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Model Aging and Oxidation Effects on Varietal, Fermentative, and Sulfur Compounds in a Dry Botrytized Red Wine

Bruno Fedrizzi,*[,‡,§] Giacomo Zapparoli,[∥] Fabio Finato,[§] Emanuele Tosi,[⊥] Arianna Turri,[§] Michela Azzolini,[⊥] and Giuseppe Versini[§]

[‡]University of Padova, Chemical Sciences Department, via Marzolo 1, 35131 Padova, Italy

[§]Unione Italiana Vini Società Cooperativa, viale del Lavoro 8, 37135 Verona, Italy

[∥]University of Verona, Biotechnologies Department, Strada Le Grazie 15, 37134 Verona, Italy

[⊥]Centro per la Sperimentazione in Vitivinicoltura, Provincia di Verona, Servizio Agricoltura, Via delle Pieve 64, 37029 San Pietro in Cariano, Verona, Italy

ABSTRACT: From harvest until wine arrives to the consumer, oxygen plays a crucial role in the definition of the final aroma. In the present research, the effect of the model oxidative aging on a dry red *Botrytis* wine, such as Italian Amarone, was considered. Amarone wine was submitted to model oxidative aging and then analyzed with two different approaches (SPE-GC-MS and HS-SPME/GC-MS). The same sampling plan was adopted to study the model aging of the same Amarone wine in anaerobic conditions. The HS-SPME/GC-MS method was applied to investigate for the first time the effect of the oxidative aging on a vast number of fermentative sulfur compounds. This research highlighted peculiar evolutions for several volatile compounds. In particular, benzaldehyde showed a sensitive increment during the oxidative aging, with a rate much higher than that reported for non-*Botrytis* red wines. On the other hand, several sulfides (dimethyl sulfide, 3-(methylthio)-1-propanol, etc.) disappeared after just 15 days of oxidative aging. A wine oxidation marker such as 3-(methylthio)-propanal was not found in any of the oxidized wines; conversely methionol-*S*-oxide was tentatively identified. This evidence has not been mentioned in the literature. A possible involvement of grape withering process and *Botrytis* in these mechanisms was supposed: a dry red wine, produced from the same but without any grape withering process and *Botrytis* infection (e.g., Bardolino wine), was submitted to oxidative aging and analysis. This red wine showed an evolution similar to those reported in the literature for dry red wines but significantly different from the Amarone wine.

KEYWORDS: Oxidative aging, aroma compounds, sulfur volatiles, headspace−solid phase microextraction, solid phase extraction

INTRODUCTION

Oxidation processes have always been considered as a crucial phenomena in winemaking from harvesting to storage.[1−4] In particular, wine oxidation is either responsible for important spoilages [1,5] or could have a pivotal role in some particular wine styles.[6−8] Oxygen contributes significantly to wine development by impacting the color, aroma, and sensorial properties of red and white wines.[9,10]

The amount of oxygen introduced in wine depends strictly on the winery practices and the right oxygen management allows modulating chemical and sensorial profiles of wine. Mild oxygenation processes can improve the quality of wine, stabilizing the phenolics, and is widely used in wineries as alternative or complementary methods (i.e., micro-oxygenation) for wine aging.[11] The transfer of slow amounts of oxygen during barrel aging favors positive modifications of color and aroma that characterize aged wines. The oxidative aging practice is important on determining the peculiar aroma profiles of wine like Madeira and Sherry wines.[12,13]

Several fermentative compounds (fatty acid ethyl esters, acetates, and fatty acids) decrease with oxidative aging, while others, such as sotolon and furfural derivatives, increase. Moreover, the whole aroma profile can change, due to variations of the C13 norisoprenoids content.[14,15] In the case of aging in oak wood, the oxidation processes contribute also to the variation on the content of compounds such as vanillin derivates, volatile phenols, and lactones.[16]

Amarone wine is a dry red wine produced in Valpolicella area (Verona, Italy) by withered grapes of *Vitis vinifera* Cv. Corvina and Rondinella grape varieties. During the grape dehydration (traditionally 4−6 months) *Botrytis cinerea* infection usually occurs, even if its level depends on seasonal conditions and withering technology;[17,18] generally, the wine is left in oak barrels for at least 3 years. This aging process is undoubtedly important to confer the peculiar flavor and aroma that make Amarone wine known around the world. In spite of the oxidation that can occur throughout the whole winemaking process, from the withering stages through to aging, it is during this latter phase that oxidative reactions seem to provide the wine with a particular typicality which makes Amarone unique. Because there is a lack of information about the phenomena occurring in this wine during its maturation, investigations to elucidate the contribution of oxidation on Amarone wine volatiles are required.

The aim of the present study is to examine the effect of model oxidative aging in Amarone wine, by analyzing fermentative, varietal, and sulfur compounds. The accelerated wine oxidation was carried out at 30 °C in order to amplify the evolution of volatile compounds. D'Auria et al. [19] reported as the simple transition

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Table 1. Retention Time, Identification, and Quantification Ions and Calibration Parameters[a]

analytes	RT (min)	quant ion (m/z)	qualif ions (m/z)	R^2	L_D(μg/L)
dimethyl sulfide[b]	4.76	62	45, 47	0.991	0.16
dethyl sulfide[b]	8.52	75	61, 90	0.993	0.11
S-methyl thioacetate[b]	14.42	90	43, 47	0.994	0.31
S-ethyl thioacetate[b]	15.40	104	43, 60	0.995	0.20
dimethyl disulfide[c]	15.01	94	64, 79	0.991	0.06
diethyl disulfide[c]	18.00	122	66, 94	0.994	0.07
2-(methylthio)-1-ethanol[d]	22.61	92	47, 61	0.993	0.21
3-(methylthio)-1-propanol[d]	24.72	106	58, 61	0.996	1.65
4-(methylthio)-1-butanol[d]	26.04	120	61, 102	0.995	0.54
dimethyl sulfoxide[e]	29.07	63	78, 61	0.991	0.75

[a] RT, retention time; quant ion, quantitation ion; qualif ions, qualifier ions. [b] Dimethyl-d_6 sulfide; RT, 4.73 min; quant ion, 68; qualif ions, 50, 66 as IS. [c] Dipropyl disulfide; RT, 18.68 min; quant ion, 108; qualif ions 66, 150 as IS. [d] 3-(Methylthio)-1-hexanol; RT, 26.57; quant ion, 148; qualif ions, 61, 75 as IS. [e] Dimethyl-d_6 sulfoxide; RT, 28.83; quant ion, 66; qualif ions, 84, 64.

from 20 to 30 °C induced significant modifications on the composition of some fermentative compounds of important Italian red wines, Amarone included.

In the present paper, the impact of oxidative processes, combined with model aging, on the chemical quality of this botrytized wine will be discussed.

MATERIALS AND METHODS

Chemicals. 1-Hexanol, 2-phenyl ethanol, benzyl alcohol, benzaldehyde, phenyl acetaldehyde, furfurol, 5-methyl furfural, linalool, α-terpineol, citronellol, 4-terpineol, 1-octen-3-ol, β-damascenone, vanillin, homovanillyl alcohol, homovanillic acid, methyl vanillate, ethyl vanillate, acetovanillone, syringaldehyde, syringol, and acetosyringone were purchased by Sigma-Aldrich (Milan, Italy). The sulfur compounds considered were: dimethyl sulfide (DMS), dimethyl sulfoxide (DMSO), diethyl sulfide (DES), dimethyl disulfide (DMDS), diethyl disulfide (DEDS), *S*-methyl thioacetate (MTA), *S*-ethyl thioacetate (ETA), 2-(methylthio)-1-ethanol (MTE), 3-(methylthio)-1-propanol (i.e., methionol; MTP), and 4-(methylthio)-1-butanol (MTB). Dimethyl-d_6 sulfide (DMS-d_6), dimethyl-d_6 sulfoxide (DMSO-d_6), dipropyl disulfide (DPDS), and 3-(methylthio)-1-hexanol (MTH) were used as internal standards (IS). All the sulfured standards had a purity of ≥98% and were supplied by Sigma-Aldrich (Milan, Italy) and Lancaster (Milan, Italy). All the other volatile molecules (i.e., ethyl 4-hydroxybutyrate, *N*-methylbutyl acetamide, *N*-ethylphenyl acetamide, endiol, TDN, vitispiranes, actinidols, 3-oxo-α-ionol, propiovanillone, butyrovanillone) were tentatively identified by using the compounds kindly provided by Prof. Adolf Rapp (Bundesforschungsanstalt fur Rebenztlchtung, Geilweilerhof, Germany) and Dr. Raymond Baumes (Institut National de la Recherche Agronomique, Montpellier, France).

Experimental Plan. *Vinification.* The wine Amarone della Valpolicella (17% alcohol strength, pH 3.5, 5.8 g/L total acidity as tartaric acid, 9 g/L sugar content) used for the trials was produced during the 2008 vintage from *Vitis vinifera* Cv. Corvina and *Vitis vinifera* Cv. Rondinella grapes, partially dried in natural conditions for four months using the traditional over-ripening technique in the Valpolicella area (Verona, Italy). With this technique, the grapes are naturally withered on mats or racks in fruit drying rooms without the use of conditioned chambers where temperature and relative humidity are strictly controlled. Contrarily to the former, the latter process reduces drastically the mold infection of grapes. Grape berries (2000 kg) were crushed and the resulting juice and pomaces were transferred into a 2500 L stainless steel tank. Fermentation was carried out according to traditional red winemaking.[20] Then 50 mg/L of SO_2 were added before the inoculation with a commercial yeast strain (VRB, Lallemand Inc., Montreal, Canada)) at the concentration of (4−5) × 106 CFU/mL. The alcoholic fermentation was conducted in a local winery with temperatures ranging between 12 and 16 °C. Must aeration and cap management were carried out by pumping-over operations. At the end of the alcoholic fermentation (about 21 days), the wine was devatted and clarified by natural sedimentation for two days in a winery room where the temperature (10−14 °C) was not controlled.

Experimental Design. The wine was manually bottled at room temperature in 750 mL dark glass bottles. To test for model aging, the bottles were filled to the top (750 mL) with the Amarone wine and then kept under nitrogen, while to check for model oxidative aging dependence, wine (375 mL) was put into bottles (750 mL) with 375 mL ullage volume of air. All bottles were sealed with a screw cap using a manual bottling machine and were stored in a room at a controlled temperature of 30 °C (±0.5). The control was a 750 mL bottle filled up to the top with the same Amarone wine, sealed with a screw cap under nitrogen, and analyzed at bottling.

The model aging and the model oxidative aging dependence on the level of several aroma compounds was measured at 15, 30, and 60 days. Three separate bottles were analyzed for each sampling time, control included.

To check for postharvest withering process of grape berries and *Botrytis* infection dependence on the content of volatile compounds, a commercial Bardolino wine (2009 vintage) was taken into account. This wine is produced from fresh grapes of the same varieties used for Amarone wine production. The model oxidative aging was repeated (375 mL of wine in 750 mL dark glass bottles at 30 °C), analyzing the wine after 0, 15, and 30 days. The samples were submitted to solid phase extraction (SPE) GC-MS and head space solid phase microextraction (HS-SPME) GC-MS analysis, and even in this case three different bottles were analyzed for each sampling time.

All the HS-SPME and SPE analyses were performed in triplicate.

SPE-GC-MS Analysis of Volatile Compounds. The ENV+ cartridges (6 mL volume, 1 g sorbent) for SPE extraction were purchased from Isolute (IST Ltd., Mid Glamorgan, UK). The analysis of the aroma compounds was performed according to Fedrizzi et al.[21] The extraction was performed on an automated solid phase extraction apparatus (Aspec XL, Gilson Inc.; Middleton, WI, USA). The cartridge was first activated with methanol (10 mL) and then rinsed with Milli-Q water (10 mL). The sample (58 mL, wine/Milli-Q water, 1:1 v/v) was eluted through the SPE cartridge, then the cartridge was rinsed with 10 mL of distilled water. The analytes were recovered with dichloromethane (9 mL), dried with sodium sulfate, and concentrated to about 200 μL under a gentle stream of nitrogen.

GC-MS analyses were carried out on a GC 6890N gas chromatograph equipped with a DB-Wax capillary column (60 m × 320 μm ID × 0.25 mm film thickness, Agilent Technologies, Milano, Italy) and coupled with a MS 5975B mass spectrometer (Agilent Technologies).

Table 2. Mean (ppb) and Standard Deviation (SD) of the Fermentative and Varietal Compounds Analyzed and Tukey's Test Results to Test for Amarone Wine Model Aging and Model Oxidative Aging Effects[a]

	control			15d N_2[b]			30d N_2[b]			60d N_2[b]			control			15d OX[b]			30d OX[b]			60d OX[b]		
analyte	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD
hexanol[c]	1988	ns	236	1875	ns	38	1892	ns	57	1916	ns	105	1988	ns	236	1976	ns	109	1851	ns	37	1915	ns	182
2-phenyl ethanol[c]	48506	ns	7026	43993	ns	1483	43058	ns	848	44301	ns	2100	48506	ns	7026	43803	ns	1510	42454	ns	1100	42881	ns	3402
benzyl alcohol[c]	558	ns	19	723	ns	180	767	ns	100	789	ns	265	558	ns	19	806	ns	17	786	ns	51	738	ns	461
ethyl 4-hydroxybutyrate[d]	4340	a	799	3624	a	234	3515	a	156	3145	b	9	4340	a	799	3572	a	108	3409	a	179	3228	b	19
benzaldehyde[c]	89.9	a	6.1	63.8	b	1.4	63.1	b	1.6	61.1	b	0.7	89.9	a	6.1	154.4	b	7.6	152.3	b	5.1	165.6	b	3.8
phenyl acetaldehyde[c]	7.1	a	0.7	5.3	b	0.7	2.7	c	0.4	2.1	c	0.8	7.1	a	0.7	24.5	b	1.1	21.8	c	1.3	17.6	d	0.7
furfurol[c]	154.7	a	2.7	174.9	b	1.2	195.9	c	3.5	241.5	d	6.5	154.7	a	2.7	181.9	b	3.2	202.3	b	4.1	264.5	c	15.2
5-methyl furfural[c]	3.3	a	0.0	4.2	b	0.1	4.8	c	0.0	6.4	d	0.2	3.3	a	0.0	3.5	a	0.1	4.1	b	0.2	5.2	c	0.1
N-methylbutyl acetamide[d]	2219	ns	55	2868	ns	385	3037	ns	204	3075	ns	860	2219	ns	55	3316	ns	91	3131	ns	52	3045	ns	113
N-ethylphenyl acetamide[d]	402	ns	9	381	ns	12	393	ns	9	408	ns	23	401.9	a	9.5	403.6	a	8.2	383.0	a	13.9	453.6	b	32.6
linalool[c]	11.4	a	0.1	9.9	b	0.3	9.8	bc	0.5	9.0	c	0.3	11.4	a	0.1	8.5	b	0.9	8.1	bc	0.2	7.2	c	0.4
α-terpineol[c]	9.9	a	0.3	10.9	ab	0.6	12.0	bc	0.7	12.5	c	0.2	9.9	a	0.3	11.0	b	0.5	11.3	b	0.5	11.7	b	0.2
citronellol[c]	7.7	a	0.2	6.7	b	0.3	5.7	c	0.1	5.6	c	0.3	7.7	a	0.2	5.6	b	1.0	5.0	b	0.9	5.0	b	0.1
endiol[d]	21.2	a	0.4	28.6	b	1.0	30.8	b	0.5	34.2	c	1.9	21.2	a	0.4	31.7	b	3.0	31.1	b	2.0	32.7	b	2.7
4-terpineol[c]	39.3	ns	1.2	38.6	ns	0.5	38.4	ns	1.1	38.5	ns	1.0	39.3	ns	1.2	36.7	ns	1.6	37.4	ns	0.3	36.8	ns	0.8
1-octen-3-ol[c]	9.0	ns	0.3	9.3	ns	0.1	9.3	ns	0.4	9.5	ns	0.5	9.0	a	0.3	9.2	a	0.1	9.9	ab	0.3	10.4	b	0.7
β-damascenone[c]	4.6	ns	0.4	5.0	ns	0.8	4.9	ns	0.1	5.3	ns	0.2	4.6	ns	0.4	4.2	ns	0.1	4.2	ns	0.3	4.0	ns	0.1
TDN[d]	0.6	a	0.1	0.6	a	0.0	0.7	a	0.0	1.2	b	0.2	0.6	a	0.1	0.5	a	0.0	0.6	a	0.1	0.9	b	0.0
vitispiranes[d]	4.7	a	0.1	6.4	b	0.3	7.3	b	0.7	9.9	c	0.3	4.7	a	0.1	4.9	a	0.7	5.0	a	0.2	6.9	b	0.1
actinidols[d]	14.5	a	0.7	20.7	b	0.9	24.4	c	0.1	29.3	d	1.5	14.5	a	0.7	21.0	b	1.1	24.5	c	0.2	28.5	d	1.4
3-oxo-α-ionol[d]	115.1	a	0.4	116.9	a	1.9	119.9	ab	2.6	134.2	b	11.5	115.1	a	0.4	121.1	a	3.9	115.2	a	4.3	135.7	b	9.5
vanillin[c]	16.4	a	1.1	15.5	a	1.6	19.3	ab	1.4	22.1	b	2.2	16.4	a	1.1	161.8	b	7.1	162.9	b	1.5	190.5	c	6.3
homovanillyl alcohol[c]	293	ns	11	281	ns	16	278	ns	1	306	ns	22	292.9	a	11.3	293.7	a	18.2	273.0	a	13.8	330.3	b	25.0
homovanillic acid[c]	43.6	a	0.3	37.3	b	2.0	38.8	b	1.7	40.4	ab	2.2	43.6	a	0.3	41.7	ab	2.2	36.2	b	2.9	42.3	ab	3.0
methyl vanillate[c]	17.3	a	0.3	17.4	a	0.5	17.9	ab	0.1	20.2	b	1.8	17.3	ns	0.3	17.9	ns	0.3	17.0	ns	1.6	19.7	ns	1.8
ethyl vanillate[c]	245	ns	4	238	ns	7	237	ns	8	261	ns	23	245.4	a	4.0	307.9	bc	12.5	294.9	b	9.9	339.5	c	23.3
acetovanillone[c]	195	ns	5	198	ns	8	200	ns	2	206	ns	5	195.4	a	4.6	205.3	a	4.1	201.2	a	3.8	227.5	b	15.0
propiovanillone[d]	22.6	ns	1.3	23.5	ns	0.6	23.8	ns	0.6	25.3	ns	2.4	22.6	ns	1.3	23.8	ns	0.9	22.9	ns	0.9	22.6	ns	1.9
butyrovanillone[d]	78.6	a	3.4	37.1	b	5.3	32.5	b	2.3	50.8	c	3.4	78.6	a	3.4	36.5	b	2.9	34.1	b	5.1	40.9	b	4.4
syringaldehyde[c]	22.8	a	1.5	20.0	a	4.6	33.2	b	3.7	24.6	a	1.6	22.8	a	1.5	292.0	b	10.8	202.2	c	15.1	312.1	b	41.7
syringol[c]	13.2	a	0.7	13.0	a	0.7	13.8	ab	0.5	18.4	a	3.7	13.2	a	0.7	12.9	a	0.5	13.7	ab	1.9	17.6	b	2.5
acetosyringone[c]	20.8	ns	2.2	19.5	ns	1.0	19.6	ns	0.4	21.1	ns	2.1	20.8	a	2.2	28.3	b	1.8	26.0	ab	2.7	32.5	b	2.6

[a] Values with the same letter do not differ significantly in the Tukey's test, $p < 0.05$. n.s., not significant. [b] 15d N_2, 30d N_2, 60d N_2:15, 30, and 60 days respectively of model aging. 15d Ox, 30d Ox, 60d Ox: 15, 30, and 60 days respectively of model oxidative aging. [c] Identification based on purchased reference compounds. [d] Tentative identification. The two data set (e.g. model aging and model oxidative aging) were considered separately in performing the Tukey's test.

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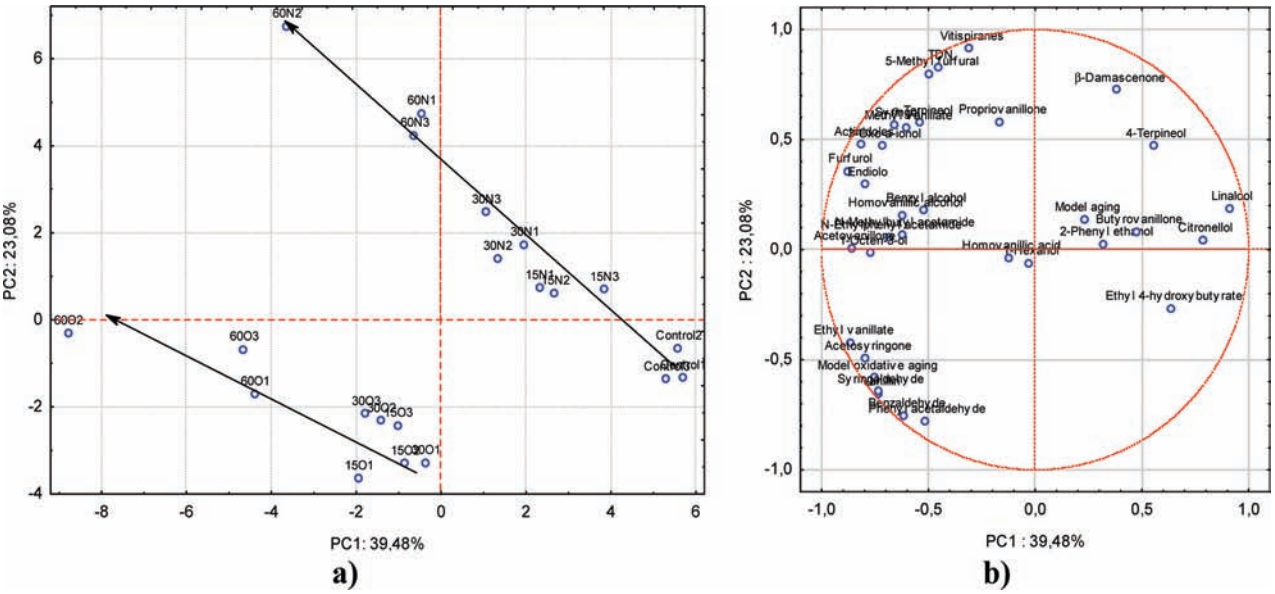

Figure 1. PCA data treatment of the volatile aroma compounds. (a) Biplot of the scores and (b) biplot of the loadings. Control; 15N1, 15N2, 15N3: 15 days model aging under nitrogen; 30N1, 30N2, 30N3: 30 days model aging under nitrogen; 60N1, 60N2, 60N3: 60 days model aging under nitrogen; 15O1, 15O2, 15O3: 15 days model oxidative aging; 30O1, 30O2, 30O3: 30 days model oxidative aging; 60O1, 60O2, 60O3: 60 days model oxidative aging. Model oxidative aging: 375 mL ullage volume of air; three replicated bottles.

The oven temperature program adopted was: 50 °C (4 min), 4 °C/min to 240 °C, 240 °C (16 min).[21] Helium was used as carrier gas at a flow-rate of 1.5 mL/min. The temperature of transfer line and GC injector were 200 and 250 °C, respectively. The electron impact energy and the MS source were 70 eV and 230 °C, respectively. All the analyses were carried out in SCAN mode, using the National Institute of Standards and Technology (NIST) library and reference compounds to confirm the identification. A response factor equal to 1 toward the internal standard (1-heptanol), as commonly performed in the analysis of flavor compounds, was adopted for the quantitative analysis.

HS-SPME/GC-MS Determination of Fermentative Sulfur Compounds. Fermentative sulfur compounds (i.e., molecules originated from the yeast metabolism during the wine fermentation) were analyzed according to a previously published method.[22] The choice of the best fiber to study the quoted fermentative sulfur compounds was made according to our previous experiences and to literature data.[22−24] The fiber chosen was a carboxen−polydimethylsiloxane−divinylbenzene (CAR-PDMS-DVB; 50/30 mm, 2 cm long). The sampling was carried out with the MPS2 Autosampler (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). The SPME holder for automated sampling, and the fibers were purchased from Supelco (Bellefonte, PA, USA). The fibers were conditioned before the use according to the producer's instructions. The sample (5 mL) was transferred into a 20 mL vial, and 2 g of NaCl was added. HS-SPME sampling was carried out at 35 °C for 30 min.

GC-MS apparatus was a GC 6890N (Agilent Technologies) equipped with a DB-Wax capillary column (60 m × 320 μm ID × 0.25 mm film thickness, Agilent Technologies) and coupled with a MS 5975B mass spectrometer (Agilent Technologies). Gas chromatography conditions were: GC injector temperature 250 °C; injection in splitless mode for 1 min; oven temperature program, 35 °C (5 min), 1 °C/min to 40 °C, 10 °C/min to 250 °C. Helium was used a carrier gas (flow 1.5 mL/min).

The chromatographic analyses were carried out in single ion recording (SIR) mode. Identification of the analytes and internal standards was achieved by coinjecting the pure reference compounds and using the NIST library; mass fragments adopted for the quantification are according to Fedrizzi et al.[22]

Table 3. Analytical Data of the Fermentative Sulfur Compounds Considered[a]

	average	MIN	MAX	SD
dimethyl sulfide	6.9	0.9	17.8	5.9
dethyl sulfide	2.3	0.5	5.4	1.5
S-methyl thioacetate	2.2	1.5	3.4	0.6
S-ethyl thioacetate	1.7	1.2	2.4	0.4
dimethyl disulfide	9.7	7.1	15.9	2.1
diethyl disulfide	0.9	0.5	1.6	0.3
2-(methylthio)-1-ethanol	15.4	0.5	29.2	12.5
3-(methylthio)-1 -propanol	874.5	11.7	1696.6	760.1
4-(methylthio)-1-butanol	77.2	1.7	145.8	57.0
dimethyl sulfoxide	28.1	18.4	45.3	1.1

[a] MIN: minimum; MAX: maximum; SD: standard deviation.

A calibration curve for each analyte was prepared according to the internal standard method. Validation was performed on a dry red wine (13% alcohol strength v/v) treated twice with charcoal (3 g/L) to remove any sulfur compounds detectable by the proposed HS-SPME/GC-MS method as reported elsewhere.[22,23] Linearity and sensibility were verified in the concentration ranges typical of red wines.[23] Calibration curves were prepared using seven concentration levels and five replicate solutions per level; detection limit (L_D) was calculated (Table 1) according to Hubaux−Vos procedure.[25]

SPE-GC-MS Quantification of DMSO. DMSO analysis was performed by slightly modifying the method published by Segurel et al.[26] Hydromatrix (100 g; Varian, Palo Alto, CA, USA) was combined with NaCl (60 g; Carlo Erba, Milan, Italy) and then put at 250 °C for 6 h. This mixture (2 g) was then packed into a glass cartridge (9 mL) and then activated with methanol (5 mL) and rinsed with water (5 mL). Ten mL of the wine sample, spiked with 20 ppb of the internal standard (DMSO-d_6), were eluted through the cartridge. After loading the wine the column was rinsed with Milli-Q water (5 mL) and then eluted with CH_2Cl_2 (10 mL). The extract was concentrated gently to dryness under

Table 4. Mean and Standard Deviation (SD) of the Fermentative Sulfur Compounds Analyzed and Tukey's Test Results to Test for Amarone Wine Model Aging and Model Oxidative Aging Effects[a]

	control			15d N_2			30d N_2			60d N_2		
analyte	mean (ppb)		SD	mean (ppb)		SD	mean (ppb)		SD	mean (ppb)		SD
dimethyl sulfide	7.6	a	1.4	8.3	a	0.8	13.1	b	2.2	18.1	c	1.6
dethyl sulfide	3.2	ns	0.3	3.0	ns	0.3	4.1	ns	1.2	3.5	ns	0.5
S-methyl thioacetate	1.6	a	0.0	2.1	a	0.0	2.8	b	0.3	3.0	b	0.4
S-ethyl thioacetate	1.4	a	0.2	1.7	a	0.1	2.2	b	0.1	2.2	b	0.2
dimethyl disulfide	9.7	ns	0.6	8.9	ns	0.6	12.9	ns	2.8	11.3	ns	1.7
diethyl disulfide	1.0	ns	0.1	0.8	ns	0.1	1.2	ns	0.3	1.1	ns	0.2
2-(methylthio)-1-ethanol	27.6	ns	2.4	26.9	ns	2.7	23.2	ns	3.4	25.6	ns	1.3
3-(methylthio)-1-propanol	1448	ns	25	1602	ns	82	1445	ns	113	1558	ns	147
4-(methylthio)-1-butanol	136	ns	10.1	124	ns	7.7	119	ns	14.1	119	ns	3.2
dimethyl sulfoxide	20.5	a	1.0	24.3	b	0.8	25.4	b	0.5	26.3	b	0.6
	control			15d Ox			30d Ox			60d Ox		
analyte	mean (ppb)		SD	mean (ppb)		SD	mean (ppb)		SD	mean (ppb)		SD
dimethyl sulfide	7.6	a	1.4	1.1	b	0.2	1.2	b	0.1	1.1	b	0.3
dethyl sulfide	3.2	a	0.3	0.7	b	0.1	0.7	b	0.0	0.7	b	0.1
S-methyl thioacetate	1.6	a	0.1	1.7	a	0.2	1.8	a	0.2	2.2	b	0.3
S-ethyl thioacetate	1.4	ns	0.2	1.4	ns	0.2	1.4	ns	0.2	1.4	ns	0.2
dimethyl disulfide	9.7	ns	0.6	8.5	ns	1.1	8.6	ns	1.1	7.8	ns	0.9
diethyl disulfide	1.0	a	0.1	0.7	a	0.1	0.7	a	0.1	0.6	b	0.1
2-(methylthio)-1-ethanol	27.6	a	2.4	2.7	b	0.2	1.4	b	0.1	0.6	b	0.2
3-(methylthio)-1 -propanol	1448	a	25	35	b	6	22	b	3	13	b	1
4-(methylthio)-1-butanol	136	a	10.1	23	b	3.1	17	b	2.5	2	c	0.5
dimethyl sulfoxide	20.5	a	1.2	35.1	b	0.4	39.9	c	0.3	44.9	d	0.7

[a] Values with the same letter do not differ significantly in the Tukey's test, $p < 0.05$. n.s.: not significant. 15d N_2, 30d N_2, 60d N_2:15, 30, and 60 days respectively of model aging. 15d Ox, 30d Ox, 60d Ox: 15, 30, and 60 days respectively of model oxidative aging.

nitrogen and finally redissolved in 200 μL of methanol and injected for GC-MS analysis.

GC-MS analyses were carried out on a GC 6890N (Agilent Technologies) gas chromatograph equipped with a DB-Wax capillary column (60 m × 320 μm ID × 0.25 mm film thickness, Agilent Technologies) and coupled with a MS 5975B mass spectrometer (Agilent Technologies). The oven temperature program was: 35 °C (4 min), 4 °C/min to 150 °C (3 min), 40 °C/min 240 °C (5 min). Helium was used as carrier gas at a flow-rate of 1.5 mL/min. The temperature of transfer line and GC injector were 200 and 250 °C, respectively. The electron impact energy and the MS source were 70 eV and 230 °C, respectively. All the analyses were carried out in SIR mode; Table 1 reports the fragments used for quantification and the detection limit of this method.

Statistical Analysis. Tukey's test was applied to check for the influence of model aging and model oxidative aging treatments on the molecules considered, while principal component analysis (PCA) was used to identify possible clustering according to either oxidation or aging phenomena. The data were statistically evaluated and plotted using STATISTICA v7.1 (Statsoft Italia Srl, Padova, Italy) and Origin v7.0 (OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION

Oxidation is considered one the most important and most critical parameters to be taken into account in wine production. In particular, a good understanding of the dependence of this phenomena on the content of peculiar aroma compounds is pivotal for wines subjected to long aging.

In the present paper, the effect of model oxidative aging on a noble Italian red wine, such as the Amarone wine, was investigated. This particular wine is produced with partially dried grapes infected by *Botrytis cinerea*;[27] the peculiar postharvest technology that characterizes the traditional Amarone winemaking could play an important role in the oxidation mechanisms studied in this wine, and therefore this peculiar situation of this wine must be taken into account.

Fermentative and Varietal Compounds Analysis. Table 2 reports the volatile compounds taken into account for both model aging (30 °C for 15, 30, and 60 days with the samples kept under nitrogen to exclude any presence of oxygen) and model oxidative aging (30 °C for 15, 30, and 60 days with 375 mL ullage volume of air), their mean values, and the results of the Tukey's test.

1-Hexanol, benzyl alcohol, and 2-phenyl ethanol are molecules strictly correlated to the winemaking technology applied. In particular, 1-hexanol is correlated to skin contact and pressure applied to the grape at pressing,[28,29] while benzyl alcohol and 2-phenyl ethanol are mainly fermentative compounds originating from yeast metabolism.[30,31] No influence of model aging or oxidative aging on the level of these compounds was observed.

Ethyl 4-hydroxybutyrate, as a representative of the fruity esters class, is produced by yeast metabolism and its concentration is in equilibrium with the cyclic form, i.e. γ-butyrolactone. It showed a decrement during both model aging and oxidative aging as a consequence of the chemical equilibrium with the γ-lactone and

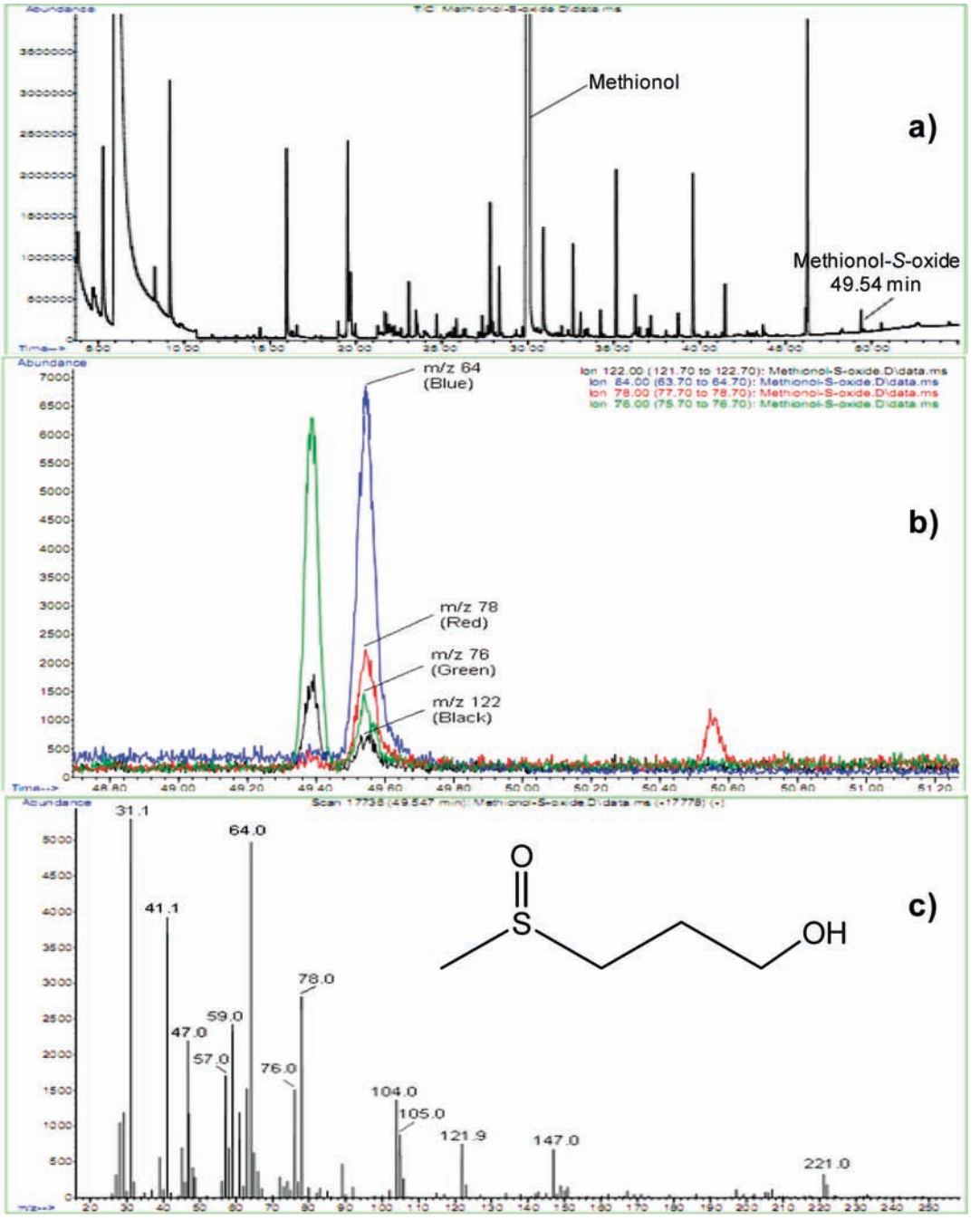

Figure 2. Tentative identification of methionol-*S*-oxide in the crude reaction. (a) Total ion chromatogram; (b) extracted ion chromatogram at methionol-*S*-oxide putative retention time; (c) methionol-*S*-oxide mass spectra.

its possible hydrolysis. According to the Tukey's test, such changes became statistically significant only after 60 days at 30 °C.

Benzaldehyde concentration in the control sample appeared to be lower than in Amarone wines previously analyzed;[32] this molecule is a marker of *Botrytis cinerea* infection,[33] and the measured contents are consistent with an infection of this fungus on the berries processed. The variation of its level in the wines is different depending to the treatment adopted: no changes were noticed during model aging, while a significant increment was detectable in the first 15 days (15d Ox) of oxidative aging, with no further evolution in the following days. No information on such behavior are reported in the literature for wines, even if it is reported that particular enzymatic pathways could be activated by the presence of the *Botrytis* infection.[34]

Phenyl acetaldehyde showed a quite similar behavior compared to the previous aldehyde. In particular, a slight decrease can be observed during model aging under nitrogen while the oxidative aging study showed a preliminary increment in the first 15 days followed by depletion during the other 45 days. The degradation kinetics appear different in the two aging experiments, with a higher rate when oxygen is taking part in the reaction. This evidence could also imply particular enzymatic processes, which could be faster in aerobic conditions.

Furfurol and 5-methyl furfural content, which originate from carbohydrate residue reactions,[35] showed in all the experiments increments during model aging and model oxidative aging.

N-3-(Methylbutyl)-acetamide and *N*-3-(ethylphenyl)-acetamide, both originating from amino acid metabolisms,[36,37] have a strong pungent scent and they can be important off-flavors in red wine. No changes were measured for these molecules in both model aging and oxidative aging.

Monoterpenes and C13-norisoprenoids in our experiment appeared to be only slightly affected by model aging and oxidative aging as is also reported elsewhere.[35] 4-Terpineol levels found in our samples are consistent with grape berries bearing *Botrytis cinerea* infection, even if its level appears a little lower than that found in older products.[32]

Some benzenoids were also quantified in these experiments: no important differences were observed in both model aging and oxidative aging for most of the benzenoid derivatives measured. Nevertheless, the content of vanillin varied strongly in oxidized wine, increasing more than 10 times compared to the control. Similarly, syringaldehyde level increased drastically during oxidative aging.

This data set was also submitted to PCA analysis (Figure 1) to point out the compounds responsible for the differences between the control, the aged, and the oxidized wines. The first two components (PC1, PC2) collected 62.56% of the total variability of the system.

The biplot of the scores (Figure 1a) pointed out a clear separation between the wines aged under nitrogen and the wines aged in an oxidative environment (375 mL ullage volume of air). Furthermore, it is possible to observe that these two treatments affected differently the wine evolution as it is possible to observe that the wines belonging to the two groups are aligning along ideal straight lines with different slopes.

The analysis of the loadings plot (Figure 1b) permits to observe that oxidized products are highly correlated with benzenoid compounds (i.e., ethyl vanillate, acetosyringone, benzaldehyde, etc.) as also indicated by the position of the "model oxidative aging" loading; wines aged under nitrogen are mostly represented by linalool, citronellol, 4-terpineol, and β-damascenone. The trends highlighted by the two arrows show that the species mostly correlated with aging are C13-norisoprenoids, furfurol, and 5-methyl furfural.

Sulfur Compounds Analysis. Sulfur presents a wide redox chemistry,[38] and organic sulfur compounds can undergo oxidative/reductive reactions in a wine environment. Even if the contribution to wine aroma is not completely understood for most of these molecules, it is clear that this class of compounds plays a ubiquitous and crucial role in the aroma definition of many wines and wine styles.[24]

The fermentative sulfur compounds quantified in the Amarone wines submitted to model aging and model oxidative aging (Table 3) appeared to be mostly in the ranges reported in the literature.[23,39−41]

The data highlights of some peculiar behaviors of the investigated molecules. In particular, Table 4 shows the average concentration and Tukey's test results for the samples submitted to model aging (750 mL bottle volume filled up to the top, sealed under nitrogen and left at 30 °C for 0, 15, 30, and 60 days) and model oxidative aging (375 mL ullage volume of air, sealed and left at 30 °C for 0, 15, 30, and 60 days).

An increment for some fermentative sulfur compounds with aging was already reported in the literature;[23,26] in particular, in the present paper, DMS was confirmed to increase with aging. A very slight increment can be observed for DMDS and DEDS, even though such increments are not statistically significant according to the Tukey's test. Conversely, no increment was found for the other sulfide analyzed, i.e. DES. The increment of DMS has always been correlated to *S*-methyl methionine degradation,[26,42,43] even if no connection has been demonstrated in wine yet. As for disulfides, no clear correlation between their increment and aging has been explained in wine matrices either.

Table 5. Mass Fragmentation and Relative Abundance of the Tentatively Identified Methionol-*S*-oxide

MS fragment (m/z)	Krammer et al. (1997) %	current research %
31	100	100
64	96	92
41	79	72
78	59	51
47	49	44
59	48	47
76	36	34
57	34	35
104	30	27
61	26	22
122	16	15
105	15	17

The effect of oxygen on some fermentative sulfur compounds has been previously taken into account,[44] even if in that case only three sulfur compounds were considered. In the present research, the effect of oxygen was verified on a bigger number of fermentative sulfur compounds. The compounds studied were chosen according to their chemical class, biogenesis pathway, and sensory contribution. The oxygen effect is unknown for most of these molecules; furthermore, the selection of a dry wine produced from withered berries infected with *Botrytis cinerea* introduces an important variable in studying the oxidation mechanisms.

To test for the possible oxygen effects, the bottles were left at 30 °C for 0 (i.e., control), 15, 30, and 60 days, respectively with an ullage volume of 375 mL of air. To ensure a good accuracy in the sample preparation, three different bottles for each sampling point were submitted to analysis.

The data in Table 4 show the outcome of the forced oxidation; as proved by the Tukey's test, it is noticeable as some species were strongly affected by oxidation. In particular DMS, DES, MTE, MTP, and MTB concentration dropped after 15 days; after this time, DMS and DES level remain constant, while MTE, MTP, and MTB concentration kept decreasing at a slower rate. DMDS slightly decreased with oxidation while DEDS did not show any change along the whole time sampled. 3-(Methylthio)-propanal was not detected, and 3-(methylthio)-propionic acid remained constant during the entire experiment considered (data not shown).

To account for the disappearance of the sulfide species, the *S*-oxidized forms of the relevant molecules were taken into account. In particular, DMSO was quantified in the wine by slightly modifying a previously published paper.[26] The method adopted provided a good sensibility, and the data obtained (Table 4) were in agreement with the results found by previous authors.[26,45]

DMSO showed a significant increment during oxidative aging, differently from that found in dry red wine;[26] no clear correlation

Table 6. Mean (ppb) and Standard Deviation (SD) of Fermentative, Varietal, and Sulfur Compounds Analyzed and Tukey's Test Results to Test for Bardolino Wine Model Oxidative Aging Effects[a]

	control			15d OX			30d OX		
analyte	mean		SD	mean		SD	mean		SD
hexanol[b]	1204	a	2.3	913	b	4	940	c	5.2
2-phenyl ethanol[b]	30326	a	2105	33824	b	693	36237	b	279
benzyl alcohol[b]	376	ns	4.6	396	ns	107	442	ns	52
ethyl 4-hydroxybutyrate[c]	1302	a	64	1188	b	112	1374	a	11
benzaldehyde[b]	26.9	a	1.5	37.8	b	2.1	52.0	c	1.1
phenyl acetaldehyde[b]	16.4	a	0.2	57.7	b	1.2	40.7	c	2.9
furfurol[b]	106.4	a	0.9	203.2	b	18.3	308.0	c	5.9
5-methyl furfural[b]	2.1	a	0.1	3.9	b	0.2	5.0	c	0.2
N-methylbutyl acetamide[c]	1985	ns	55	1572	ns	496	1833	ns	211
N-ethylphenyl acetamide[c]	283	a	16	149	b	9	178	b	7
linalool[b]	12.1	a	0.2	11.1	bc	0.6	10.0	c	0.7
α-terpineol[b]	12.5	ns	1.1	15.8	ns	6.9	12.9	ns	0.3
citronellol[b]	3.5	a	0.7	2.7	ab	0.3	1.8	b	0.3
endiol[c]	36.4	a	1.6	32.1	b	2.4	41.0	a	3.0
4-terpineol[b]	0.7	a	0.2	1.0	ab	0.1	1.3	b	0.5
1-octen-3-ol[b]	9.0	ns	0.0	7.2	ns	0.5	6.3	ns	2.3
β-damascenone[b]	1.5	ns	0.5	1.7	ns	0.2	1.7	ns	0.1
TDN[c]	0.7	ns	0.1	0.9	ns	0.1	0.6	ns	0.1
vitispiranes[c]	2.8	a	0.2	5.4	b	0.3	4.4	b	1.4
actinidols[c]	10.9	a	0.4	19.1	b	2.9	28.9	c	0.5
3-oxo-α-ionol[c]	89.5	a	3.8	52.3	b	3.3	59.5	b	7.2
vanillin[b]	14.4	a	0.7	37.5	b	2.2	104.0	c	12.0
homovanillyl alcohol[b]	229	a	11	108	b	8	131	b	4
homovanillic acid[b]	35.4	a	0.9	12.7	b	2.1	13.6	b	1.6
methyl vanillate[b]	15.0	a	0.1	12.5	b	0.2	15.0	a	1.0
ethyl vanillate[b]	95	a	3.8	96	a	5	121	b	4
acetovanillone[b]	196	a	9.9	145	b	7	170	b	9
propiovanillone[c]	26.2	a	1.4	17.0	b	0.4	20.4	c	0.7
butyrovanillone[c]	52.2	a	2.7	20.4	b	5.3	44.3	a	0.4
syringaldehyde[b]	11.4	a	1.3	16.1	a	5.3	59.1	b	6.2
syringol[b]	6.5	a	0.2	8.9	a	1.6	13.5	b	0.3
acetosyringone[b]	17.0	a	0.6	9.9	b	0.2	13.7	c	1.5
dimethyl sulfide[b]	4.5	a	0.3	5.7	b	0.1	8.8	c	0.1
dethyl sulfide[b]	2.2	ns	0.2	2.4	ns	0.1	2.5	ns	0.2
S-methyl thioacetate[b]	1.1	ns	0.1	1.3	ns	0.2	1.2	ns	0.1
S-ethyl thioacetate[b]	1.0	ns	0.1	1.3	ns	0.2	1.1	ns	0.1
dimethyl disulfide[b]	5.5	ns	0.2	4.6	ns	0.4	6.4	ns	0.6
diethyl disulfide[b]	1.5	ns	0.1	1.3	ns	0.1	1.8	ns	0.2
2-(methylthio)-1-ethanol[b]	22.3	a	1.1	25.7	b	0.5	29.2	c	0.4
3-(methylthio)-1-propanol[b]	985	a	17	1363	b	43	1371	b	22
4-(methylthio)-1-butanol[b]	103	a	5.0	95	b	10.0	121	b	17.0
dimethyl sulfoxide[b]	13.2	ns	5.0	15.2	ns	2.2	16.2	ns	1.0

[a] Values with the same letter do not differ significantly in the Tukey's test, $p < 0.05$. ns, not significant. 15d Ox, 30d Ox: 15 and 30 days respectively of model oxidative aging. [b] Identification based on purchased reference compounds. [c] Tentative identification.

between DMSO and DMS kinetics can be drawn. In previous papers,[26,45] any putative relationship between DMSO and DMS was considered to be a complex one as other DMSO precursors are likely to play a role in the DMSO formation during model oxidation. In particular, it is believed that methionine-*S*-sulfoxide and other hypothesized but yet not known precursors might have a pivotal role in the formation DMSO in grape and wine.[26]

Another *S*-oxidized species tentatively identified in the oxidized Amarone wine is methionol-*S*-oxide. This molecule was first recognized in wines from *Vitis vinifera* L. cv. Scheurebe by Krammer and co-workers.[46] A qualitative reference standard was obtained by submitting a water/ethanol 90:10 solution (100 mL) spiked with methionol (1 ppm) to a forced oxidation, according to the conditions reported in the literature.[47] Then 5 mL of this

solution were then transferred to a SPME 20 mL vial and submitted to HS-SPME/GC-MS analysis in SCAN mode. This reaction led to several products, but at 49.54 min, it was possible to recognize a peak with mass fragmentation identical to that reported by Krammer et al. (Figure 2).

Table 5 reports the data for methionol-*S*-oxide fragmentation reported by Krammer et al.,[46] and the MS data obtained for the methionol-*S*-oxide tentatively identified in our experimental conditions. The fragmentation pattern is in perfect agreement, providing us with the retention time of methionol-*S*-oxide. Even though a precise quantification was not performed, it was possible to observe a significant increment of the tentatively identified methionol-*S*-oxide signal during the forced oxidative aging, while such an increment was not observed during the model aging under nitrogen. Such behavior would be in agreement with that found for DMS with the formation of the relevant *S*-oxide form (i.e., DMSO), suggesting a possible similar pathway.

According to the literature, this molecule could have a strong impact on wine aroma as it is described as having cheesy/putrid attributes.[46] No information on its sensory threshold is available.

A connection among the particular evolution profiles observed for sulfur compounds and other aroma compounds in the oxidative aging of Amarone wine, the withering process of grapes, and the presence of the *Botrytis* infection was envisaged. To check for this hypothesis, a dry red wine produced from fresh grapes of the same varieties such as Bardolino wine was analyzed. The wine (375 mL) was put into a dark glass bottle (375 mL ullage volume of air) and stored at 30 °C for 0 (i.e., control), 15, and 30 days, respectively. These samples were then submitted to SPE-GC-MS and HS-SPME/GC-MS analyses. Table 6 reports the mean values of measured fermentative, varietal, and sulfur compounds and the Tukey's test results.

Benzaldehyde increment in the first 15 days appeared slower in Bardolino wine. Also other benzenoids showed an increment in Bardolino, which is far slower than that observed for the oxidative aging of Amarone.

Noteworthy is the behavior of sulfur compounds in the wine produced from fresh grapes. MTP does not decrease during oxidative aging, while in Amarone wine it was almost all depleted after the first 15 days. The same behavior can be observed for other sulfide species (DMS, DES, MTE, and MTB).

This particular difference for sulfur compounds evolution between *Botrytis* and non-*Botrytis* red wine confirms how crucial the grape withering process is on the aroma formation of Amarone wine. Zamboni et al.,[48] elucidating the molecular mechanisms of Corvina grape withering, revealed that berry dehydration triggers a number of different responses including those involved in several metabolic processes. Hence, the modification of berry composition due to the post harvest dehydration, favored by *Botrytis cinerea* when present, greatly conditions the fermentation products. Due to the potential importance of fermentative sulfur compounds on the aroma complexity and quality of red wines in connection with their extremely low sensory threshold, more investigations are required.

The current research permitted to highlight particular oxidative evolutions that have not been reported for other wines. In this work, by choosing a dry red wine obtained from withered grape berries bearing a *Botrytis* infection, a compound never identified in sweet and dry *Botrytis* white wine is reported. This compound (e.g., methionol-*S*-oxide) could play a pivotal role in the aroma definition of wines even if more studies are necessary.

It was noticeable that some benzenoid derivatives in the Amarone (i.e., benzaldehyde, phenyl acetaldehyde, syringaldehyde, and vanillin) showed a peculiar evolution during oxidative aging if compared to the evolution measured in the Bardolino. Moreover, most noteworthy, all sulfide species were significantly depleted after the first 15 days in the Amarone oxidative aging, while a completely different behavior was observed for the Bardolino. All these evidence suggest a potential involvement of the withering process and of *Botrytis* in these pathways.

Finally, according to the informal sensory analysis performed on our samples by a group of Amarone winemakers, oxidative aging seems to provide the wine with the peculiar typicality which makes Amarone one of the most renowned wines in the world.

AUTHOR INFORMATION

Corresponding Author

*Phone: +39 045 8200901. Fax: +39 045 8331805. E-mail: bruno.fedrizzi@unipd.it.

DEDICATION

We dedicate this work to Dr. Giuseppe Versini, who recently passed away. All of us are deeply indebted to Dr. Versini for his fundamental teachings and support throughout these years.

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