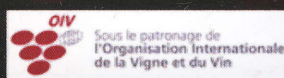


WINE ACTIVE COMPOUNDS 2014

Proceedings of the Third Edition of the International
Conference Series on Wine Active Compounds

Edited by Pr. Régis Gougeon
Université de Bourgogne

WAC 2014
March 26, 27, 28, 2014



Oenological tannins as a source of thiol precursors

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Keywords: oenological tannins; thiol precursors; LC-MS

1. INTRODUCTION

Oenological tannins are widely used as adjuvants in winemaking. Their contribution to colour stability, mouthfeel and aroma profile [1, 2] has been studied in the past, while the possibility that grape tannins could also provide varietal aroma precursors, such as the *S*-glutathionylated and *S*-cysteinyllated precursors of thiols, was only recently demonstrated [3]. In a second experiment, the effect of the pre-fermentative introduction of thiol precursors, via tannins addition, on the 3-mercaptohexano-1-ol and 3-mercaptohexyl acetate levels in the finished wine was verified [Larcher 2014, submitted]. All the wines supplemented with grape tannins showed higher level of free thiols than the one without tannins. This evidence supports the hypothesis that oenological tannins could be used to adjust the final level of tropical thiols.

2. MATERIALS AND METHODS

2.1 Chemicals and materials

Formic acid (FA) and acetonitrile (ACN) of HPLC grade were provided by Sigma-Aldrich (Milan, Italy). (R/S)-3-*S*-cysteinylhexan-1-ol (Cys-3MH), (R/S)-3-*S*-glutathionylhexan-1-ol (GSH-3MH) and their labelled forms *d*₃-(R/S)-3-*S*-cysteinylhexan-1-ol (*d*₃-Cys-3MH), and *d*₃-(R/S)-3-*S*-glutathionylhexan-1-ol (*d*₃-GSH-3MH) were supplied by Buchem B.V. (Apeldoorn, The Netherlands). 4-*S*-Cysteinyl-4-methylpentan-2-one (Cys-4MMP), 4-*S*-glutathionyl-4-methylpentan-2-one (GSH-4MMP) were synthesised following already published protocols [4, 5]. Ultrapure water was in lab-produced using an Ultrapure Water System (Arium Pro UV DI; Sartorius, Göttingen, Germany). Fifty-eight tannin samples from on the Italian market were

collected at local distributors and wineries. They were classified according to the label specifications declared by producers or distributors. Tannin sample (50 mg) was first moistened with some drops of methanol, dissolved in 45 mL methanol/water (70:30) solution, and added of the internal standards *d*₃-Cys-3MH and *d*₃-GSH-3MH. The solution, filtered through a 0.22-μm PVDF syringe filter (Millex-GV; Millipore, Tullagreen, Ireland), was transferred into a 2 mL glass vial for the LC-MS analysis.

2.2 LC-MS

The precursors were analysed using an UPLC Acquity (Waters Corporation, Milford, US), coupled to a Xevo TQ MS mass spectrometer (Waters) for all the experiments. The chromatographic module consisted of an Acquity UPLC HSS T3 C18 column (1.8 μm, 2.1 mm x 100 mm; Waters), set at 40 °C. The eluents, water (A) and ACN (B) both added of 0.1% formic acid, were used at a flow rate of 0.45 mL/min. The solvent B gradient was: 5% (2 min), to 100% (in 5 min), at 100% (1 min), then to 5% (in 0.01 min) and at 5% (2 min). The sample injection volume was of 5 μL. MS experiments were performed in positive ion mode setting the capillary voltage at 2.5 kV. Desolvation and collision gasses were respectively nitrogen (1000 L h⁻¹) and argon (0.20 mL min⁻¹). Desolvation and source temperature were set at 500 °C and 150 °C, respectively. The experimental conditions used for Product ion (PI) experiment in the identification of thiol precursors were: cone voltage potential and collision energy were set respectively at 16 V and 10 eV for Cys-3MH; 26 V and 12 eV for GSH-3MH; 16 V and 10 eV for Cys-4MMP; and 16 V and 14 eV for GSH-4MMP. For the method validation, linearity was studied at five calibration levels,

between 0.1 and 30 $\mu\text{g/l}$, and the correlation coefficients (R^2) for the deuterated thiol precursors were always higher than 0.98.

3. RESULTS AND DISCUSSION

3.1. Identification of 3-S-cystenylhexan-1-ol and 3-S-glutathionylhexan-1-ol in tannin

The LC-MS/MS experiments were organised in two phases: the first, with the direct infusion of the reference Cys-3MH, GSH-3MH, Cys-4MMP and GSH-4MMP, to optimise the fragmentation of the thiol precursors for the MRM experiments. The second, to record the Product Ion (PI) mass spectra (figure 1) and optimise of the chromatographic separation of the labelled and unlabelled precursors. GSH-3MH was present in all of the analysed samples, whilst Cys-3MH in most of them. No 4MMP precursors were above the detection limit of the method (0.01 mg kg^{-1} for both precursors).

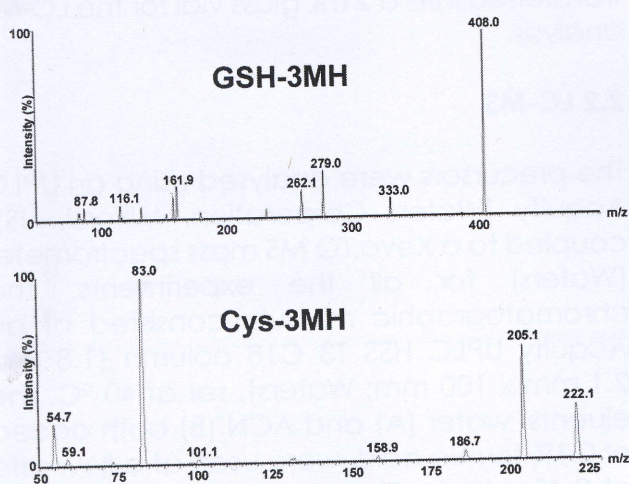


Figure 1. Product Ion Mass Spectra of GSH-3MH and Cys-3MH in grape tannin.

3.2. Thiol precursors in commercial tannins

As reported in table 1, grape skin tannins showed to be very rich of thiol precursors (min = 0.232, median = 10.1 and max = 138 mg kg^{-1} for GSH-3MH; 0.33, 9.1, and 200 mg kg^{-1} for Cys-3MH). Also the contents found in some grape seed tannins (min = 0.01, median = 0.61, and max = 33.6 mg kg^{-1} for GSH-3MH; 0.01, 0.40, and 6.2 mg kg^{-1} for Cys-3MH) were of technological interest. The precursor contents found in the two grape products were statistically different (Mann-Whitney U-test, $p < 0.05$) and consistent with the distribution of these molecules inside

the grape berry [6, 7]. Oak tannins (labelled as ellagic tannins) were also rich of thiol precursors with contents of 1.24 mg kg^{-1} of GSH-3MH and 0.246 mg kg^{-1} of Cys-3MH. Conversely, the other botanical sources were generally poor or absent of these precursors, and traces, when present, were reasonably due to cross-contaminations happened along the production process. On the basis of this evidence we suggest an innovative use of tannin for supplementing thiol precursors and in this way possibly changing the free 3MH and 3MHA content in wine after fermentation. Moreover, other researches indicate a possible direct contribute of the precursor Cys-3MH in the retronasal sensory perception of 3MH [8]. This could prefigure new scenarios for the addition of precursors-rich tannins as tropical-note flavouring agents in unfermented food and alcohol-free beverages. Regarding the abundance of Cys-3MH and GSH-3MH in grape products, their content ratio was significantly depending on the specific origin. The values ranged from 0.18 to 2.0 for grape seed tannin and from 0.23 to 1.5 for grape skin tannins. Finally, if we hypothesise a standard addition (200 mg L^{-1}) of a precursors rich tannin (e.g. GSH-3MH = 138 mg kg^{-1} ; Cys-3MH = 200 mg kg^{-1}), and we estimate a 3% molar conversion of precursors to the free forms during fermentation this would theoretically increase 3MH of up to 1 $\mu\text{g L}^{-1}$, potentially impacting the sensory characteristics of wine.

Tannin (N)	GSH-3MH (mg/kg)			Cys-3MH (mg/kg)		
	min	mean	max	min	mean	max
Grape skin (9)	0.232	34.3	138	0.329	32.8	200
Grape seeds (7)	0.008	5.19	33.6	0.006	1.103	6.17
Oak (ellagic) (1)		1.24			0.246	
Grape skin (enocianin) (2)	0.591	0.615	0.639	0.204	0.315	0.425
Tara (1)		0.170			<0.001	
Acacia (Brazilian) (1)		0.053			0.007	
Cherry tree/mimosa 80:20 (1)		0.048			0.015	
Tree fruits (4)	0.006	0.033	0.105	<0.001	0.035	0.135
Tea (green) (1)		0.026			<0.001	
Oak (American) (2)	<0.001	0.015	0.029	<0.001	<0.001	0.002
Chestnut (3)	0.004	0.012	0.023	<0.001	0.004	0.010
Quebracho (4)	0.002	0.011	0.019	<0.001	0.006	0.017
Acacia (Gambier) (1)		0.009			0.027	
Oak (toasted) (5)	<0.001	0.009	0.02	<0.001	0.008	0.019
Oak (French) (2)	0.003	0.007	0.01	<0.001	<0.001	0.002
Oak (4)	0.002	0.005	0.009	<0.001	0.003	0.006
Gall (3)	<0.001	0.005	0.012	<0.001	<0.001	0.003
Oak (French, toasted) (2)	0.004	0.005	0.005	<0.001	0.002	0.003
Officinal plants (1)		0.004			<0.001	
Gall (Turkish, <i>Quercus infectoria</i>) (1)		0.004			<0.001	
Tea (1)		0.002			0.003	
Gall (Chinese, <i>Rhus semialata</i>) (2)	<0.001	<0.001	0.002	<0.001	0.002	0.003

Table 1. GSH-3MH and Cys-3MH content in 58 commercial grouped by botanical origin.

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