant to mold diseases and consequently with lower operating costs. Precursors are also present in their juices (LARCHER *et al.*, 2014), and the release of 3SH through specific commercial enzymes could contribute to overall aroma.

Table 1: Thiol precursors in juice in relation to the clarifying agent used. (Values with the same letter are not statistically different in Tukey's HSD test, $p<0.05$; n.s. = non significant).

	GSH-3SH (μg/L)			Cys-3SH (μg/L)		
Treatment	**Mean (n=19)**	**S.D.**	**sign.**	**Mean (n=19)**	**S.D.**	**sign.**
Control	344	73	a	80	53	a
Bentonite (1 g/L)	336	75	a	79	53	a
Charcoal (1 g/L)	276	66	b	73	46	b
PVPP (1 g/L)	344	74	a	80	53	a

In conclusion, of the clarifying agents used in this experiment, only charcoal proved able to significantly reduce 3-sulfanylhexanol precursors in juice. Nevertheless, in the light of the usually lower doses of these products adopted for juice in modern white winemaking, the low conversion ratios of the precursors to the corresponding free thiols (ROLAND *et al.*, 2011a), and the limited percentage changes observed in this experiment, it can be deduced that the clarifying agents used affect the content of thiol precursors in a technologically and sensorially negligible manner, despite the low sensory threshold of the relative derivatives in free and acetate form.

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Conclusions

The high variability of the grape juices used in the experiment resulted in only charcoal slightly diminishing the concentration of precursors (regardless of matrix), and the remaining juices were meanly unaltered after the treatment with bentonite and PVPP. This means that, even when using very high levels of clarifiers, the effect on the aromatic potential - specifically in regard to thiol precurors - is technologically and sensorially negligible. These results are of primary technological importance for Gewrürztraminer, as its varietal characteristics (high protein and phenols content along with coloured skin) frequently make treatment of juice with the above-mentioned clarifying agents necessary.

SECTION 5. INVESTIGATIONS CONCERNING FERMENTATION PHASE

5.1. Prefermentation addition of grape tannin increases the varietal thiols content in wine

5.2. Importance of polyfunctional thiols on semi-industrial Gewürztraminer wines and the correlation to technological treatments

SECTION 5.1

Prefermentation addition of grape tannin increases the varietal thiols content in wine

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Aim of the work

Following our previous works that have quantified the presence of S-gluthationilated and S-cysteinylated precursors in commercial tannins, the present study was aimed to verify the impact of a prefermentative supplementation of tannins rich in varietal thiol precursors on the final content of the free molecules, making use of two cultivars already known to be aromatically characterised by this family of compounds. To this end, 17 Müller Thurgau and 15 Sauvignon Blanc non-devatted grape juices were fermented at lab scale with and without the tannin supplementation at room temperature. Juices were analysed for their precursor content and wines for their free thiol content

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Pre-fermentation addition of grape tannin increases the varietal thiols content in wine

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ABSTRACT

The recent finding that grape tannin may contain significant amount of S-glutathionylated (GSH-3MH) and S-cysteinylated (Cys-3MH) precursors of the varietal thiols 3-mercapto-1-hexanol and 3-mercaptohexyl acetate, characteristic of Sauvignon blanc wines, offers new opportunities for enhancing the tropical aroma in fermented beverages. In this study this new hypothesis was investigated: Müller Thurgau (17 samples) and Sauvignon blanc (15 samples) grapes were fermented with and without addition of a selected grape tannin. As expected, the tannin-added juices were higher in precursors, and they produced wines with increased free thiols. Preliminary informal sensory tests confirmed that in particular the Sauvignon wines produced with the tannin addition were often richer with increased "fruity/green" notes than the corresponding reference wines. This outcome confirms that grape tannin addition prior to fermentation can fortify the level of these compounds.

1. Introduction

3-Mercapto-1-hexanol (3MH) and 3-mercaptohexyl acetate (3MHA) have been intensively studied in the last 20 years (Roland, Schneider, Razungles, & Cavelier, 2011) and are one of the key components of Sauvignon blanc distinctive flavour (Benkwitz et al., 2012; Parr, Green, White, & Sherlock, 2007; Parr, Valentin, Green, & Dacremont, 2010).

Past and current research has been devoted to understanding how different biogenic pathways could alter the transport, conversion and release of different thiol species (Harsch & Gardner, 2013; Roncoroni et al., 2011; Subileau, Schneider, Salmon, & Degryse, 2008a; Subileau, Schneider, Salmon, & Degryse, 2008b; Winter, van der Westhuizen, Higgins, Curtin, & Ugliano, 2011). To the best of our knowledge only two pathways involving either a yeast β-lyase enzyme (Holt et al., 2011; Roncoroni et al., 2011) or a more complex mechanism *via* the addition of H_2S to (*E*)-2-hexenal (Harsch et al., 2013; Schneider, Charrier, Razungles, & Baumes, 2006) have so far been identified to be able to produce free thiols. The recent publication by Harsch et al. (2013) shed new light on a biogenic pathway previously underestimated, increasing our understanding on the *de novo* synthesis of these two molecules.

Furthermore, the effect of viticulture and winemaking on the levels of these molecules (Allen et al., 2011; Chone et al., 2006; Masneuf-Pomarede, Mansour, Murat, Tominaga, & Dubourdieu, 2006; Mattivi et al., 2012; Peyrot des Gachons et al., 2005) has also been studied; contrasting results were obtained, with no clear indication of the effect of maceration, oxidative and reductive grape processing, SO_2 addition, etc., on the final value of free thiols. At this stage still only a small percentage of the total amount of free 3MH and 3MHA is explained by the pathways identified (Roland et al., 2011), leaving much room for uncertainty in the wine industry on how to control and manipulate these molecules.

Plant-derived tannins have been extensively studied since the beginning of the 20th century; they have been classified into three groups according to their chemical structure and physical characteristics: hydrolysable tannins (gallotannins), ellagitannins and flavonoids. Oenological tannins are added at different stages of the winemaking protocol because of the contribution they give to the overall wine profile. Previous studies have focused on the effect of these adjuvants on colour stability, mouthfeel and aroma profile.

Our recent finding that oenological tannins could be an external source of thiol precursors (Larcher, Tonidandel, Nicolini, & Fedrizzi, 2013b) has prompted our attention to the possibility of introducing controlled amounts of S-glutathionylated and S-cysteinylated precursors before fermentation as an additional exogenous source of free thiols.

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In this study we are presenting the first results obtained from this attempt: samples from two international grape varieties (i.e. Müller Thurgau and Sauvignon blanc) were sampled in the vintage 2012 and fermented with and without prior tannin addition. The grape juices were analysed for their content in thiol precursors while 3MH and 3MHA levels were measured in the final wines.

2. Materials and methods

2.1. Chemicals

LC–MS grade formic acid (FA) and acetonitrile (ACN), ⩾97% potassium metabisulfite, ⩾98% L-ascorbic acid, ⩾98% reduced glutathione (GSH), and anhydrous ⩾99% dimethyl carbonate were supplied by Sigma (Milan, Italy). d_3-(R/S)-3-S-Cysteinyl-1-hexanol (d_3-Cys-3MH), and d_3-(R/S)-glutathionylhexan-1-ol (d_3-GSH-3MH) were provided by Buchem B.V. (Apeldoorn, The Netherlands), along with unlabelled Cys-3MH and GSH-3MH.

Ethyl propiolate (ETP) and 1-hexanethiol (1HT) were supplied by Sigma, 3-mercapto-1-hexanol (3MH) by Alfa Aesar (Karlsruhe, Germany) and 3-mercaptohexyl acetate (3MHA) by Endeavour (Daventry, UK). MilliQ water was produced using an Arium Pro UV DI system (Sartorius, Göttingen, Germany).

2.2. Sample preparation

Seventeen Müller-Thurgau (MT) and 15 Sauvignon blanc (SB) samples of ripe grape from Trentino (Italy) were manually harvested in 2012. The compositional profiles of the juices obtained for the two white grape varieties are reported in Table 1.

On the same day, after a short storage at cool temperature (<20 °C; <8 h) and the removal of any rotten berries, the grape bunches were reduced in 3-berry clusters and subdivided into 2 homogeneous subsamples of 2 kg each. The 2 subsamples were then submitted to 2 different winemaking protocols identified as 'REF' and 'TAN'.

According to the REF protocol, to the grape berries (2 kg), 160 mg of L-ascorbic acid and 160 mg of potassium metabisulfite (PMS) were added; the grapes were manually crushed in sealed 40 × 40 cm polyethylene plastic bags and left to macerate in skin-contact for 24 h at 20 °C. The juice was then manually squeezed and transferred under argon protection to a 200-mL glass bottle and inoculated with 50 g/hL VIN13 yeast strain (Anchor Wine Yeast, Stellenbosch, South Africa). Fermentation temperature was kept between 17 °C and 19 °C until sugar consumption (<2 g/L) and no significant differences were found between the 2 protocols for the fermentation completion (Wilcoxon matched pairs test, $p < 0.05$; <20 days). After fermentation, PMS (200 mg/L) was added to the wine and left for 5 days at 2–4 °C until the lees were completely settled. Wine was then transferred under argon to 250-mL glass bottles, filled to the top and closed with metal crown caps equipped with polyethylene sealing gaskets. The samples were stored at 2 °C for 3 months before analysis. The same protocol was also used for the preparation of the TAN samples, with the only exception of an addition of 1.6 g grape tannin (powder form; Epitan, Perdomini, Italy) to the grape berries (2 kg) before crushing. This grape tannin dose lies within the range (from few grams up to 100 g/hL) suggested for wine treatments by Vivas, Vivas De Gaulejac, and Nonier, (2002).

2.3. Instrumental analysis

2.3.1. LC–MS/MS analysis of thiol precursors in musts and tannin

Quantification of thiols precursors was performed using an Waters Acquity UPLC equipped with a Xevo TQ MS mass spectrometer (Waters Corporation, Milford, MA), injecting 5 μL sample on an Acquity UPLC HSS T3 C18 column (1.8 μm film thickness, 2.1 mm × 100 mm; Waters). Flow rate was set at 0.45 mL/min and column temperature at 40 °C. Experimental conditions were based upon Larcher et al., (2013a). Eluent A was a 0.1% formic acid aqueous solution and eluent B was 0.1% formic acid ACN. Analyte separation was obtained using the following gradient for solvent B: 5% for 2 min, raised to 100% in 5 min, then held at 100% for 1 min, and back to 5% in 0.01 min. Column reconditioning was performed holding B at 5% for 2 min before each injection. MS isotopic dilution analysis was performed in positive ion mode (capillary voltage, 2.5 kV) and with argon (0.20 mL/min; gas) and nitrogen (1000 L/h) as collision and desolvation gas, respectively. Cone voltage potential, collision energy and other performance characteristics of the method are given in Table 2.

In order to perform the thiol precursors analysis, 5 mL of must were sampled with a 10-mL syringe and rapidly infused in methanol (20 mL) previously chilled at −20 °C. The solution was spiked with the internal standards d_3-GSH-3MH and d_3-Cys-3MH, both at 35 μg/L, and filtered through 0.22 μm filters (Millex-GV, Millipore, Carrigtwohill, Ireland) before the LC–MS injection. For the musts analyses the samples were diluted 5 times with methanol, while tannin was prepared dissolving 50 mg in 70% methanol aqueous solution, and filtered through 0.22 μm filters (Millex-GV, Millipore), as previously reported (Larcher et al., 2013b).

The measure precision, expressed as R.S.D. and evaluated repeating 7 times the analysis of a wine spiked with 3MH (1 μg/L) and 3MHA (0.1 μg/L), was for both thiols always less than 10%.

2.3.2. GC–MS/MS analysis of free thiols in wine

The thiols extraction and analysis was performed by slightly adapting the method recently published by Herbst-Johnstone, Piano, Duhamel, Barker, and Fedrizzi, (2013). To the wine sample (50 mL), 1-hexanethiol (0.5 mL at 0.25 mg/L), and a 100 mM ethanolic ETP solution (1 mL) were added; the mixture was then stirred for 2 min at 500 rpm. Wine solution pH was adjusted to 10.0 ± 0.1 by means of 10 N NaOH additions. The mixture was stirred for 10 min at 500 rpm, followed by centrifugation for 10 min at 6000 rpm. The sample was loaded onto an SPE C18 cartridge (DSC-18 SPE tube; Supelco, Bellefonte, PA) previously activated

Table 1
Composition of Müller-Thurgau (MT; $N = 17$) and Sauvignon blanc (SB; $N = 15$) musts from Trentino (Italy) grapes.

Cultivar	Brix (°)	pH	Total acidity (as g/L of tartaric acid)	Density at 20 °C	Tartaric acid (g/L)	Malic acid (g/L)	Potassium (mg/L)	Yeast assimilable nitrogen (mg/L)
MT								
min	15.4	3.2	3.9	1.0648	2.7	2.5	0.92	15
median	19.2	3.4	4.7	1.0815	3.2	3.8	1.70	145
Max	20.5	3.6	6.2	1.0879	4.1	5.1	2.07	222
min	17.9	3.1	4.5	1.0766	3.0	3.3	1.19	32
SB								
median	21.5	3.2	7.1	1.0928	3.7	5.1	1.37	106
Max	22.8	3.5	9.6	1.0986	4.1	7.9	1.79	247

Table 2
Characteristics of the LC–MS/MS and GC–MS/MS methods.

	RT (min)	Calibration range (μg/L)	Linearity (R^2)	Ion mode	Cone Voltage (V)	Quantifying trace; CE (eV)	1° qualifying trace; CE (eV)	2° qualifying trace; CE (eV)
Thiols Precursors (LCMS)								
Cys-3MH	3.36 - 3.39[a]	0.1 to 30	0.982	ESI+	16	222>83; (14)	222>205; (10)	222>101; (12)
GSH-3MH	3.72 - 3.76[a]	0.1 to 30	0.989	ESI+	26	408>162; (22)	408>262; (16)	408>116; (34)
d_3-Cys-3MH	3.36 - 3.39[a]			ESI+	16	225>86; (14)	225>208; (10)	225>76; (32)
d_3-GSH-3MH	3.72 - 3.76[a]			ESI+	26	411>162; (22)	411>265; (16)	411>116; (34)
Derivatized free Thiols (GCMS)								
3MH-ETP	18.25	0.01 to 10	0.990	EI+	/	232>127; (10)	232>141; (10)	187>87; (15)
3MHA-ETP	19.05	0.01 to 10	0.987	EI+	/	274>127; (15)	274>141; (10)	229>187; (10)
1HT-ETP (ISTD)	16.45			EI+	/	216>128; (10)	216>142; (15)	171>87; (10)

[a] (S)- and (R)-diasteroisomer, respectively.

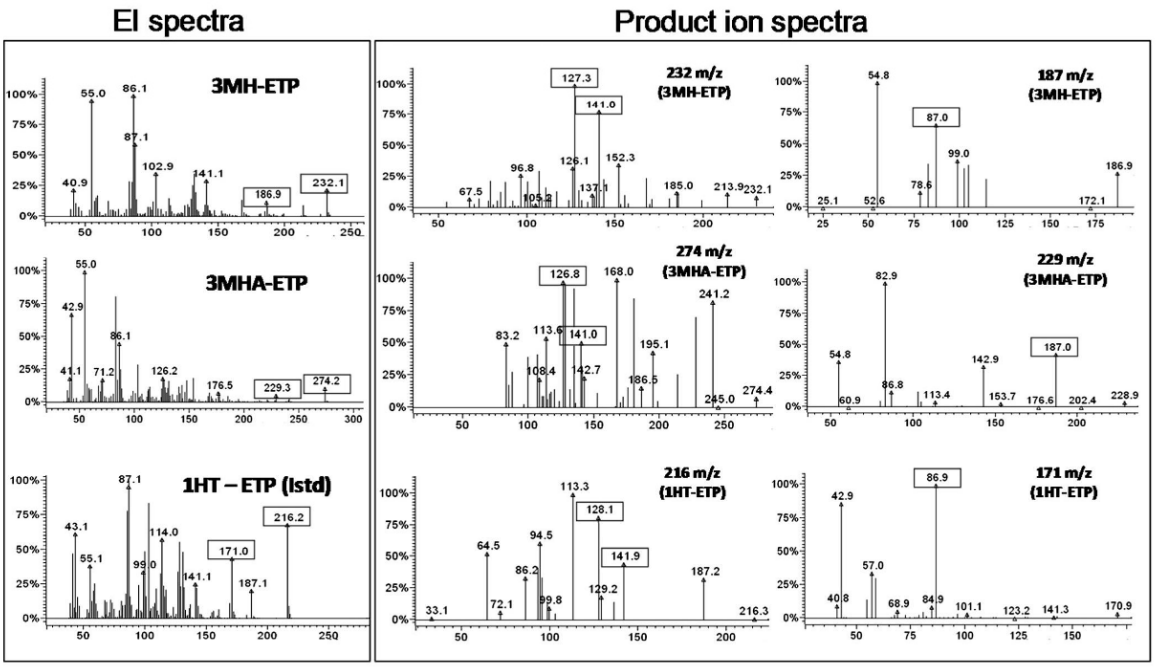

Fig. 1. Electron Impact (EI) spectra of the ethyl propiolate (ETP) adducts of 3-mercapto-1-hexanol (3MH-ETP), 3-mercaptohexyl acetate (3MHA-ETP) and 1-hexanethiol (1HT-ETP) and the relevant product ion spectra.

with methanol (10 mL) and washed with ultrapure water (10 mL). The cartridge was rinsed with water (10 mL) and then the analytes were collected by passing through 10 mL of CH_2Cl_2. The organic phase was dried with Na_2SO_4 and concentrated to about 30 μL under a gentle stream of nitrogen.

Quantification was performed using a Varian 450 gas chromatograph (Middelburg, Netherlands) equipped with Varian 300 triple quadruple mass spectrometer (Walnut Creek, CA). The analytical column was a 30 m × 0.25 mm × 0.25 μm DB5-MS capillary column (J&W, Agilent Technologies Italia, Milan, Italy). The injection volume (splitless mode: 1 min) was 1 μL, and injector temperature was set at 250 °C. The carrier gas (helium) flow was 1 mL/min. The oven temperature program was as follows: 70 °C held for 3.5 min, raised to 210 °C at a rate of 10 °C/min. The oven temperature was then increased to 300 °C at 15 °C/min, and was held at 300 °C for 2 min. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode, at ionisation energy of 70 eV and using argon as the collision gas (1.8 mTorr).

The EI spectra and the MRM experiments were carried out on a model wine solution (12% vol, 6 g/L tartaric acid) added with 3MH, 3MHA and 1HT (2 mg/L) derivatised and extracted according the abovementioned procedure. The EI spectra of 3MH-ETP (MW = 232), 3MHA-ETP (MW = 274) and 1HT-ETP (MW = 216) and the relevant product ion spectra are shown in Fig. 1.

The high abundance of the molecular ion $[M]^+$ and the $[M–OCH_2CH_3]^+$ fragment are observed for all three molecules under study. For this reason these ions were chosen as parent ions in the MS/MS experiments. In order to choose the best transition a daughter ion scan was performed.

3MH-ETP product ion spectrum of molecular ion $[M]^+$ (m/z 232), shows two main fragments at m/z 141 and at m/z 127 belonging to $[M–(H_2O)–(C(O)OCH_2CH_3)]^+$ and $[M–(CH_3OH)–(C(O)OCH_2CH_3)]^{+\cdot}$ respectively. The ion at m/z 187 ($[M–OCH_2CH_3]^+$) mainly generated a fragment at m/z 87 relevant to the species $[HSCHCHCO]^+$.

As far as it concerns the fragmentation of the 3MHA-ETP molecular ion (m/z 274), the fragment ions at m/z 141 and 127 were chosen; these fragments belong $[M–(CH_3C(O)OH)–(C(O)OCH_2CH_3)]^+$ and $[M–(CH_3C(O)OCH_3)–(C(O)OCH_2CH_3)]^+$, respectively. As second qualifying transition was m/z 229 ($[M–OCH_2CH_3]^+$) to m/z 187 (tentatively identified as $[M–OCH_2CH_3–(CH_2CO)]^+$).

Table 3
3-S-cysteinylhexan-1-ol (Cys-3MH) and glutathionylhexan-1-ol (GSH-3MH) content distribution of Müller-Thurgau (MT) and Sauvignon Blanc musts (SB) added (TAN) and not added (REF) of grape tannin. (Min = minimum content; Max = maximum content).

	Treatment	Min.	25th percentile	Median	75th percentile	Max.
MT (17)						
GSH-3MH (μg/L)	REF	26	57	72	98	132
GSH-3MH (μg/L)	TAN	203	235	260	271	324
Cys-3MH (μg/L)	REF	10	17	23	26	38
Cys-3MH (μg/L)	TAN	42	56	62	68	83
SB (15)						
GSH-3MH (μg/L)	REF	118	142	158	190	268
GSH-3MH (μg/L)	TAN	283	325	339	383	424
Cys-3MH (μg/L)	REF	28	67	72	81	97
Cys-3MH (μg/L)	TAN	80	97	110	122	155

Table 4
3-Mercaptohexanol (3MH) and 3-mercaptohexyl acetate (3MHA) content distribution of Müller-Thurgau (MT) and Sauvignon Blanc (SB) wines produced from musts added (TAN) and not added (REF) of grape tannin. (Min = minimum content; Max = maximum content).

	Treatment	Min.	25th percentile	Median	75th percentile	Max.
MT (17)						
3MH (μg/L)	REF	0.11	0.40	0.69	0.79	1.17
3MH (μg/L)	TAN	0.64	1.12	1.67	1.86	3.28
3MHA (μg/L)	REF	<0.01	<0.01	0.01	0.01	0.02
3MHA (μg/L)	TAN	<0.01	0.01	0.02	0.03	0.06
SB (15)						
3MH (μg/L)	REF	0.23	0.29	0.34	0.65	1.32
3MH (μg/L)	TAN	0.46	0.75	1.50	1.97	2.09
3MHA (μg/L)	REF	<0.01	<0.01	0.01	0.02	0.05
3MHA (μg/L)	TAN	0.01	0.01	0.03	0.07	0.11

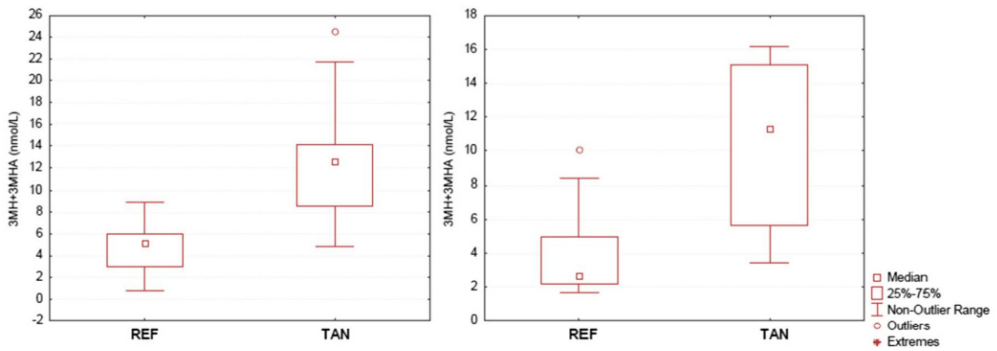

Fig. 2. Distribution of the total molar content of 3-mercapto-1-hexanol (3MH) and 3-mercaptohexyl acetate (3MHA) in Müller-Thurgau (left) and Sauvignon Blanc (right) wines produced from musts added (TAN) and not added (REF) with grape tannin.

Table 2 summarises the analytical parameters adopted and the methods' performance.

2.4. Data analysis

Box plots and statistical tests were carried out using STATISTICA v. 8.0 (StatSoft Inc., Tulsa, OK).

3. Results and discussion

3.1. Thiol precursors in must

Analysis of the thiol precursors in the oenological tannin used in the TAN protocol gave a concentration of 150 mg/kg for GSH-3MH and 36 mg/kg for Cys-3MH. These results are consistent with those found in commercial grape tannins by Larcher et al., (2013b).

Table 3 shows the thiol precursors contents measured in the 32 musts prepared according to the REF (no tannin addition) and the TAN (with pre-fermentative tannin addition) protocols, before the yeast inoculum. The concentration of natural precursors measured in the REF samples for MT was in good agreement with that found by Roland et al., (2010a) in the MT's parent Riesling, while for SB, GSH-3MH levels were similar to those found by Allen et al., (2011) and Capone and Jeffery, (2011). Cys-3MH in SB was also comparable to that found by Capone and Jeffery (2011).

The addition of a consistent amount of enological tannin to the grape samples before their crushing produced, in the relevant musts, an average increase of both GSH-3MH (177 μg/L) and Cys-3MH (38 μg/L), changing significantly (Sign and Wilcoxon Matched Pairs tests; $p < 0.001$) the precursors content in the juices of both varieties. This would support the hypothesis of the effectiveness of grape tannin addition for ameliorating the thiol precursor contents in musts before fermentation.

The differences found between the expected precursors contents (calculated considering the tannin contribution and the natural content of the must) and the concentrations experimentally measured in musts could be related to the difficult dissolution of the solid tannin added to the grapes before their crushing. Additionally, the manual pressing of the grapes, performed in sealed plastic bags for ensuring maximum protection against oxygen, generated slightly different juice yields, that could have an impact on the final precursor concentrations.

3.2. Free thiols in wine

Table 4 reports the contents of free thiols measured in the wines produced according to the TAN and REF protocols. The levels of 3MH and 3MHA in SB are in general agreement with the concentrations reported by others (Capone, Sefton, & Jeffery, 2011; Fedrizzi, Versini, Lavagnini, Nicolini, & Magno, 2007; Roland, Vialaret, Razungles, Rigou, & Schneider, 2010b; Tominaga, Murat, & Dubourdieu, 1998), while those found in MT are comparable to the findings of Fedrizzi et al., (2007).

According to the Tukey's test, the concentration of 3MH in MT ($p < 0.001$) and in SB ($p < 0.001$) as well as that of 3MHA in MT ($p < 0.001$) and in SB ($p < 0.05$) is significantly higher in TAN wines. Also, the sum of the molar concentrations of the two thiols (Fig. 2) was significantly higher (Tukey's test, $p < 0.001$) in the wines obtained from must with added grape tannin (median content of 5.16 in REF *vs.* 12.6 nmol/L in TAN for MT wines; 2.69 *vs.* 11.3 nmol/L for SB). Regarding the potential sensory impact and referring to the threshold given by Tominaga et al., (1998), the observed median differences in sulfur free forms between TAN and REF wines corresponded to 19 units of Odour Activity Value (*OAV* = concentration/sensory threshold) in SB and 16 in MT for 3MH, whilst 4 *OAV* in SB and 2 in MT for 3MHA.

The median molar conversion yield of 3MH precursors (as sum of the S-cysteinylated and S-glutathionylated forms) into free thiols (as sum of 3MA and 3MHA) for MT wines was 1.85% and 1.37% and for SB wines it was 0.36% and 0.85%, for REF and TAN samples, respectively. The conversion yields obtained for the different varieties and treatments are in agreement with those reported in the literature (Subileau, Schneider, Salmon, & Degryse, 2008; Winter et al., 2011) with only a small amount of these two precursors being converted into 3MH and 3MHA.

The free thiols increase observed for the TAN wine appears to be connected to the pre-fermentative addition of oenological tannins to the grape juices. Nonetheless, it must be remembered that other factors could have contributed to these results, which we are not able to measure with this experiment. The precautions taken after the fermentation (the use of argon and screw caps for the bottling) seem to suggest thiols oxidation phenomena were reasonably unlikely to have taken place.

Because of the limited availability of wine, an in-depth sensory investigation was not carried out. Nevertheless, 7 expert judges found that for 10 out of 15 SB and 9 out of 17 MT, the TAN wines were significantly ($p < 0.02$; Roessler, Pangborn, Sidel, & Stone, 1978), more "fruity/green" than the corresponding REF wines through preliminary paired sensory tests. No significant differences were found for the other samples, probably also as a consequence of the presence of sulfur off-flavours caused by the strictly reductive conditions applied in the experiment that did not permit the usual wine devattings, with the inevitable presence of residual wine lees after bottling. This first sensory investigation provides important insight into the potential effect of the pre-fermentative addition of oenological tannins. Further sensory studies will be required to fully elucidate the contribution of these adjuvants to the overall wine sensory profile.

4. Conclusions

This paper presents for the first time the possibility to utilise oenological tannins as a pre-fermentative source of free thiols. The differences due to the tannin treatment seem to be able to contribute to the fruity/green flavour of the finished wines. Conversion yields and absolute concentrations of the free thiols seem to support this hypothesis and are in agreement with those reported in the literature for these precursors.

This evidence could open new avenues to the possibility of modulating thiol concentrations by the manipulation of these adjuvants prior to fermentation.

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Conclusions

This experiment carried out in lab scale, demonstrated that the prefermentation supplementation of juice with grape skin tannin rich in precursors of 3MH enhanced the concentration of these compounds in the juice while the thiol content of the corresponding wines was similarly augmented. These results indicates the possibility to modulate wine thiolic aroma through technological prefermentation options. Sensory tests confirmed the presence of a more fruity/green aroma in wine from juice supplemented with tannin rich in precursors but the analysis cannot be considered exhaustive due to the limited availability of wine for each sample. Therefore, further results from semi-industrial scale experiments are necessary to confirm the technological significance of this approach before a potential industrial scale up.

SECTION 5.2

Importance of polyfunctional thiols on semi-industrial Gewürztraminer wines and the correlation to technological treatments

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Abstract

Thiol compounds responsible for tropical fruit assicated aroma have been extensively studied over the last twenty years. The occurrence of their non-aromatic precursors in grapes and musts is reported largely mainly for the cultivar Sauvignon Blanc. The presence of these thiols as precursors or free molecules in grape, juice and wine has been reported in several different varieties, suggesting that they are more or less ubiquitous both for *Vitis spp.* and interspecific hybrids. The biosynthetic pathways resulting in these compounds are yet to be completely elucidated but, in the meantime, industry needs to improve technological knowledge to better manage winemaking steps to enhance the variety-dependent aroma of wine.

In this work we studied the implications of the use of grape skin tannins - rich and poor in thiol precursors - on the final content of 3-mercaptohexan-1-ol (3MH) and its acetate (3MHA) in wine and the effect in terms of sensory appreciability. The evaluation of 36 vinifications carried out in a semi-industrial scale permitted us to prove that only a tannin originally rich in precursors (*High*), when added to juice at the beginning of fermentation, enhanced both the concentration of precursors in the juice and the final concentration of aromatic thiols in the resultant wine. The 3MH and 3MHA developed as a consequence of the juice supplementation with tannin *High* and increased pleasantness and typicality of Gewürztraminer wines. A later supplementation with tannin *High* at the end of the alcoholic fermentation was sensorally not effective.

Introduction

In the last twenty years, since Darriet and his co-workers (1993) firstly reported the presence of a precursor of 4-methyl-4-mercaptopentan-2-one (4MMP) in the grape juice, the research regarding thiolic compounds has been extensive, mainly focusing on 3-mercaptohexan-1-ol (3MH), its acetate (3MHA) and 4MMP (Tominaga *et al.*, 1998). These molecules are characterised by a extremely low organoleptic threshold and a strong tropical fruity aroma (Tominaga *et al.*, 1998). From the beginning, research has focused on Sauvignon Blanc, probably the *Vitis vinifera* variety most characterised by the above mentioned thiols which significantly contribute to define wine quality perception, but their occurrence has also been observed in a number of other cultivars, both in juices as non volatile precursors (Capone *et al.*, 2010) and in wines as a free molecules (Capone *et al.*, 2011), where they are seemingly ubiquitous (Concejero *et al.*, 2014)

The non aromatic precursors were initially considered the main source of thiols in wines but this did not explain all the resulting molecules after alcoholic fermentation. Other pathways for *de novo* precursors formation (Schneider *et al.*, 2006) and free molecules (Schneider *et al.*, 2006; Harsch *et al.*, 2013; Duhamel *et al.*, 2015) have been proposed but still this cannot explain the final content in wines and further research is necessary to fully understand all mechanisms involved. Numerous studies have been conducted on the evaluation of the aromatic potential of grapes and its dependence on agronomical and climatic features (Peyrot des Gachons *et al.*, 2005; Allen *et al.*, 2011) along with the technological solutions to be applied during harvest (Capone and Jeffery, 2011) and grape processing (Murat *et al.*, 2001; Swiegers *et al.*, 2009; Mattivi *et al.*, 2012; Larcher *et al.*, 2015). The increase of precursors in juice is sometimes positively correlated to the final content of 3MH and 3MHA in wine (Kobayashi *et al.*, 2010). Nevertheless, the relationship between precursors and free derivatives is not completely elucidated (Pinu *et al.,* 2012) and a high variability due to matrix effects (Pinu *et al.,* 2014) has been observed, along with others factors.

The presence of 3MH and 3MHA in Gewürztraminer wines and the content of their precursors in grapes and juice have been previously reported (Tominaga *et al*., 2000; Roland *et al.*, 2010; Concejero *et al.*, 2014). More recent studies showing the high concentration of precursors in Gewürztraminer pomace (Roman Villegas *et al.*, 2016) described the thiolic aromatic potential of this variety, whose characteristic aroma is, up to now, been mostly defined by terpenes - mainly geraniol (Mandery, 1983; Marais, 1987) and *cis* rose oxide (Versini *et al.,* 1999; Koslitz *et al.* 2008) - and, to a lesser extent and exclusively in wine, by vinylphenols (Versini, 1985; Grando *et al.*, 1993). The present study is aimed to set the importance of this “new” thiol-related aroma on the quality

perception of Gewürztraminer wines, considering the role played by thiols in Sauvignon Blanc and confirming - in a real vinification process on semi-industrial scale – the preliminary evidences on the possible use of oenological grape tannins as a potential source of thiol precursors (Larcher *et al.*, 2015).

Materials and methods

Chemicals

Sigma-Aldrich supplied methanol (≥ 99,8%; Ukraine), ethanol (≥ 99,8%; Ukraine), formic acid and acetonitrile (LC-MS grade; Italy), magnesium sulfate (≥97%; USA), sodium chloride (≥99,8%; Denmark), sodium-3-carboxy-3,5-dihydroxy-5-oxo-pentanoate hydrate (≥99%; Germany), trisodium-2-hydroxypropane-1,2,3-tricarboxylate dihydrate (≥99%; Belgium) and 1-hexanethiol (≥95%; Germany). Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one; ≥98%) was from Cayman Chemical Company (Ann Arbor, Michigan USA). 3-mercaptohexan-1-ol (3MH, ≥98%), 3-mercaptohexyl acetate (3MHA; ≥98%) and 4-methyl-4-mercaptopentan-2-ol (4MMPOH; ≥98%) were supplied by (Endeavour, Daventry, UK) and 4-methyl-4-mercaptopentan-2-one (4MMP; ≥98%) by Frutarom Ltd (Reinach, Switzerland). 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) and 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) and the corresponding labelled forms d_3-(R/S)-3-S-cysteinylhexan-1-ol and d_3-(R/S)-glutathionylhexan-1-ol were supplied by Buchem B.V. (Apeldoorn, the Netherlands).

Experimental designs and samples preparation

Three different batches for each one of the two varieties used - Sauvignon Blanc (SB) and Gewürztraminer (GWT) - were harvested in Trentino (Italy) for the experimental design. Each grape batch (500 Kg) was crushed-destemmed (Ares 15, OMAC s.r.l., Corridonia, MC, Italy) and pressed (Up 600, Willmes Anlagentechnik Gmbh, Lorsch, Germany) in the Experimental Winery of E. Mach Foundation at S. Michele all'Adige (TN, Italy). Juice was cold settled (10°C, 36 h) right after supplementation with sulfur dioxide (SO_2, 50 mg·L^{-1}), pectic enzyme (Zymaflore P110 L, 15 µL·L^{-1}; Perdomini, S. Martino Buonalbergo, Italy) and argon blanketing. After settling, the racked juice was sampled and divided into 3 homogeneous fractions: Control, *High* and *Low* corresponding respectively to no treatment and treatments (500 mg·L^{-1}) with a tannin rich and poor in 3MH precursors. Following, treatments were split in 2 tanks separately supplemented with 300 mg·L^{-1} of VIN13 (Anchor Wine Yeasts, Cape Town, South Africa) and VL3 (Laffort, Bourdeaux, France) commercial yeast strains, both known for their ability to give free thiols (Murat *et al.*, 2001; Howell *et al.*, 2004; Swiegers *et al.*, 2009). Alcoholic fermentation was carried at 18-20°C until depletion of sugars, then wines were sulphited (80 mg·L^{-1}) and

cold settled between 0 and 4 °C for 15 days, prior to devatting. All juice and wine processing steps ran under argon blanketing. Samples were kept at 2°C until analysis.

A second experimental design was implemented using the sole control wines fermented with the VIN13 strain, with the purpose of determining the effectiveness of a later supplementation with tannins. At the end of the alcoholic fermentation (sugars < 2-3 $g \cdot L^{-1}$), an aliquot of these wines was sampled and added (after alcoholic fermentation treatment, After AF) with 500 $mg \cdot L^{-1}$ of treatment *High* tannin and kept at 20°C for 7 days. All operations were performed under argon blanketing. Wines were then processed as described above.

Tannin choice

Two grape tannins, whose botanical origin was ascertained using the method proposed by Malacarne *et al.* (2016), were chosen within several commercial products previously analysed for their thiol precursor contents. The choice was based on previous works that reported the different composition of 3MH precursors in tannins (Larcher *et al.*, 2013b) and their ability to transfer precursors into grape juice (Larcher *et al.*, 2015). The tannin selected for the treatment *High* (Enartis Tan Skin; Esseco, Novara, Italy) had a balanced high concentration of 3-S-glutathionyl mercaptohexan-1-ol and 3-S-cysteinyl mercaptohexan-1-ol: 162.4 and 125.0 $mg \cdot Kg^{-1}$ respectively; the corresponding values for treatment *Low* (Tannin Grape; Erbslöh, Geisenheim, Germany) were 0.3 and 0.1 $mg \cdot Kg^{-1}$, respectively. Treatment *Low* was used in order to depurate results from the antioxidant effect linked to tannins, that could either increase reductive scents in the final wines or limit the thiol loss due to oxidation reactions.

Sample preparation

For precursors analysis, 25 mL of juice was introduced into 25 mL of methanol already chilled and kept at -20°C until analysis. For free thiols analysis, 35 mL of wine, 5 mL of ethanol and 100 µg of internal standard (1-hexanethiol), were mixed, supplemented with magnesium sulphate (12 g), sodium chloride (4 g), sodium-3-carboxy-3,5-dihydroxy-5-oxo-pentanoate hydrate (1.5 g) and trisodium-2-hydroxypropane-1,2,3-tricarboxylate dehydrate (3 g) and stirred on a multireax mixer (2500 r.p.m, 10 min; Multi Reax Vortex, Heidolph Instruments GmbH, Schwabach, Germany). The sample was then centrifuged (4.500 r.p.m., 5 min; IEC CL31 Multispeed centrifuge, Thermo Electron Industries S.A.S. Z.I., France) and 2 mL of the supernatant were derivatized with 150 µL of ebselen (Ebs) solution (600 mg/L) and stirred again for 5 min.

Prior to analysis, sample were filtered through a polyvinylidene difluoride syringe filter (0,22 µm; Millex-GV, Millipore, Tullagreen, Ireland).

Ultra High Performance Liquid Chromatography – Mass Spectrometer (UHPLC-MS) essays

An Aquity Ultra *High* Pressure Liquid Chromatography (UHPLC Waters Corporation, Milford, MA, USA) coupled to a Xevo TQ MS mass spectrometer (Waters Corporation) was used for the analysis of 3MH precursors in commercial tannins and juices with the conditions previously reported by Larcher *et al.* (2013b and 2013a respectively). The same equipment was used for the analysis of Ebs-derivatized volatile thiols in wines adapting the method proposed by Vichi and co-workers (Vichi *et al.,* 2015). These adducts were chromatographically separated on a Waters C18 BEH column (100 mm x 2.1 mm x 1.7 µm) at 0.450 $mL \cdot min^{-1}$ flow rate, using a gradient of H_2O + 0.1% formic acid (FA) as eluent A, and methanol + 0.1% FA as eluent B. Initial rate (80% A and 20% B) was maintained for 0.25 min, then increased to 100% B in 7 min, keeping that rate for further 2 min. The electrospray ion source (ESI) worked at 120°C on positive mode (capillary voltage: 0.70 KV) with nitrogen as nebulization gas at 1000 $L \cdot h^{-1}$ and 500 °C. Cone voltage potential was 16 eV for 3MH-Ebs and Mhex-Ebs and 18 eV fo 3MHA-Ebs. Collision energy for all adducts was set to 18 eV.

Method validation

Linearity of the method was checked in a 5-2500 $ng \cdot L^{-1}$ range for 3MH-Ebs and 3MHA-Ebs. Linearity showed R^2 values always over 0.99 for each thiol compound studied. Limit of quantification was set at 1 $ng \cdot L^{-1}$.

Fourier transform infrared spectroscopy (FTIR) measurements

30 mL of juice, previously centrifuged (5000 r.p.m., 5 min) and filtered (25 mm×0.45 µm cellulose acetate syringe cartridge; Alltech, Deerfield, IL, USA), were analysed for °Brix, pH, titratable acidity, tartaric acid, malic acid and potassium with a WineScan™ FT 120 Type 77310 (Foss Electric A/S Hillerød, Denmark), calibrated with the official methods (Organisation Internationale de la Vigne et du Vin 2013). Wines were equally prepared for the analysis of ethanol, pH, glycerol, titratable and volatile acidity, as well as tartaric and malic acids.

Sensory and statistical analysis

Sensory analysis was only carried out on the cold stabilised, devatted and sulfited wines produced with the VIN13 strain. The panel was made up with expert producers of GWT and SB wines in Trentino and belonging to the commission for the ascertaining of the sensory qualification of Trentino Designation of Controlled Origin. Before sensory analysis, judges were trained for the recognition of the grapefruit-like thiol aroma using a neutral Chardonnay singularly spiked or not with 3MH (300 $ng \cdot L^{-1}$), 3MHA (15 $ng \cdot L^{-1}$) and then with both molecules together, maintaining the same concentrations as above. The selection of judges has been done on the basis of their ability to recognise the above sensation applying a duo-trio test, using a GWT spiked with 200 $ng \cdot L^{-1}$ and 10 $ng \cdot L^{-1}$ of 3MH and 3MHA respectively and the corresponding unspiked wine. 18 judges (15 males and 3 females) were selected to perform sensory analysis of the intensity of the grapefruity scent of wines using a 0-10 cm non-structured scale sheet. Wines were only evaluated by orthonasal olfaction to avoid possible afteraromatic interferences (Starkenmann *et al.* 2008; Starkenmann and Niclass, 2011). Following this, judges have been asked in a re-randomized series to evaluate the typicality of the wines.

Statistical analysis was performed with Statistica 9.0 software (StatSoft Inc., Tusla, OK, USA), applying the procedures each time declared. The significance of the results of the sensory test has been set according to Roessler *et al.* (1978).

Results

Tannin and juice composition

Grapes were picked on the same day for each variety during the 2013 harvest, in accordance with the compositional characteristics required in the region by industrial wineries. Table 1 reports the basic quality control parameters of the batches used, showing typical cultivar values for a cold growing season, as 2013 in Trentino. The initial concentration in 3MH precursors in control juices (table 1) perfectly matched with the reported values for the same varieties in different areas (Roland *et al.*, 2010; Pinu *et al.*, 2012). The content of GSH-3MH in GWT and SB was not statistically differentiated: 82.7±26.4 $\mu g \cdot L^{-1}$ and 102.9±15.7 $\mu g \cdot L^{-1}$, respectively, even though SB was meanly 20 $\mu g \cdot L^{-1}$ richer. Differences between varieties were instead significant for Cys-3MH (Fisher's LSD test, $p<0.05$, $n=3$) whose concentration in SB more than doubled GWT's (27.5±0.6 $\mu g \cdot L^{-1}$ vs. 13.1±2.4 $\mu g \cdot L^{-1}$). This significance was also found for the total precursor content, described as the molar sum of precursors (262±65 and 377±40 $nmol \cdot L^{-1}$ for GWT and SB respectively). Both varieties were statistically richer in GSH-3MH than Cys-3MH, in accordance with previous papers (Capone *et al.*, 2010; Capone *et al.*, 2011).

Effect of the treatment with grape tannins on the content of precursors and volatile thiols

Treatment *Low*, as expected, did non increase the final concentration of GSH-3MH and Cys-3MH (table 1) and juices spiked with this tannin were not distinguishable for any thiol precursor studied from the corresponding untreated samples, neither in GWT nor in SB. Treatment *High* however significantly augmented the natural concentration of any precursor in both cultivars (Anova main effects; source: batch, treatment; Fisher's LSD test, p<0,05).

For the entire dataset, the mean increase in juice due to the treatment *High* was 52.8 and 67.0 $\mu g \cdot L^{-1}$ respectively for GSH-3MH and Cys-3MH. These values are sufficiently in line with the theoretical augmentation (81.2 $\mu g \cdot L^{-1}$ and 62.5 $\mu g \cdot L^{-1}$) thus confirming the hydrosolubility of precursors and the efficacy of a juice supplementation with a "thiolic" tannin in the final precursor content (Larcher *et al.*, 2015), regardless matrix effects. The observed increase in Cys-3MH (as the decrease in GSH-3MH) could be due to the endogenous enzymatic activity, already reported in *Vitis vinifera* (Peyrot des Gachons *et al.*, 2002b)

Regarding the basic analytical composition of the wines, there were no detectable differences (table 2) between treatments for any of the parameters studied neither in GWT nor SB, thus confirming the correct homogenisation of the initial batches. The impact caused by treatments on the final concentration in wine of free thiols is also shown; treatment *Low* determined no significant differences on 3MH and 3MHA if compared to control wines, either for GWT or SB. The lower 3MH concentration found in treatment *Low* wines for every couple of samples *Low*-Control could be ascribed to the increase of simple phenols (in particular, catechin, epicatechin and caftaric acid) caused of the tannin supplementation, of which quinones have already been reported to react with 3MH (Nikolantonaki *et al.*, 2010; Laurie *et al.*, 2012). Treatment *High* increased significantly 3MH in both varieties meanwhile 3MHA augmented statistically only in GWT; SB wines did not show a significant difference for this parameter, even if mean values in treatment *High* almost doubled the other two treatments. This latter data is not surprising being the final 3MHA not dependent on the concentration of 3MH (Swiegers *et al.*, 2009) but on the yeast strain and fermentation conditions (Thibon *et al.*, 2008; Subileau *et al.*, 2008a). The efficacy of the juice treatment with tannins rich in precursors on the final concentration of aromatic thiols in wine was previously reported for lab scale fermentations of non-devatted juices (Larcher *et al.*, 2015), which does not reflect customary practices in wine industry. Here we confirmed those results, applying a more faithful to reality process necessary to validate potential sensory conclusions.

Effect of the tannin addition moment

The concentration of 3MH and 3MHA in the wines fermented with VIN13 and supplemented with tannin *High* at the end of alcoholic fermentation (After AF) was respectively 355±75 and 4±1 $ng \cdot L^{-1}$ in GWT and 935±402 and 64±45 $ng \cdot L^{-1}$ in SB. These concentrations were statistically lower (Wilcoxon Matched Pairs Test, $p<0.01$) if compared to a prefermentative supplementation of the same tannin (see table 2). The results could be partially explained by the inhibitory effect of alcohol on the membrane permeability of *Saccharomyces cerevisiae* (Thomas and Rose, 1973; Ding *et al.*, 2009) and/or on the residual enzymatic activity in wines, to our knowledge not yet specifically reported for β-lyase. The lower content of free thiols in the treatment After AF could be partially justified by the decrease in the liberation rate of 3MH during the first half of alcoholic fermentation as found by Concejero *et al.* (2016). This decrease does not depend on a lack of precursors, since several authors reported the presence of 3MH precursors in wines (Capone *et al.*, 2010, Concejero *et al.*, 2016). Nonetheless After AF supplementation significantly increased the 3MH content when compared to the corresponding control wine (Wilcoxon Matched Pairs Test, $p<0.05$). The augmentation ranged between 25% and 127%, thus indicating that a change of the technological conditions applied (concentration, time and/or temperature) could enhance the final grapefruit aroma of wines. The After AF augmentation of 3MH was higher in GWT (58%) than in SB (39%); differences could be possibly related to a higher enzymatic activity in GWT, due to its higher pH (Table 2). 3MHA however did not follow any clear trend, as expected, since yeasts acetylate fundamentally during the early stages of the fermentation (Concejero *et al.*, 2016), and resulted meanly in a 21% and 23% loss in GWT and SB respectively. The overall effect of the After AF treatment increased anyway the odour activity values (OAV) - calculated by dividing the concentration found by the organoleptic threshold reported for each molecule (Tominaga *et al.*, 1996) - for GWT (+38%) but was negligible in SB (-0,7%), which is strongly affected by the loss in 3MHA caused by the treatment.

Yeast strain effect and free-to-bound ratio

Regarding the basic quality control parameters of wines, only the volatile acidity in SB and the glycerol in GWT presented a small but significant difference between yeast strains (Table 2), remaining however within ranges of technological correctness. A trend of VL3 and VIN13 yeast strains to give respectively higher values of volatile acidity and glycerol was previously observed by Guzzon *et al.* (2011), Masneuf-Pomarède *et al.* (2006), and Nicolini *et al.* (2009). Differences were not found for the final concentration of 3MH or 3MHA, neither for GWT nor SB. This finding, that at first glance contradicts previous literature (Howell *et al.*, 2004; Masneuf-Pomarède *et al.*, 2006; Swiegers and Pretorious, 2007) is justified as both yeasts have extensively demonstrated - amongst commercial strains - a great ability to free

thiols (Murat *et al.*, 2001; Swiegers *et al.*, 2009, Kobayashi *et al.*, 2010). Yeast strains did not presented any difference in the acetylation ratio − 4.1% for the overall dataset - but the varieties did (Fisher's LSD test, $p<0.05$): GWT (2.2%) showed a smaller ratio than SB (6.1%). Within each cultivar, the variability found for this ratio - much greater in SB - is attributable to the batch and not to the initial content of precursors, despite the treatment.

The free-to-bound ratio, intended as the ratio between total free thiols and total 3MH precursors, both expressed as $mol \cdot L^{-1}$, ranged from 0.4 to 2.2% for the whole experiment, with a mean value of 0.91% complying with previous works (Murat *et al.*, 2001a; Subileau *et al.*, 2008b; Kobayashi *et al.*, 2010; Winter *et al.*, 2011; Pinu *et al.*, 2012; Larcher *et al.*, 2013b). The mean ratio is not statistically differentiated between treatments, but between cultivars (Fisher LSD test, $p<0.05$), with Sauvignon Blanc's (1.3%) more than doubling Gewürztraminer's (0.57%), giving further evidence of the great importance of the matrix effect on this value. The concentration of precursors in juice is significantly correlated (fig. 1; Pearson correlation test, $p<0.05$) with that of thiols in wine for GWT (r=0.92) but not SB (r=0.64), confirming the contrast with previous data reported for Koshu (Kobayashy *et al.*, 2010) and SB (Pinu *et al.*, 2012).

Wine sensory analysis

In the light of the already known role of thiols and the positive correlation between free thiols and the quality perception of SB wines (Lund *et al.*, 2009), the sensory analysis was limited to the sole GWT wines fermented with VIN13. Statistical analysis showed that only treatment *High* significantly augmented the grapefruit-like sensation (Fisher's LSD test, $p<0.05$, Fig. 2) in agreement with the increase in free thiols. This "additional" aroma formed as a result of the supplementation with tannin rich in precursors, did not penalise the odour quality of wine but, on the contrary, enhanced the overall orthonasal appreciation and typicality perceived by panelists. These results correlate sensory quality parameters to an increase of OAV's in GWT wines (meanly from 5 to 16 OAV's respectively for control and treatment *High* wines) and could be exploited for winemaking in terms of blending of wines or managing better prefermentative steps of grapes, which pomaces are known to contain a huge quantity of precursors (Román Villegas *et al.*, 2016).

Conclusions

Definetly, prefermentative supplementation with a tannin rich in 3MH precursors has proved to be effective at increasing the precursor concentration of juices. This treatment has also augmented the concentration of free thiols in wine. The supplementation of the same tannin after fermentation further

resulted in an increase of the thiol concentration in wine although at a lower extent than in prefermentation phase. The later treatment affected Gewürztraminer wines more than Sauvignon Blanc wines. The increased concentration of 3-mercapto-1-hexanol and 3-mercaptohexyl acetate in treated wines was positively correlated to an enhanced perception of grapefruit-like wine aroma, overall appreciation and typicality of Gewürztraminer. This work has further confirmed the huge matrix effect on the expression of thiols in wine.

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Table 1. Chemical composition of Gewürztraminer and Sauvignon Blanc juices (GSH-3MH: 3-S-glutathionyl mercaptohexan-1-ol; Cys-3MH: 3-S-cysteinyl mercaptohexan-1-ol; TSS: Total soluble solids; YAN: yeast assimilable nitrogen) .

Batch	TSS (°Brix)	pH	Titratable acidity ($g \cdot L^{-1}$)	Tartaric acidity ($g \cdot L^{-1}$)	Malic acid ($g \cdot L^{-1}$)	Potassium ($mg \cdot L^{-1}$)	YAN ($mg \cdot L^{-1}$)	GSH-3MH ($\mu g \cdot L^{-1}$)			Cys-3MH ($\mu g \cdot L^{-1}$)		
								Control	Low	High	Control	Low	High
Gewürztraminer													
A	23.6	3.47	3.9	4.15	2.83	1984	200	112	115	175	15	15	79
B	23.1	3.38	4.0	4.14	3.01	1768	190	76	75	133	13	13	82
C	23.4	3.48	3.9	3.45	2.97	1827	259	60	62	113	11	12	74
Sauvignon Blanc													
A	20.6	3.22	5.8	4.58	4.03	1264	162	85	84	131	27	28	93
B	20.0	3.28	6.3	5.70	3.64	1650	166	114	117	160	27	27	94
C	19.1	3.30	4.6	4.59	2.22	1144	207	110	103	162	28	27	101

Table 2. Chemical composition of wines split by cultivar *vs* treatment and *vs* yeast strain (3MH: 3-mercaptohexan-1-ol; 3MHA: 3-mercaptohexyl acetate). Different letters highlight significant differences (Factorial anova; sources of variance: treatment & yeast strain; Fisher's LSD test, $p<0,05$)

	Treatment								Yeast strain				
	Control			Low			High			VIN13			VL3		
	Mean (n=6)	Std. Dev.		Mean (n=6)	Std. Dev.		Mean (n=6)	Std. Dev.		Mean (n=9)	Std. Dev.		Mean (n=9)	Std. Dev.	
Gewurztraminer															
3MH (ng·L^{-1})	195	74	b	175	72	b	558	128	a	350	224		269	181	
3MHA (ng·L^{-1})	5	2	b	5	2	b	20	5	a	11	8		9	8	
Alcohol (% vol)	14.66	0.18		14.69	0.21		14.67	0.19		14.66	0.17		14.69	0.20	
pH	3.57	0.15		3.58	0.14		3.57	0.13		3.57	0.12		3.57	0.15	
Titratable acidity (g·L^{-1})	4.1	0.5		4.1	0.4		4.1	0.4		4.0	0.3		4.2	0.5	
Volatile acidity (g·L^{-1})	0.31	0.17		0.32	0.15		0.32	0.14		0.28	0.13		0.35	0.16	
Sugars (g·L^{-1})	1.3	0.4		1.2	0.2		1.4	0.3		1.3	0.3		1.3	0.3	
Tartaric acid (g·L^{-1})	0.99	0.17		1.03	0.18		1.00	0.21		0.98	0.16		1.03	0.20	
Malic acid (g·L^{-1})	1.85	0.26		1.75	0.23		1.76	0.22		1.71	0.21		1.86	0.22	
Glycerol (g·L^{-1})	6.9	0.6		6.8	0.6		6.8	0.4		7.2	0.3	a	6.4	0.4	b
Sauvignon Blanc															
3MH (ng·L-1)	642	208	b	536	204	b	1168	313	a	769	391		795	364	
3MHA (ng·L-1)	67	49		45	34		114	88		90	82		61	43	
Alcohol (% vol)	12.44	0.20		12.47	0.16		12.48	0.19		12.49	0.20		12.43	0.15	
pH	3.25	0.08		3.27	0.07		3.26	0.07		3.24	0.08		3.28	0.05	
Titratable acidity (g·L^{-1})	5.4	0.7		5.3	0.6		5.3	0.7		5.3	0.7		5.4	0.6	
Volatile acidity (g·L-1)	0.16	0.07		0.18	0.07		0.17	0.07		0.13	0.04	b	0.21	0.07	a
Sugars (g·L-1)	2.3	2.4		2.0	1.5		1.4	0.6		1.4	0.6		2.4	2.2	
Tartaric acid (g·L-1)	1.89	0.11		2.07	0.15		1.95	0.15		2.00	0.16		1.95	0.14	
Malic acid (g·L-1)	2.14	0.33		1.99	0.37		2.04	0.35		2.02	0.36		2.10	0.33	
Glycerol (g·L-1)	5.3	0.4		5.1	0.3		5.3	0.3		5.3	0.4		5.1	0.3	

Figure 1. Correlation between the sum of the 3-S-glutathionyl mercaptohexan-1-ol (GSH-3MH) and 3-S-cysteinyl mercaptohexan-1-ol (Cys-3MH) in juice and the sum of the total free thiols in wine 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA). Significance has been set by Pearson correlation (n.s.= not significant; *** = p<0.001).

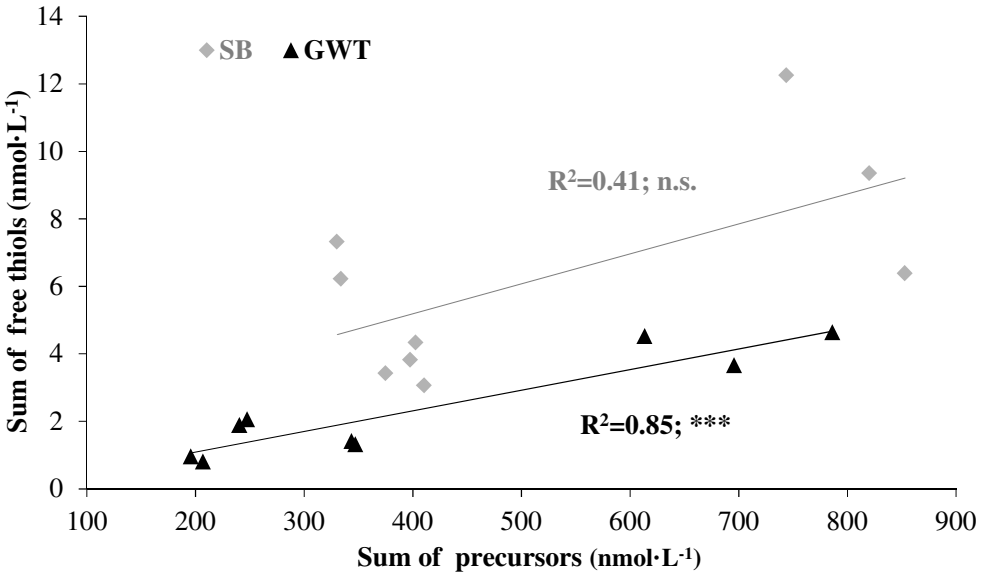
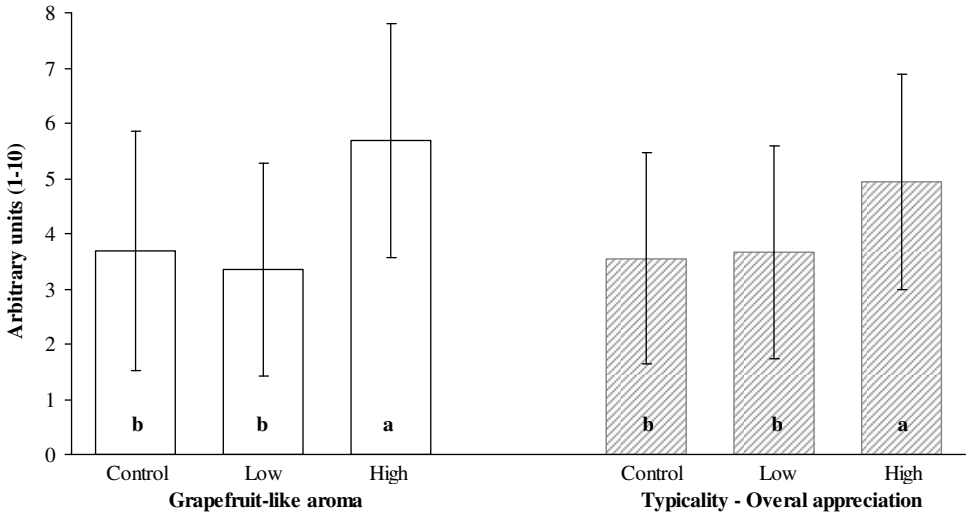


Figure 2. Intensity (mean ± standard deviation; N=54) of the grapefruit-like scent and typicality evaluated by 18 judges for Gewürztraminer wines fermented with the VIN13 strain depending on the treatment (Control, Low and High, corresponding to no tannin supplementation and treatment with tannin poor or rich in precursors, respectively). Histograms with the same letter are not statistically different in Fisher's LSD test, $p < 0.05$).



SECTION 6. GEWÜRZTRAMINER BY-PRODUCTS VALORISATION

6.1. Novel technologycal strategies to enhance tropical thiol precursors in winemaking by-products

SECTION 6.1

Novel technologycal strategies to enhance tropical thiol precursors in winemaking by-products

Román Villegas, T., Tonidandel, L., Fedrizzi, B., Larcher, R., & Nicolini, G.*

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Aim of the work

The food and beverage chain cannot avoid the increasing concern found all over the industry about waste management, which in regards to wine industry exceeds 4 million tons per year. In this work we wanted to verify some prerequisites for new strategies of utilisation and valorisation of grape marcs, the most important winemaking by-product. In particular, we investigated the possibility to improve the compositional characteristics of marcs in view of their possible use as a source for the enological adjuvants industry in the production of flavoured or even "varietal" tannins, as well as for the production of beverages and spirits. Pomace samples of Gewürztraminer, Müller Thurgau and Sauvignon Blanc were macerated for several hours with and without the presence of SO_2, an additive commonly used in winemaking. Further non-varietal marcs, collected directly from industrial wineries, were ground in the presence of vine leaves or bunch stems. Samples were analysed for their concentration in thiol precursors.

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Novel technological strategies to enhance tropical thiol precursors in winemaking by-products

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ABSTRACT

Grape pomace is a winemaking by-product that can be used to extract oenological tannins. Recently, some grape skin tannins were shown to contain very high amounts of two polyfunctional thiol precursors (3-*S*-glutathionylhexan-1-ol, 3-*S*-cysteinylhexan-1-ol) whose free forms are responsible for appreciated tropical-like flavours. This study shows that an oxidative treatment (no SO_2) of white grape pomace and the presence of grape leaves and stems can increase the content of the above mentioned precursors. Moreover, it shows significant differences between Sauvignon Blanc, Gewuerztraminer and Mueller-Thurgau grape pomace for the 3-mercaptohexan-1-ol precursors and 4-*S*-cysteinyl-4-methylpentan-2-one. The grape cultivar is crucial, but the technological ability of enhancing the level of the volatile thiol precursors simply by treating the grape marc in different ways is a promising and powerful tool for the production of potentially flavouring tannins intended for food and beverage industry.

1. Introduction

The wine industry is producing a high amount of horticultural by-products, with an estimated value of 4 Mt/y worldwide (Corbin et al., 2015; FAO, 2015). Grape pomace is mainly constituted of berry skin and seeds, which are extremely rich in polyphenols and represent the starting material for the extraction of grape tannins (European Commission, 2003, 2004; Laufenberg, Kunz, & Nystroem, 2003; Teixeira et al., 2014).

Oenological tannins are a complex mixture in which polyphenolic compounds play a major role. They have been used in different fields like leather, adhesives, ore flotation, cements as well as pharmacy and medicine (Pizzi, 2008). Moreover, they are adjuvants commonly employed in the food and beverage industry for their ability to stabilise colour, enhance flavour complexity and modulate taste (Haslam, 2007; Vivas, 2000; Vivas, Bourgeois, Vitry, Glories, & deFreitas, 1996; Vivas & Glories, 1996; Vivas, Nonier, & Gaulejac, 2004; Vivas, Nonier, & Vivas de Gaulejac, 2003). Several biological sources can be used to extract these products and, among them, grape skin and seeds are gaining an increasing role given their ability to tailor the food and beverage sensory characteristics (Cliff, Stanich, Edwards, & Saucier, 2012; Sonni, Chinnici, Natali, & Riponi, 2011).

In the specific context of wine and beverages, tropical sensory notes are currently highly desired. The main molecules responsible for these aromas are polyfunctional thiols (3-mercaptohexan-1-ol, 3MH; 3-mercaptohexyl acetate, 3MHA; 4-mercapto-4-methylpentan-2-one, 4MMP) produced during fermentation from non-volatile *S*-glutathionyl and *S*-cysteinyl precursors or through other biosynthetic pathways involving H_2S and (*E*)-2-hexenal not completely elucidated (Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009; Harsch et al., 2013; Peyrot des Gachons, Tominaga, & Dubourdieu, 2002; Schneider, Charrier, Razungles, & Baumes, 2006; Tominaga, Peyrot des Gachons, & Dubourdieu, 1998; Winter, van der Westhuizen, Higgins, Curtin, & Ugliano, 2011).

We recently found that some commercial grape skin tannins can contain remarkable amounts of Cys-3MH and GSH-3MH (Larcher, Tonidandel, Nicolini, & Fedrizzi, 2013) and that their addition to juice prior to fermentation can increase the content of 3MH and 3MHA in wine (Larcher et al., 2015). This evidence prompted us to focus our attention on technological options to increase the level of the non-volatile thiol precursors in grape marc.

Some agronomical variables, *e.g.* type and timing of nitrogen fertilisation of vineyard or water and nitrogen deficit (Chone et al., 2006; Peyrot des Gachons et al., 2005), as well as varietal differences seem to affect the accumulation of thiol precursors (Larcher et al., 2013; Roland et al., 2011; Roland, Vialaret, Razungles, Rigou, & Schneider, 2010). To the best of our knowledge,

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the possibility of enhancing the level of the non-volatile thiol precursors by merely acting on grape pomace treatment has never been reported. This could be a powerful tool for the production of potentially flavouring tannins, with possible applications both in beverage production and food processing, as well as to add value to wine by-products. The possibility that these hydrophilic precursors could be rapidly hydrolysed in the mouth (Starkenmann et al., 2008) originating aromatic species, independently from a fermentation process, could represent a further interesting technological opportunity.

In the present work different technological strategies are presented. In particular, the effect of the supplementation of grape pomace with SO_2, grape leaves and stems on the concentration of Cys-3MH and GSH-3MH was evaluated. The former treatment employs a commonly utilised winemaking additive and represents a strategy easily extendable to several food and beverage industrial processes. The addition of leaves and stems in grape pomace mimics the outcome of the machine harvested grape where significant amount of matter other than grape is usually present.

2. Materials and methods

2.1. Chemicals

Ultrapure water was produced with an Arium Pro UV DI Ultrapure Water System (Sartorius, Göttingen, Germany). Liquid chromatography-mass spectrometry grade formic acid (FA) and acetonitrile (ACN), ⩾97% potassium metabisulfite, ⩾98% L-ascorbic acid, ⩾98% reduced GSH, anhydrous ⩾99% dimethyl dicarbonate and ⩾99.9% methanol were supplied by Sigma-Aldrich (Milan, Italy). Buchem B.V. (Apeldoorn, the Netherlands) supplied d_3-3-*S*-cysteinylhexan-1-ol (d_3-Cys-3MH) and d_3-3-*S*-glutathionylhexan-1-ol (d_3-GSH-3MH), along with unlabelled 3-*S*-cysteinylhexan-1-ol (Cys-3MH) and 3-*S*-glutathionylhexan-1-ol (GSH-3MH). 4-*S*-cysteinyl-4-methylpentan-2-one (Cys-4MMP) and 4-*S*-glutathionyl-4-methylpentan-2-one (GSH-4MMP) were synthesised at the School of Chemical Sciences of the Auckland University following the protocols reported in the literature (Fedrizzi et al., 2009; Tominaga, Peyrot des Gachons, & Dubourdieu, 1998).

2.2. Sample preparation

2.2.1. General

Two experiments, named "SO_2" and "Stems/Leaves", have been carried out at the Experimental Winery of the Edmund Mach Foundation, San Michele all'Adige (Italy) during the vintage 2013.

2.2.2. SO_2 experiment

Pomace samples of Mueller-Thurgau (MT; N. = 10; juice composition, mean values: total soluble solids 16.6 °Bx, pH 3.18, titratable acidity 6.0 g/L as tartaric acid), Gewurztraminer (TR; 24; 23.5, 3.40, 4.1) and Sauvignon Blanc (SB; 12; 22.0, 2.94, 8.3) were obtained after crushing-destemming (Ares 15, OMAC s.r.l., Corridonia, MC, Italy) and pressing (3.5 bar; 20L Hydropress, Spiedel GmbH., Offerdingen, Germany) grapes harvested according to the industrial plans of the commercial wineries of the region, with no SO_2. Each pomace sample, made up of 500 g, was divided into 2 equal aliquots named "SO_2" and "Control", respectively. To the first aliquot, Milli-Q water (50 ml) containing 80 mg of SO_2 (as potassium metabisulfite) was added. The second one was supplemented with 50 ml Milli-Q water and 25 mg NaN_3 to drastically reduce microbial activity. After a 5-h maceration at room temperature (16°–23 °C), 100 g sample was ground with methanol (80 ml) to stop enzymatic reactions and kept at −20 °C until analysis.

2.2.3. Stems/leaves experiment

Non mono-varietal white grape pomace (N = 16, ~30 kg each), sampled directly from the hopper at the end of the pressing cycle at industrial ripeness of juice (22.3 ± 1.1 °Bx), were collected from different wineries in Trentino (Italy). Each sample was divided into 3 aliquots: one supplemented with 3 grape leaves per 100 g pomace, another with 3 stems per 100 g pomace and the last aliquot without any supplementation (Control). Finally, the samples were ground and stored as above until analysis. The amount of leaves and stems adopted was merely chosen to verify, on a qualitative level, the effect of the treatment in the thiol precursors production.

Before analysis, the sample was remixed and a 15 g aliquot was taken and spiked with an H_2O:CH_3OH (1:1; v:v; 15 ml) solution. Then, it was supplemented with d_3-Cys-3MH (1.57 μg) and d_3-GSH-3MH (1.64 μg) as labelled internal standards, stirred (10 min), centrifuged (4000 rpm × 5 min; Centrifuge 4226, ALC International s.r.l., Milan, Italy) and finally the supernatant was filtered (0.22 μm) and injected.

2.3. Chemical and statistical analysis

LC-MS/MS quantification of thiol precursors was carried out using an UPLC Acquity coupled with a Xevo TQ MS mass spectrometer (Waters Corporation, Milford, USA). A 5 μl sample was injected onto an Acquity UPLC HSS T3 C18 column (1.8 μm film thickness, 2.1 mm × 100 mm; Waters). Flow rate was set at 0.45 ml/min and column temperature at 40 °C. MS isotopic dilution analysis was performed in positive ion mode (capillary voltage, 2.5 kV), with argon (0.20 ml/min) and nitrogen (1000 L/h) as collision and desolvation gas, respectively. Other characteristics of the method are specified in a previous work (Larcher et al., 2013).

Box plots and statistical tests were carried out using STATISTICA v. 8.0 (StatSoft Inc., Tulsa, OK).

3. Results and discussion

3.1. SO_2 experiment

The thiol precursors measured in the 92 grape pomace samples determined according to the protocol discussed above, ranged between 23 and 3478 μg/kg and 55–4432 μg/kg for the Cys-3MH

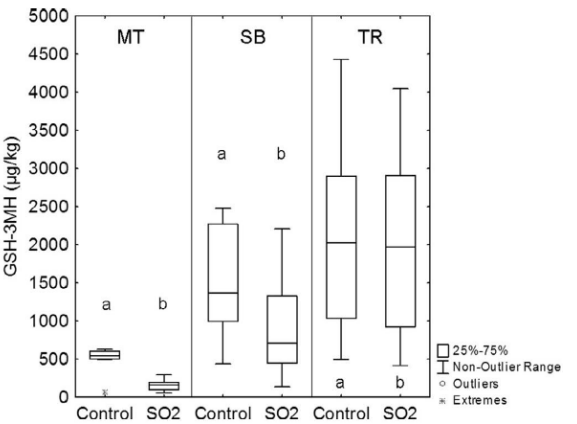

Fig. 1. 3-*S*-glutathionylhexan-1-ol (GSH-3MH) content in Mueller-Thurgau (MT), Sauvignon Blanc (SB) and Gewuerztraminer (TR) grape pomace processed with (SO_2) or without (Control) supplementation of 320 mg/kg SO_2. Box plots with different letters are statistically different (Sign test, $p < 0.05$).

and GSH-3MH, respectively (Figs. 1 and 2). Cys-4MMP ranged from below the limit of detection (LOD, 0.1 μg/kg) to 4.4 μg/kg, while GSH-4MMP was under its LOD (0.1 μg/kg) in all samples.

As far as it concerns the control samples, cultivar differences ($p < 0.05$) were observed by applying both parametric (Unequal Number HSD Tukey's test) and non parametric approaches (Mann-Whitney *U* test; Kolmogorov-Smirnov test), all tests giving consistent results. For GSH-3MH, TR had a significantly higher mean content (2186 μg/kg) than MT (509 μg/kg), while SB held a non significant intermediate position (1519 μg/kg). For Cys-3MH, the TR samples (1576 μg/kg) were statistically different from SB (742 μg/kg) and MT (257 μg/kg). TR was found to be particularly rich in 3MH non-volatile precursors, confirming that previously found in grape juice by Roland and coworkers (Roland, Schneider, Guernevé, Razungles, & Cavelier, 2010). Cys-4MMP was significantly higher in SB (mean value: 1.5 μg/kg) than in MT (0.5 μg/kg) and TR (0.1 μg/kg).

The effect of the addition of SO_2 as potassium metabisulfite to the grape pomace on the concentration of the varietal thiol precursors, was studied for a potential subsequent production of oenological tannins and a wine waste valorisation. The Sign test was applied on the 10, 12 and 24 pairs of samples obtained for MT, SB and TR grape samples, respectively.

Data in Fig. 1 show that the supplementation with SO_2 significantly reduces GSH-3MH formation in all varieties, even if to a different extent. Cys-3MH is also impacted by SO_2 in MT and SB, while no effect was observed in TR (Fig. 2). The effect of SO_2 on the thiol precursors content in grape pomace is in agreement with that observed by Capone and Jeffery (2011) for the machine harvested samples. The reduction on the glutathionyl and cysteinyl precursors being formed under the "SO_2" regimes could be ascribed to the inhibition of the enzymes responsible for the conjugation between GSH and (*E*)-2-hexenal and the further degradation of the GSH-3MH to Cys-3MH as well as the formation of stable sulfonic acid from the reaction between (*E*)-2-hexenal and the bisulfite anion (Duhamel et al., 2015).

As far as it concerns the statistically significant parameters, compared to the control the SO_2 treatment reduced the 3MH precursors' contents to ~35%, ~60% and ~90% in MT, SB and, TR, respectively. On the other hand, the results obtained from this experimental plan do not allow dissection of the contribution of the different technological ripening conditions for that specific season (i.e. Bx, pH and, TA) and potential varietal differences. Concerning Cys-4MMP, no statistical differences were found due to the treatments.

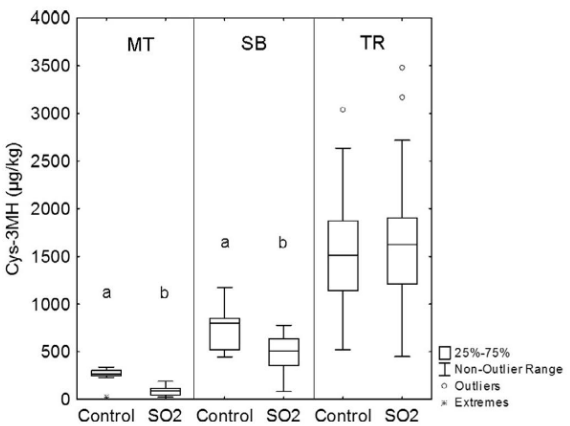

Fig. 2. 3-*S*-cysteinylhexan-1-ol (Cys-3MH) content in Mueller-Thurgau (MT), Sauvignon Blanc (SB) and Gewuerztraminer (TR) grape pomace processed with (SO_2) or without (Control) supplementation of 320 mg/kg SO_2. Box plots with different letters are statistically different (Sign test, $p < 0.05$).

3.2. Stems/leaves experiment

The effect of the presence of leaves and stems on the pomace non-volatile thiol precursors was investigated using Main Effects ANOVA, with batch and treatment as source of variance, and with the Fisher's LSD test. Leaves and stems caused a marked and statistically significant increase of the 3MH precursors (Table 1). This is consistent with the known richness of these plant tissues in C_6 alcohols and aldehydes (Joslin & Ough, 1978; Hashizume & Samuta, 1997). Moreover, it is supported by the recent evidence that thiol precursors are also present in other grape vine tissues (Kobayashi et al., 2010). On average, leaves increased the content in GSH-3MH and Cys-3MH by about 3.4-fold, while stems increased them by 2.5 and 3.7-fold, respectively. No differences were found for Cys-4MMP.

Considering that the addition of the leaves, in terms of weight, was approximately half that of stems, the data reported in Table 1 could be explained in light of the higher levels of hexenal reported in leaves by Hashizume and Samuta (1997). In fact (*E*)-2-hexenal has been reported to be able to react with GSH to originate the 3MH precursors (Capone & Jeffery, 2011). The fact that grape leaves and stems are rich in several classes of aroma compounds has been known for several years. In particular, grape leaves have been reported to contain monoterpenes (*e.g.* 1,8-cineole), sesquiterpenes (*e.g.* rotundone), shikimates, pyrazines, and C_{13}-norisoprenoides (Wirth, Guo, Baumes, & Günata, 2001; Capone, Jeffery, & Sefton, 2012), while stems have been shown to be particularly rich in pyrazines (Hashizume & Samuta, 1997), representing a possible additional contribution to the aroma complexity of winemaking by-products.

4. Conclusions

Significant differences for pomace-extracted thiol precursors have been found among different grape varieties. Gewuerztraminer proved to be particularly rich in thiol precursors, suggesting a potential application of this variety for flavouring tannin extractions. Compared to a more oxidative protocol, SO_2 addition to pomace seems to reduce the amount of non-volatile thiol precursors extracted from the grape pomace while the intentional or casual presence of leaves and stems causes significant increases in pomace for 3MH precursors. This finding could be particularly interesting with a view to using marc obtained from mechanically harvested grape.

The choice of the variety is confirmed to be pivotal, but the technological ability of enhancing the level of non-volatile thiol precursors by merely treating grape pomace in different ways is a promising and powerful tool for the production of potentially flavouring tannins intended for the food and beverage industry.

Table 1
Effect of the presence of leaves and stems on the pomace content in *S*-glutathionyl and *S*-cysteinyl precursors (GSH- and Cys-) of 3-mercaptohexanol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP). Mean values (N = 16) and significance at Fisher's LSD test, $p < 0.05$. Values with different letters are statistically different; n.s. = non significant.

Treatment	GSH-3MH (μg/kg)		Cys-3MH (μg/kg)		Cys-4MMP (μg/kg)	
Control	813	c	341	b	0.26	n.s.
Leaves	2784	a	1159	a	0.30	n.s.
Stems	2063	b	1255	a	0.31	n.s.

Compliance with ethics requirements

This study does not contain any studies with human or animal subjects.

Conflict of interest

None.

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Conclusions

The absence of SO_2 always favoured the presence of significantly higher concentrations of thiol precursors except for 3-S-cysteinyl mercaptohexan-1-ol in Gewürztraminer marcs. The experimental design showed the noteworthy richness and aroma potentiality of Gewürztraminer marcs compared to Müller Thurgau and, surprisingly, to Sauvignon Blanc, a cultivar deeply studied for the importance of thiols in the aroma of its wines.

The treatment of grape marcs with leaves and stems resulted in a several-fold increase of both 3-S-glutathionyl mercaptohexan-1-ol and 3-S-cysteinyl mercaptohexan-1-ol, with leaves showing a higher effect per mass unit.

We proved that some industrially applicable options can improve the characteristics of grape by-products, hypothesising their usages in the wine adjuvant, beverages and spirits industry.

SECTION 7. CONCLUSIONS OF THE THESIS

The work has highligthed new strategies to improve quality and to give further value to Gewürztraminer wine and its by-products. It focused on this variety - native to Trentino-South Tyrol Region (Italy) – in view of its increasing, but already noteworthy, role played in the regional economy.

The thesis has proved that some varietal thiols – i.e. 3-mercaptohexan-1-ol and its acetate – fit and enhance the tipicality and the grapefruit-like note of Gewürztraminer. In the light of this evidence, it has been deepen the agronomic and technogenic variability of these compounds and their precursors: 3-S-cysteinyl mercaptohexan-1-ol and 3-S-glutathionyl mercaptohexan-1-ol

The organic and non occasional analysis of a number of Gewürztraminer grapes and wines from Trentino, permitted to characterise the composition of the variety, in which no precursors of 4-mercapto-4-methylpentan-2-one have been found. In particular, it has been reported for the first time, at the best of our knowledge:

- the existence and the range of the thiol precursors' variability in Gewürztraminer clones;
- the accumulation kinetics of 3MH precursors in Gewürztraminer during grape ripening;
- the distribution of 3-mercaptohexanol precursors between marcs and juice measured in semi-industrial samples and the varietal richness of these marcs compared to Sauvignon Blanc's.

The observed richness of marcs, prefigures their usage as a possible source for the extraction of variety flavouring tannins intended for the food and the beverage industry. To this aim, it has been proven that the supplementation of marcs with leaves or stems, and the absence of SO_2 augmented the content of 3-mercaptohexan-1-ol precursors.

As regards the technogenic variability in Gewürztraminer:

- it has been verified at industrial scale the extraction kinetic during pressing, observing a remarkable increase in precursors indicatively over 800 mbar, even after a previous short skin-contact;
- it has been stated the efficacy of the prefermentative skin-contact maceration.

Regarding maceration, it has been also proved that oxydizing conditions increase the glutathion-3-mercaptohexan-1-ol, despite a high variability between varieties. Furthermore, it has been demonstrated that:

- a juice treatment with the main enological adjuvants - i.e. bentonite, charcoal and PVPP - does not technologically deplete the precursors concentration;
- on the contrary, the supplementation of grape skin tannins rich in 3-mercaptohexan-1-ol precursors augments their concentration in juice, both in lab and semi-industrial scale, leading to wines richer in volatile thiols.

The present thesis has increased the knowledge about the potentialities and aromatic performances of Gewürztraminer cultivated in the Alpine area, confirming in any case, the existence of remarkable matrix effects. The experimental designs, characterised by a prevailing industrial logic, have permitted to investigate several phases of the vine-to-wine chain. This approach does not permit to evaluate the specific contribution of the different formation pathways of 3-mercaptohexan-1-ol and its precursors, nonetheless, the obtained results provide new tools to manage better winemaking processes aimed to enhance the quality of Gewürztraminer wine aroma.

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