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Evolution of phenolics and glutathione in Verdicchio wine obtained with maceration under reductive conditions

Giuseppe Di Lecce [a], Emanuele Boselli [b,*], Giuliano D'Ignazi [c], Natale G. Frega [b]

[a] Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Barcelona, Spain
[b] Department of Agricultural, Food and Environmental Sciences, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy
[c] Terre Cortesi Moncaro, Montecarotto, Italy

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ABSTRACT

The aim of this study was to evaluate the influence of different skin contact percentage (0, 150 and 600 g L^{-1} of pomace), on the phenolic content and on the reduced glutathione levels in white Verdicchio musts and in the derived wines by using reductive conditions in an industrial winery. The results were compared with those from a control must obtained without maceration. The samples were taken after pressing, during alcoholic fermentation and after six months of storage. The skin contact in low-oxygen atmosphere lead to an increase of the phenolic content and glutathione in must and white wine. At the end of fermentation, the macerated samples with pomace at 600 g L^{-1} showed higher reduced glutathione and phenolic content, with respect to the other samples. Glutathione and tyrosol were positively correlated with yeast activity but then decreased during ageing. Ethyl caffeate increased in all samples during fermentation and wine ageing, thus confirming its origin as a condensation product between caffeic acid and ethanol after the hydrolysis of caftaric acid.

1. Introduction

Reductive winemaking has been used for the production of high quality white wines since a couple of decades. It consists of the maceration of the must of white grapes in a low-oxygen atmosphere obtained through the addition of carbon dioxide, nitrogen, argon or their mixture (Di Lecce, Arranz, Estruch, & Lamuela-Raventós, 2011). Maceration is usually conducted in the presence of antioxidants such as ascorbic acid and sulphur dioxide (Antonelli, Arfelli, Masino, & Sartini, 2010), and allows to obtain white wines with an increased content of aroma compounds and precursors, such as the pleasant varietal thiols (Dubourdieu, Tominaga, Masneuf, Peyrot des Gachons, & Murat, 2006), and phenolic antioxidants (Darias-Martin, Rodriguez, Diaz, & Lamuela-Raventos, 2000; Falqué & Fernandez, 1996; Ough, 1969).

There is a tight functional connection between phenols and glutathione (GSH), a tripeptidic antioxidant present in grapes (Adams & Liyanage, 1993), musts (Cheynier, Souquet, & Moutounet, 1989; Dubourdieu & Lavigne-Cruege, 2003), and white wines (Lavigne, Pons, & Dubourdieu, 2007). The thiolic group of GSH can reduce the ROS (reactive oxygen species) and regenerate quinones to the native phenolics (Singleton, Zaya, Trousdale, & Salgues, 1984).

Several studies have demonstrated that GSH levels in different varieties of grape may range from 17.3 to 114 mg kg^{-1}, whereas the content of different musts was observed to range between 1 and 20 mg L^{-1} (Cheynier et al., 1989). GSH accounts for about 10 g kg^{-1} of dry weight of *Saccharomyces cerevisiae* and represents more than 95% of the low molecular mass thiol pool. It is delivered by yeasts at the end of alcoholic fermentation (Elskens, Jaspers, & Penninckx, 1991). The GSH level in white wine depends on the content in the musts and on the quality of the ethanolic fermentation; it decreases during ageing (Lavigne et al., 2007). The very strong reactivity of the reduced GSH with both oxygen and oxidized phenolic compounds probably explains why little research has been done into assaying reduced GSH in must and wine.

In previous works, caffeic acid, GSH and sulphur dioxide slowed the decrease of several volatile esters and terpenes in white wine (Roussis, Lambropoulos, & Tzimas, 2007). As a commercial application, producers of ingredients on industrial scale provide formulas of encapsulated or inactivated yeasts releasing certified amounts of glutathione (in the order of 20–30 g kg^{-1} of the formulation weight) as a fermentation nutrient and/or as a preserver (antioxidant) of the aromatic profile after fermentation.

The phenolic composition and concentration play a crucial role in determining the flavour sensory profile of white wine depending

* Corresponding author. Tel.: +390712204923; fax: +390712204980.
E-mail address: e.boselli@univpm.it (E. Boselli).

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on the *cultivar* used, on the agronomical conditions and practices such as maturity stage (De Simón, Hernández, Estrella, & Gómez-Cordovés, 2005), seasonal conditions (Ojeda, Andary, Kraeva, Carbonneau, & Deloire, 2002), production area (Broussaud, Cheynier, Asselin, & Moutounet, 1999), and fruit yield (Mazza, 1995). Flavan-3-ols are responsible for the astringent taste, bitterness and the structure of wines (Singleton & Esau, 1969), and the browning reactions in grape and wine (Macheix, Sapis, & Fleuriet, 1991). Hydroxycinnamic acids are the major phenolic compounds present in the pomace and their content increases with the contact time between must and the solid parts (Fuhrman, Volkova, Suraski, & Aviram, 2001).

Recent studies show that phenolic acids (e.g., caffeic acid) and flavonoids are absorbed in man and mice and are found in urine and plasma in free and deconjugated forms (Azuma et al., 2000; Choudhury, Srai, Debnam, & Rice-Evans, 1999). In a previous work (Frega, Boselli, Bendia, Minardi, & Benedetti, 2006), it was shown that ethyl caffeate (CfE) is a natural phenol present only in wine in consequence of the condensation of caffeic acid present in the berry flesh with ethanol formed after alcoholic fermentation. This compound is more lipophilic than the free form of caffeic acid (and presumably more readily absorbed by cell membranes), thus, its bioavailability is expected to be higher than non esterified phenolics such as anthocyanins or catechins. Boselli, Bendia, Di Lecce, Benedetti, and Frega (2009) have also demonstrated that CfE extracted from Verdicchio wine may exert hepatoprotective and antifibrotic effects by acting both at the cellular and molecular level in *vitro* and in *vivo*.

Verdicchio is the main local white grape variety cultivated in the Marche region (Central Italy). The aim of this work was to study the effect of maceration, at different pomace percentages (0, 150 and 600 g L^{-1}), and under reductive conditions, on the phenolic fraction and GSH level of Verdicchio must and wine, during alcoholic fermentation and after six months of ageing. Two different batches of Verdicchio grapes with different sugar content were selected for the experiment. The winemaking process was carried out on industrial scale in two batches of 300 or 10 hL.

2. Material and methods

2.1. Sampling procedure

Samples of white musts and wines were obtained from Verdicchio grapes in reductive conditions as follows: the clusters were manually harvested at the Terre Cortesi-Moncaro winery in Montecarotto (Italy) and immediately cooled to (5–10 °C) with dry ice prior to crushing. Powdered potassium metabisulfite (50–100 mg kg^{-1}) and ascorbic acid (30–50 mg kg^{-1}) were added to protect the must from oxidation.

The first batch of samples was obtained by selecting Verdicchio grapes with a total sugar content of 250 g L^{-1}. After crushing, the juice was subdivided in three aliquots: C was the control must constituted by 300 hL of must; M15 was the must subjected to maceration with 150 g L^{-1} of the pomace and M60 was the must with 600 g L^{-1} of pomace. M15 and M60 had a volume of 10 hL each, and were obtained by removing part of the must, thus increasing the pomace content up to 150 g L^{-1} and 600 g L^{-1}, respectively. Maceration was achieved only in the M samples and lasted for the entire period of the alcoholic fermentation, which was conducted at 11–15 °C. After racking, the wine was aged in a stainless steel tank for 6 months. The sampling procedure was conducted on the free run juice (j) at the beginning (s), during (m) and at the end (e) of the alcoholic fermentation and after a 6-month period of ageing of the wine (sample called 6) in a stainless steel tank. Thus, the following samples were obtained: C, sC, mC, eC and 6C for the control must; sM15, mM15, eM15 and 6M15 for the wine obtained with 150 g L^{-1} of pomace; sM60, mM60, eM60 and 6M60 for the wine obtained with 600 g L^{-1} of pomace.

The second batch of samples was obtained selecting Verdicchio grapes with a total sugar content of 26.5 g 100 mL^{-1} (Superiore samples) and the sampling was conducted on the free run juice (jS), after decantation (dS) at the beginning (sS), during (mS) and at the end (eS) of the alcoholic fermentation and after a 6-month period ageing of the wine (sample called 6S) in stainless steel tanks. The winemaking process was conducted in a 300 hL tank and the wine was maintained *sur lie* after fermentation and racking.

A readily assimilable nitrogen (APA) content of 150–160 mg L^{-1} was assured during the alcoholic fermentation of all types of wines.

2.2. Reagents and chemicals

All the solvents used were HPLC grade. *o*-Phtalaldehyde, reduced GSH, 2-aminoethanol and EDTA were purchased from Sigma–Aldrich (Milan, Italy); sodium borate and sodium acetate buffer were purchased from Fluka (Buchs, Switzerland).

Gallic acid, protocatechuic acid, caffeic acid, tyrosol, *p*-coumaric acid, (+)-catechin, ferulic acid, (−)-epicatechin and quercetin, used to confirm and quantify phenolic compounds, were purchased from Extrasynthese (Gensy, France), Fluka (Buchs, Switzerland) and Sigma–Aldrich (Milan, Italy). All the samples were filtered through Minisart RC15 (0.2 μm) regenerated cellulose syringe filters (Sartorius AG, Göttingen, D), prior to HPLC analysis.

2.3. GSH analysis

The procedure described by Park, Boulton, and Noble (2000) was used for the derivatization and analysis of reduced GSH with slight modifications: *o*-phtalaldehyde (2 mg) was dissolved in 5 mL methanol, while 2-aminoethanol (2 μL) was added to 5 mL of 0.8 mol L^{-1} solution of sodium borate (pH 7.4). An aliquot of must (150 μL) was added to 20 μL of *o*-phtalaldehyde and 2-amminoethanol. The mixture was shaken for 1 min and immediately injected into the HPLC system. The stock solution of standard was prepared in 1 mmol L^{-1} solution of sodium acetate buffer (pH 4.0) containing 0.02 mmol L^{-1} EDTA.

The HPLC system consisted of a ternary pump Varian 240 (Walnut Creek, CA, USA) and a Chrompack (250 mm × 4.6 mm) column packed with Chromspher C18, 5 μm particle size (Middelburg, Netherlands). The mobile phase A was an aqueous solution with formic acid 45 mL L^{-1} (v/v) and the mobile phase B was acetonitrile at a flow rate of 1 mL min^{-1}. The injection loop was 20 μL. The solvent gradient program was the following: at time 0 min, 10%B; at 2 min, 15%B; 6 min, 28%; 7 min, 32%; 9 min, 36%; 11 min, 44%; 12 min, 48%; 19 min, 50%; 21 min, 60%; 25 min, 68%; 28 min, 80%, 30 min, 10%. The fluorescence detector was a Jasco 821FP (Hachioji City, Japan) operating at an excitation and emission wavelength of 340 and 450 nm, respectively. All the data were acquired using a DataJet integrator by Spectra-Physics (Milan, Italy). The quantitative data was obtained by using a calibration curve of a standard solution of reduced GSH (from 0.1 to 10 mg L^{-1}). Data are reported as the average of three determinations.

2.4. Characterization of phenolic compounds in musts and wines

The separation of phenolic compounds was obtained by using the same HPLC system and solvents as described in the previous section. However, a diode array detector (DAD) and an ion trap mass spectrometer were coupled to the HPLC pump in order to identify and quantify the phenolic compounds according to a previous work (Boselli, Minardi, Giomo, & Frega, 2006). At time zero, mobile phase A was pumped isocratically for 5 min; then the

mobile phase B was increased from 0 to 15% from 5 to 40 min. Successively, the mobile phase B reached 40% in 25 min and was held for 20 min; finally, the mobile phase A returned to 100%. The HPLC flow rate was 700 μL min^{-1} and the injection loop was 20 μL. The DAD was a Varian Prostar 330 monitoring from 200 to 700 nm wavelength. The chromatograms were registered at three wavelengths: 280, 320, and 365 nm. Each wavelength was suitable for a peculiar class of compounds: 280 nm was used for hydroxybenzoic acids, flavan-3-ols and procyanidin, 320 nm for hydroxycinnamic acids and their tartaric esters, and 365 nm for flavonols. The data were acquired using the Varian Star 6.3 software. For structural elucidation, the HPLC system was coupled on-line with an LCQ ion-trap mass spectrometer (Finnigan, San José, CA, USA). Mass spectra were obtained using electro-spray negative ionization (ESI^-) of eluting HPLC peaks. The HPLC effluent was split and a flow of 0.1 mL min^{-1} entered the mass spectrometer through a steel ionization needle set at 5 kV and a heated capillary set to 200 °C. The capillary voltage was 3 V and the sheath gas flow was approx. 60 arbitrary units. The ion source and the optical parameters were optimized with respect to the negative molecular related ions of caffeic acid (m/z 179) and (+)-catechin (m/z 289); the mass range was 100–1000 m/z with a scan rate of 3 mscan s^{-1}. The tandem mass experiments were carried out with relative collision energy of 30–40%. All the data were acquired with the Excalibur software ver. 1.2 by Finnigan.

Individual compounds were quantified using a calibration curve of the corresponding standard compound. When reference compounds were not available, the calibration of structurally related substances was used. The data are reported as the average of three determinations.

2.5. Statistical analysis

The significance of differences at a 5% level ($P < 0.05$) between selected means was determined by using the 7.5.2 version of XLSTAT (Addinsoft, New York). Principal components analysis (PCA) was performed in order to get an overall overview of potential connections between the process variables, the phenolic profile, reduced GSH levels and ethanol content of the samples. The PCA was performed by using Unscrambler software (CAMO, Corvallis, USA). The data were normalized by the standard deviation of the samples.

3. Results and discussion

The grape was characterized by a pH value about 3.30–3.35. A difference of 1.5 g L^{-1} in the sugar content of the two must batches (j and jS) meant a difference of about 0.9 in the final alcohol content of the wine. The GSH levels and the phenolic individual concentrations monitored during the winemaking process and ageing of Verdicchio wine are reported in Tables 1 and 2.

3.1. Characterization of reduced GSH

The highest level of GSH was less than 2 mg L^{-1} and was reported for mM60, the must containing 600 g L^{-1} pomace sampled during fermentation (Tables 1 and 2).

The reduced GSH concentration was 0.51 mg L^{-1} immediately after pressing (j). Successively, at the start of fermentation, the GSH level increased. In the C group, the GSH levels already decreased by 32% at the middle of fermentation with respect to the initial content. Instead, the samples obtained by maceration (M15 and M60) showed a bell-shaped trend: an increase of 78% and 213% in the samples mM15 and mM60 was obtained, respectively. In macerated musts, reduced GSH level at the end of fermentation was 38% and

Table 1
Phenolic content and reduced GSH levels in Verdicchio juice, must and wine.

	j	C				M15			M60			
		sC	mC	eC	6C	sM15	mM15	eM15	6M15	sM60	mM60	eM60	6M60
Gallic acid (gal)[1]	nd	2.07 ± 0.1	5.17 ± 0.3	1.71 ± 0.1	2.75^{b} ± 0.1	5.84 ± 0.6	8.72 ± 0.3	7.99 ± 1.1	3.26^{a} ± 0.2	8.08 ± 0.5	10.6 ± 0.9	17.5 ± 1.1	2.62^{b} ± 0.0
Protocatechuic acid (protocat)[2]	nd	nd	2.99 ± 0.0	nd	1.90^{b} ± 0.0	Nd	nd	4.00 ± 0.1	5.91^{a} ± 0.2	nd	nd	10.9 ± 0.7	5.25^{b} ± 0.2
Caftaric acid (caft)[3]	19.5 ± 1.2	23.3 ± 1.1	11.8 ± 0.9	14.1 ± 1.1	$26.9^{a,b}$±3.3	18.3 ± 1.5	27.1 ± 1.7	28.6 ± 1.6	27.7^{a} ± 1.9	26.0 ± 1.1	48.2 ± 3.1	49.3 ± 2.7	23.4^{b} ± 0.9
Tyrosol (tyr)[4]	nd	4.72 ± 0.1	12.6 ± 0.7	14.5 ± 0.9	8.44^{c} ± 1.2	17.8 ± 1.1	15.0 ± 1.0	17.8 ± 0.7	26.7^{a} ± 0.9	13.0 ± 0.5	22.8 ± 0.7	18.1 ± 1.1	20.8^{b} ± 0.8
cis-coutaric acid (ccout)[5]	1.01 ± 0.0	0.90 ± 0.0	1.04 ± 0.0	1.11 ± 0.0	2.22^{b} ± 0.1	3.78 ± 0.2	4.80 ± 0.3	4.42 ± 0.2	2.79^{a} ± 0.1	4.64 ± 0.5	7.47 ± 0.9	8.68 ± 0.7	2.23^{b} ± 0.0
trans-coutaric acid (tcout)[5]	2.36 ± 0.1	0.31 ± 0.0	1.03 ± 0.0	1.23 ± 0.0	3.40^{b} ± 0.3	4.73 ± 0.3	9.51 ± 0.7	11.0 ± 0.9	4.33^{a} ± 0.2	6.37 ± 0.3	20.2 ± 1.1	33.6 ± 4.1	2.71^{c} ± 0.1
GRP[3]	13.7 ± 0.9	8.38 ± 0.3	6.03 ± 0.7	5.83±	4.37^{c} ± 0.3	9.97 ± 0.0	2.00 ± 0.0	3.94 ± 0.3	8.99^{a} ± 0.7	11.7 ± 1.1	4.96 ± 0.5	2.46 ± 0.1	6.74^{c} ± 0.1
(+)-catechin (cat)[6]	7.41 ± 0.5	2.52 ± 0.0	5.43 ± 0.5	nd	8.75^{b} ± 0.6	13.6 ± 1.1	2.65 ± 0.0	9.44 ± 0.4	13.6^{a} ± 0.9	nd	24.6 ± 2.7	30.4 ± 2.1	7.08^{c} ± 0.3
Caffeic acid (caff)[3]	3.80 ± 0.1	4.90 ± 0.3	1.64 ± 0.0	2.02 ± 0.0	3.40^{a}±	0.89 ± 0.0	nd	0.13 ± 0.0	4.18^{a} ± 0.4	2.16 ± 0.0	0.30 ± 0.0	1.60 ± 0.0	5.03^{a} ± 0.1
Fertaric acid (fert)[7]	1.46 ± 0.0	0.51 ± 0.0	0.13 ± 0.0	0.68 ± 0.0	0.56^{a} ± 0.0	0.40 ± 0.0	nd	0.23 ± 0.0	0.51^{a} ± 0.0	0.97 ± 0.0	nd	1.36 ± 0.0	0.63^{a} ± 0.0
p-coumaric acid (coum)[5]	1.17 ± 0.0	1.08 ± 0.0	0.59 ± 0.0	0.56 ± 0.0	0.07^{b} ± 0.0	0.63 ± 0.0	nd	nd	$1.66^{a,b}$±0.0	1.28 ± 0.0	0.24 ± 0.0	0.21 ± 0.0	2.61^{a} ± 0.0
(−)-epicatechin (epicat)[8]	10.7 ± 1.5	3.78 ± 0.0	nd	nd	7.95^{a} ± 0.3	2.56 ± 0.0	5.00 ± 0.3	6.24 ± 0.7	8.74^{a} ± 0.5	6.08 ± 0.3	10.6 ± 1.1	16.4 ± 1.1	4.99^{b} ± 0.3
Ferulic acid (ferul)[7]	nd	0.16 ± 0.0	0.05 ± 0.0	nd	0.54^{b} ± 0.0	0.13 ± 0.0	nd	0.08 ± 0.0	0.74^{a} ± 0.0	0.31 ± 0.0	nd	0.54 ± 0.0	0.37^{b} ± 0.0
cis-piceid (pic)[9]	0.76 ± 0.0	0.32 ± 0.0	0.63 ± 0.0	0.52 ± 0.0	1.11^{a} ± 0.0	0.96 ± 0.0	0.22 ± 0.0	1.21 ± 0.0	0.15^{b} ± 0.0	0.08 ± 0.0	nd	1.85 ± 0.0	0.60^{b} ± 0.0
Ethyl caffeate (CfE)[3]	0.27 ± 0.0	0.36 ± 0.0	0.61 ± 0.0	0.62 ± 0.0	2.10^{b} ± 0.0	0.13 ± 0.0	nd	nd	2.72^{a} ± 0.2	0.41 ± 0.0	nd	nd	2.35^{a} ± 0.1
Ethyl coumarate (CmE)[5]	0.07 ± 0.0	0.11 ± 0.0	0.28 ± 0.0	0.29 ± 0.0	0.92^{b} ± 0.0	0.12 ± 0.0	nd	nd	2.64^{a} ± 0.0	0.17 ± 0.0	nd	nd	1.22^{b} ± 0.0
trans-resveratrol (resv)[9]	nd	1.99 ± 0.0	2.90 ± 0.2	1.26 ± 0.0	Nd	1.28 ± 0.0	4.28±	2.29 ± 0.0	nd	3.53 ± 0.1	4.29 ± 0.2	0.43 ± 0.0	nd
Proanthocyanins (ProA)[6]	32.0 ± 1.8	44.6 ± 2.9	37.2 ± 2.5	40.7 ± 3.0	52.1^{b} ± 4.1	35.2 ± 2.9	39.3 ± 1.9	38.3 ± 2.1	47.7^{b} ± 2.9	36.2 ± 1.8	40.9 ± 2.3	40.0 ± 1.9	71.1^{a} ± 4.1
Sum (TP)	94.1 ± 3.6	100 ± 5.7	90.2 ± 4.1	85.1 ± 2.7	127^{b} ± 5.1	116 ± 6.3	118 ± 4.2	136 ± 5.1	163^{a} ± 7.1	121 ± 3.9	195 ± 9.7	233 ± 8.9	160^{a} ± 6.7
GSH[10]	0.51 ± 0.0	0.79 ± 0.0	0.54 ± 0.0	0.58 ± 0.0	nd	0.55 ± 0.0	0.98 ± 0.0	0.76 ± 0.0	nd	0.54 ± 0.0	1.77 ± 0.1	0.96 ± 0.0	nd

Phenolic compounds and reduced GSH quantified as: [1]mg gallic acid L^{-1}; [2]mg protocatechuic acid L^{-1}; [3]mg caffeic acid L^{-1}; [4]mg tyrosol L^{-1}; [5]mg *p*-coumaric acid L^{-1}; [6]mg (+)-catechin L^{-1}; [7]mg ferulic acid L^{-1}; [8]mg (−)-epicatechin L^{-1}; [9]mg quercetin L^{-1}; [10]mg reduced GSH L^{-1}. nd < 0.05 mg L^{-1}. The results are the mean values of three repeats. Different letters in the rows represent statistically significant differences ($p < 0.05$).

Table 2
Phenolic content and reduced GSH levels in Verdicchio Superiore juice, must and wine.

	S					
	jS	dS	sS	mS	eS	6S
Gallic acid (gal)[1]	nd	2.37 ± 0.2	11.1 ± 1.2	nd	11.5 ± 0.9	5.90 ± 0.3
Protocatechuic acid (protocat)[2]	7.72 ± 0.6	6.03 ± 0.6	7.66 ± 0.5	nd	9.77 ± 2.4	nd
Caftaric acid (caft)[3]	28.2 ± 3.7	43.9 ± 3.9	34.1 ± 2.9	35.3 ± 2.3	41.1 ± 5.7	32.9 ± 3.1
Tyrosol (tyr)[4]	nd	nd±	19.8 ± 1.7	nd	18.2 ± 1.2	14.9 ± 0.9
cis-coutaric acid (ccout)[5]	3.04 ± 0.1	2.80 ± 0.3	2.88 ± 0.2	3.30 ± 0.5	4.64 ± 0.3	4.37 ± 0.8
trans-coutaric acid (tcout)[5]	4.86 ± 0.3	8.95 ± 0.7	5.37 ± 0.2	5.67 ± 0.5	7.30 ± 0.5	8.50 ± 0.7
GRP[3]	19.4 ± 2.3	11.4 ± 0.9	7.42 ± 0.6	8.35 ± 0.9	11.5 ± 1.3	4.39 ± 0.3
(+)-catechin (cat)[6]	nd	nd	8.06 ± 0.8	11.0 ± 1.5	22.9 ± 1.1	16.0 ± 1.3
Caffeic acid (caff)[3]	nd	nd	2.48 ± 0.0	2.95 ± 0.1	3.75 ± 0.3	2.71 ± 0.1
Fertaric acid (fert)[7]	0.34 ± 0.0	1.36 ± 0.0	0.61 ± 0.0	0.94 ± 0.0	2.52 ± 0.1	0.60 ± 0.0
p-coumaric acid (coum)[5]	nd	1.41 ± 0.0	1.21 ± 0.0	0.94 ± 0.0	2.10 ± 0.0	3.05 ± 0.4
(−)-epicatechin (epicat)[8]	nd	14.1 ± 2.1	5.63 ± 0.2	11.6 ± 1.3	10.3 ± 0.8	14.7 ± 1.9
Ferulic acid (ferul)[7]	nd	0.58 ± 0.0	0.55 ± 0.0	0.47 ± 0.0	1.32 ± 0.0	0.40 ± 0.0
cis-piceid (pic)[9]	nd	3.89 ± 0.2	3.34 ± 0.0	3.25 ± 0.3	4.41 ± 0.2	1.23 ± 0.0
Ethyl caffeate (CfE)[3]	nd	nd	0.59 ± 0.0	0.69 ± 0.0	1.29 ± 0.0	1.42 ± 0.0
Ethyl coumarate (CmE)[5]	nd	nd	0.37 ± 0.0	0.44 ± 0.0	0.77 ± 0.0	1.38 ± 0.0
trans-resveratrol (resv)[9]	nd	nd	2.29 ± 0.0	1.03 ± 0.0	0.66 ± 0.0	0.20 ± 0.0
Proanthocyanins (ProA)[6]	40.5 ± 1.2	49.1 ± 6.7	42.9 ± 3.0	37.4 ± 1.9	45.5 ± 5.6	71.6 ± 4.9
Sum (TP)	104 ± 4.5	146 ± 9.1	156 ± 5.1	123 ± 3.8	199 ± 7.9	184 ± 6.1
GSH[10]	1.16 ± 0.0	0.97 ± 0.0	1.32 ± 0.2	1.37 ± 0.1	0.91 ± 0.0	nd

Phenolic compounds and reduced GSH quantified as: [1]mg gallic acid L^{-1}; [2]mg protocatechuic acid L^{-1}; [3]mg caffeic acid L^{-1}; [4]mg tyrosol L^{-1}; [5]mg *p*-coumaric acid L^{-1}; [6]mg (+)-catechin L^{-1}; [7]mg ferulic acid L^{-1}; [8]mg (−)-epicatechin L^{-1}; [9]mg quercetin L^{-1}; [10]mg reduced GSH L^{-1}. nd < 0.05 mg L^{-1}. The results are the mean values of three repeats.

77% higher compared to the initial values, unlike the control and the Superiore wine.

A similar trend was shown in the S samples (Table 2). S grapes reached a higher degree of maturity, however the must was not subjected to maceration. A decrease of reduced GSH was registered after the decantation step. After reaching a maximum at middle of fermentation, the GSH level decreased at the end of fermentation to the value obtained in the samples macerated with 600 g L^{-1} of pomace. A 6-months ageing period lead to the complete degradation of reduced GSH in all the wines.

3.2. Phenolic profile

The phenolic profile of Verdicchio Superiore (eS) obtained by HPLC-DAD (λ = 280 nm) is depicted in Fig. 1. During alcoholic fermentation, an increase of the sum of phenolics detected with HPLC (Table 1 and Fig. 1) at two different wavelengths (at 280 and 320 nm) depending on their maximum of absorbance, was registered in all samples, except in the control, which contained the lowest level of phenolics among the samples.

At the start of fermentation, sC, sM15 and sM60 samples showed a higher content of the sum of HPLC phenols with respect to the control must (j). In general (Table 1), the decrease of phenolic compounds during the fermentation of the control must was ascribable to caftaric acid, glutathionylcaftaric acid (GRP), caffeic acid and (−)-epicatechin.

In the samples subjected to skin-contact, the phenolic fraction increased (Table 1, M samples). As previously reported, the increase was higher in the samples subjected to maceration with a higher fraction of pomace. The main increase in M15 and M60 samples was shown for gallic acid, caftaric acid and *cis*- and *trans*-coutaric acid (Table 1). Caftaric, *cis* and *trans*-coutaric acid and fertaric are the cinnamic acids formed in the grape berries from the esterification of caffeic, coumaric and ferulic acid with tartaric acid, respectively. Verdicchio has been already reported as a good source of these congeners in previous work (Boselli et al., 2009). Since all the phenolic compounds are prone to oxidation, GSH can react with the quinonic form of the cinnamic acids through an electrophilic addition leading to the regeneration of the cinnamic form. Alternatively, the free forms of cinnamic acids can arise from the hydrolysis of the tartaric esters, leading to the biological precursors (caffeic, coumaric and ferulic acid).

These aspects were confirmed by an increase of caffeic acid registered after 6 months (6M15 and 6M60, Table 1) and the contemporary decrease of the tartaric esters in the macerated wines.

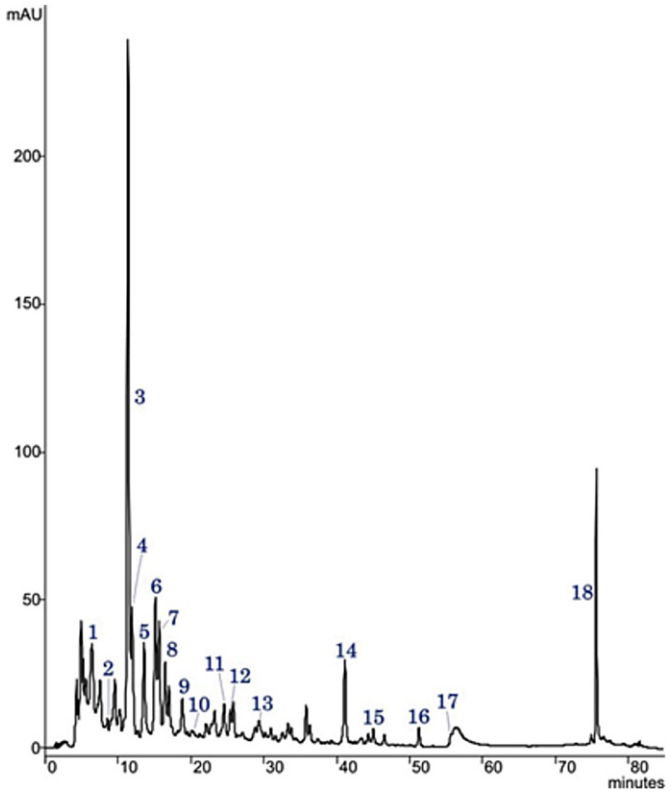

Fig. 1. Phenolic profile HPLC-DAD (λ = 280 nm, sample eS). 1) gallic acid; 2) protocatechuic acid; 3) caftaric acid; 4) tyrosol; 5) *cis*-coutaric acid; 6) *trans*-coutaric acid; 7) glutathionylcaftaric acid (GRP); 8) (+)-catechin; 9) caffeic acid; 10) fertaric acid; 11) *p*-coumaric acid; 12) (−)-epicatechin; 13) ferulic acid; 14) *cis*-piceid; 15) ethyl caffeate; 16) ethyl coumarate; 17) *trans*-resveratrol; 18) procyanidins.

In M60 group, the monomeric flavanols, (+)-catechin and (−)-epicatechin, considerably increased with respect to C and M15 samples. The flavan-3-ols are localised on the epidermis of grape and the maceration with pomace favours the migration in solution of (+)-catechin and (−)-epicatechin in the fermenting must. The eM60 showed (at the end of the fermentation) a phenolic content (233 mg L^{-1}) which was higher than eC and eM15 samples. The (+)-catechin content (Table 1) was higher only in the macerated samples. Successively, during six months of storage, catechin dimerized with formation of proanthocyanin cogeners, mainly dimer (B) and trimer (C) oligomers. Trans-Resveratrol is present in the epicarp of the grape; it was immediately available in the must and was detected in small concentration in all samples. Its concentration in the macerated musts was positively related to the percentage of pomace and the highest value was detected in M15 and M60 in the middle of fermentation (2.9 and 4.28 mg L^{-1}, respectively). In the control C, M15 and M60 samples, *trans*-resveratrol showed the same trend; it increased in the early stage of fermentation, then decreased. Probably, after the fermentation steps and during ageing of wine, *trans*-resveratrol was involved in the oxidation process and was therefore no more detectable.

After six months of storage, in 6M60 samples, a decrease of phenolic compounds was registered; this residual loss was ascribed to gallic acid, protocatechuic acid, caftaric acid, *cis*- and *trans*-coutaric acid and (+)-catechin. Instead, in 6C and 6M15, an opposite trend was observed; the sum of phenolic compounds increased by 49 and 20%, respectively. 6M15 reached about the same value of the 6M60 Verdicchio.

In Verdicchio Superiore musts (S), the content of phenolic compounds already increased in the first steps of winemaking (from jS to sS). Successively, a non linear trend was registered during fermentation and the highest concentration of phenolic compounds was detected at the end of fermentation (Table 2). The eS sample showed a higher phenolic content than eC and eM15, but lower (by 17%) than eM60. The phenolic content of Superiore decreased after ageing (6S) with respect to eS, due to the decrease of gallic acid, protocatechuic acid, caftaric and *trans*-coutaric acid, tyrosol, GRP and (+)-catechin.

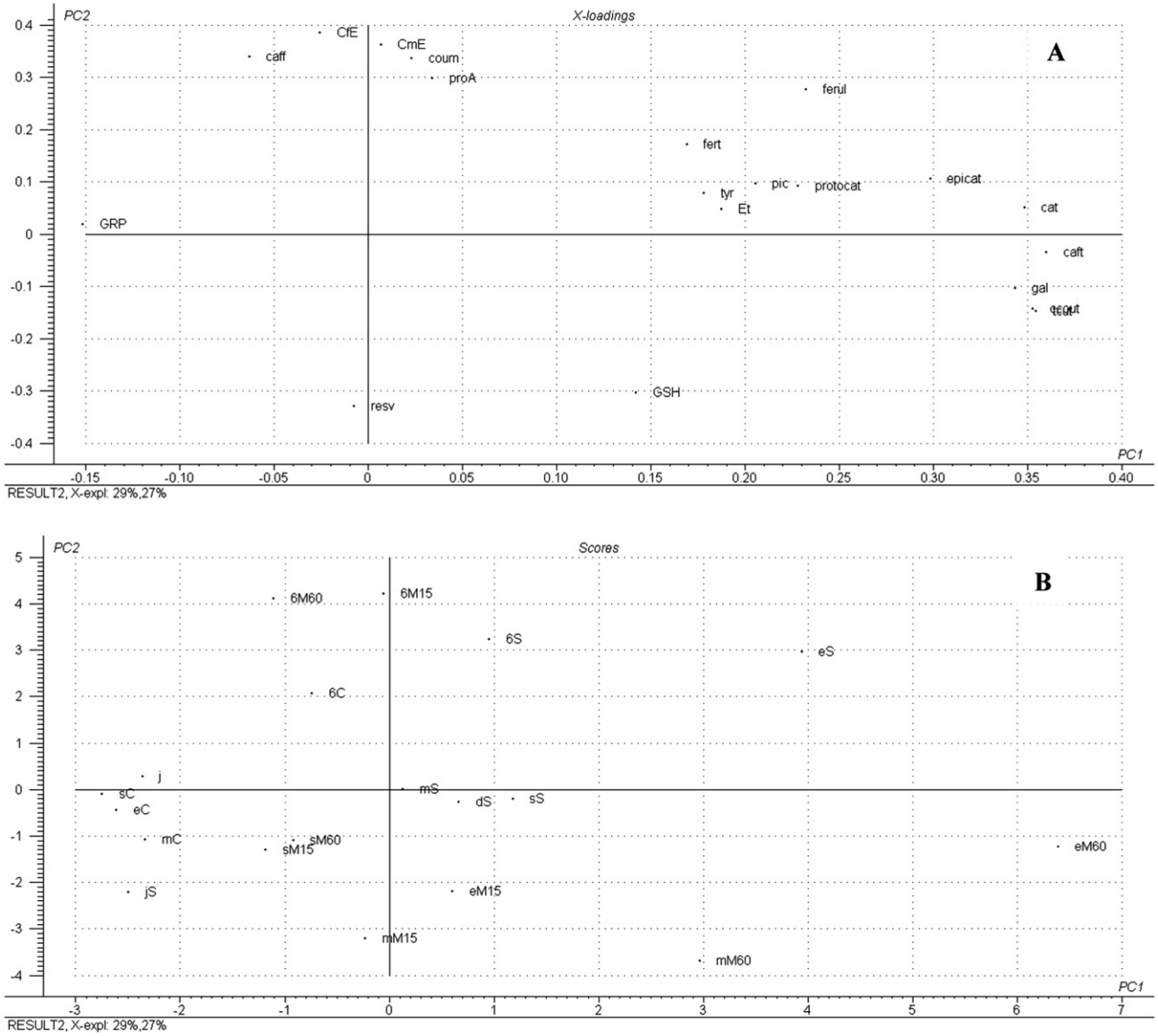

Fig. 2. PCA of the mean values reported in Table 1 plotted along the first and second principal components (p1 vs p2); A, loading plot of the variables; B, score plot of the samples.

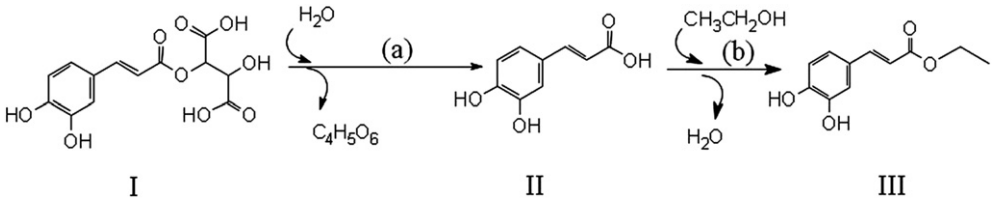

Fig. 3. Evolution of caftaric acid (I), caffeic acid (II) and ethyl caffeate (III) during alcoholic fermentation (a) and ageing of wine (b).

3.2.1. Ethyl caffeate (CfE)

Ethyl caffeate (CfE) is the condensation product of caffeic acid and ethanol and was found in Verdicchio in appreciable amounts showing significant biological activities *in vivo* (Frega et al., 2006). However, there are so far no data on the relationships among CfE, its precursors caftaric and caffeic acid and the influence of glutathione on the collateral reaction leading to the formation of GRP during the winemaking process.

In the C group, CfE increased after grape pressing in reductive conditions between the start and the end of alcoholic fermentation. CfE was absent during the fermentation in M15 and M60, probably due to the low content of free caffeic acid during fermentation, although these musts showed a higher content of caftaric acid with respect to the control. This experimental evidence shows that the direct precursor of CfE should be free caffeic acid, eventually after hydrolysis of caftaric acid. The direct formation of CfE from caftaric acid is therefore strongly limited by the steric hindrance of the latest compound.

After six months of storage, 6C, 6M15 and 6M60 showed a CfE content of 2.1, 2.7 and 2.3 mg L^{-1}, respectively, which was the highest concentration registered among the wines (Table 1). In the Superiore group (Table 2), CfE was not detected in the first step of winemaking (jS and dS samples). Successively, during fermentation, CfE increased from 0.59 to 1.29 mg L^{-1} and up to 1.42 mg L^{-1} after six month of storage. This trend is in agreement with what suggested above, because CfE increases when a higher concentration of free caffeic acid is present, such as in the 6C wine; in fact, the caftaric/caffeic ratio of Superiore wine after 6 months (12.1) is much higher than the Control after six months (7.9).

The ethyl esters of cinnamic acids showed a different fate compared to the other phenolic compounds. CfE and CmE increased during the fermentation but particularly during ageing of Verdicchio unlike all the other phenols and were well correlated with their acid precursors (as shown in the scatter plot of Fig. 2). This trend is well described in Tables 1 and 2 and was higher in the samples where the fermentation was conducted with maceration (M15 and M60).

3.3. Principal component analysis (PCA)

The first two components (PC1 and PC2) of PCA of the samples across the phenolic compounds, reduced GSH level and ethanol content, explained 56% of the total variance (Fig. 2). For the interpretation of the results, also the third component (PC3) was taken in account (16% of the total variance), however, for a better reading, only the plot obtained from the first two components were reported in Fig. 2. The loading plot (Fig. 2A) shows that the phenolics can be classified into two groups: caffeic acid, coumaric acid, their esters and proanthocyanin are higher in the wines stored for 6 months (Fig. 2B), and thus can be considered chemical markers of the ageing; CfE and CmE are well correlated with their acidic precursors, as reported above. Whereas, all the other phenolics except resveratrol are higher in the Verdicchio Superiore and in the wine macerated with 60% pomace, both just after primary fermentation. This can be due to the higher solubility and thus diffusion of phenolics from the solid parts of the grapes in a hydroalcoholic environment rather than in the must. The resveratrol content was also higher in the macerated musts (it diffuses from the pomace), but reached its maximum during the primary fermentation and then decreased.

Tyrosol showed a quite good correlation with the activity of the yeast and, therefore, with the ethanol content ($R^2 = 0.43$).

GRP was particularly higher in the musts at the start of fermentation, and then decreased. There was no correlation between GRP and GSH in the samples.

Moreover, the reduced GSH is to be found on the opposite site of the phenolic compounds which are markers of the ageing process (CfE, CmE, coum, ProA and caff); this means that the loss of reducing conditions after fermentation causes the decrease of reduced GSH, probably due to the condensation with caftaric acid and formation of GRP. This hypothesis is supported by the increase of GRP in the macerated wines during ageing, as it is reported in Tables 1 and 2.

4. Conclusion

The winemaking process under reduction combined with the skin contact allowed to obtain a fermented must with an increased content both of reduced GSH and of total phenolic compounds. The content of GSH and tyrosol increased together with the pomace percentage during the maceration phase. A bell-shaped trend of the GSH level was registered during fermentation with a maximum during the process. After six months of ageing, no more GSH was detected in the samples. The maceration under reduction conditions had also a positive influence on the content of the phenolic compounds; this increase was strictly related to the content of pomace (0–15–60%) rather than to the initial sugar content of the must obtained under reduction conditions.

The content of CfE increased in all samples during fermentation and especially during wine storage, thus confirming its origin as a condensation product between free caffeic acid and ethanol.

Caftaric acid increased in the first step of winemaking and during alcoholic fermentation. Successively, the hydrolysis of the ester bond of caftaric acid in the first six months of ageing lead to the release of free caffeic acid. In the same period (during the ageing of wine), the condensation between ethyl alcohol and caffeic acid formed CfE. This process is tentatively reported in Fig. 3.

In M15, M60 and S samples, the increase of CfE was directly related to the hydrolysis of caftaric acid to free caffeic acid.

To the best of our knowledge, this is the first time that the kinetic trends of CfE and hydroxycinnamic derivatives have been studied during the winemaking process of Verdicchio in reductive conditions.

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