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Keywords:	glutathione, HPLC, grape, winemaking

Monitoring of glutathione concentration in winemaking by a reliable HPLC analytical method

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ABSTRACT

Background and aims: Glutathione (GSH) is valued in winemaking as an effective antioxidant compound in must and white wine if its concentration exceeds a few milligrams per litre. In this paper, a previously reported analytical method for GSH quantification was further improved and applied to grape and wine samples.

Methods and Results: GSH was derivatized with p-benzoquinone and detected by HPLC/UV. The analytical method was sensitive (limit of quantification = 0.43 mg L^{-1}), linear (R^2 > 0.997), accurate (recovery = 101 % in grape juice and white wine) and repeatable (RSD = 3.1%). The monitoring of industrial-scale vinifications showed *Saccharomyces cerevisiae* had a main role in the GSH content in wine since the GSH of grape was poorly preserved following the pressing. Glutathione concentrations decreased during wine aging on the yeast lees.

Conclusion: Glutathione concentration widely changed during winemaking and its monitoring is essential for improving the wine flavor and preventing the oxidation. This method is a suitable tool for this purpose.

Significance of the study: The analytical method proposed is reliable, fast and easy-to-apply. It allows the assessment of the GSH in grape, must and wine, and the strong role of the yeast on the GSH content in the wine is reported.

Keywords: glutathione, HPLC, grape, winemaking.

Introduction

Glutathione (GSH) plays several well-known roles in winemaking. In must, it reduces the o-quinones arising from the enzymatic oxidation of hydroxycinnamoyltartaric acids, caused by the polyphenol oxidase enzyme (PPO). This hinders the formation of phenol polymers which are responsible for browning of must and wine (Salgues et al. 1986). Furthermore, GSH reduces oxidised caftaric acid to 2-S-glutathionylcaftaric acid, also known as Grape Reaction Product (GRP) (Singleton et al. 1984).

Glutathione levels higher than a few milligrams per litre in wine can effectively protect the varietal thiol compounds, by acting as a competitor for quinone reduction (Lavigne and Dubordieu 2004), since they are stoichiometrically thousands of folds higher than the amounts of varietal thiols. Other aroma compounds, such as isoamyl acetate, ethyl hexanoate and linalool, are also better protected by GSH during bottle storage (Papadopoulou and Roussis 2008, Roussis and Sergianitis 2008).

Glutathione can slow down the formation of sotolon (3-hydroxy-4,5-dimethyl-2(5H)furanone) and 2-aminoacetophenone, the major compounds responsible for the atypical aging character of white wine (Lavigne and Dubordieu 2004), especially when it is exposed to oxygen. Moreover, GSH can slow down the browning of white wine during aging and storage (Lavigne and Dubordieu 2004, Vaimakis and Roussis 1996).

The GSH concentration in wine can be affected by the GSH content in grape and conditions of must preparation, alcoholic fermentation (AF) and wine aging. Glutathione concentration up to 200 mg L^{-1} in grape juice has been reported depending on the grape cultivar, environmental conditions, viticultural practices (Cheynier et al. 1989), and the amounts of readily assimilable nitrogen in the soil (Lavigne and Dubordieu 2004). Values lower than 100 mg L^{-1} have been described for grape must

(Cheynier et al. 1989), related to exposure to oxygen, PPO activity and pre-fermentation grape skin maceration (du Toit et al. 2007, Maggu et al. 2007). The loss of GSH in must production can negatively affect the formation of precursors of the varietal thiol compounds (Roland et al. 2010) as well as the residual content of GSH during wine aging. Glutathione has been reported to be consumed by *Saccharomyces cerevisiae* at the beginning of the AF and then to be released by the yeast cell lysis (Lavigne and Dubordieu 2004, Lavigne et al. 2007). Glutathione concentration in wine is lower than in grape juice and must (up to 20 mg L^{-1}) (du Toit et al. 2007, Cassol and Adams 1995), but it can be increased by a suitable fermentative yeast strain (Rauhut 2009). Glutathione accounts for up to 0.5-1% of the dried weight of *S. cerevisiae* (Penninckx 2002) and its release in wine is affected by yeast growth conditions, such as nitrogen starvation during the AF (Lavigne and Dubordieu 2004).

The monitoring of GSH content during winemaking is essential to effectively address the winemaker toward the desired product throughout the winemaking steps as well as to preserve an high and effective antioxidant ability in the final wine since a number of factors are involved in the GSH fate. Several analytical methods have been proposed for GSH quantification in grape, must and wine (Kritzinger et al. 2013). Capillary electrophoresis and HPLC coupled with laser-induced fluorescence detection (Park et al. 2000, Lavigne et al. 2007, Janeš et al. 2010, Marchand and de Revel 2010) or mass spectrometry (du Toit et al. 2007) are usually adopted. Further approaches require atomic adsorption spectrometry (Bramanti et al. 2008) or enzymatic treatments with spectrophotometric measurement (Adams and Liyanage 1993, Cassol and Adams 1995). Such analytical methods cannot be easily applied to routine analyses in either enological laboratories or directly in cellar laboratories because of the lack of availability and cost

of the instrumentation. Moreover, some of these analytical techniques need highly qualified staff, which is an extra cost for cellar and analytical laboratories. An easy-to-apply and fast analytical method has been described by Tirelli et al. (2010) for the quantification of GSH and cysteinyl thiols in commercial yeast cell-wall fractions by derivatization with p-benzoquinone. The same analytical approach has been validated in grape juice and white wine by using an ultra-high performance chromatography equipment (Fracassetti et al. 2011), though it cannot be applied to assess the GSH concentration in grape since the grape juice analyzed was exposed to air and the copper residues on grape skin may have affected affecting the GSH concentration in juice tested. Moreover, the influence of the matrix composition on the analytical response was not fully investigated by the authors. This research aimed to assess the GSH concentration in grape, must and both red and white wine through a simple and automated high performance liquid chromatography (HPLC) method which has been intended to its use in laboratories with limited equipment. Suitable preparative conditions are mandatory for monitoring GSH concentration during must extraction and clarification since such winemaking steps are responsible of the chemical and enzymatic oxidations. The validated method was then applied to monitor the GSH concentration in triplicate fermentation and industrial scale winemaking processes in order to apply the proposed analytical approach.

Materials and Methods

Chemicals

All the chemicals were of analytical grade at least. 3-Mercaptopropionic acid (3MPA), p-benzoquinone (pBQ), potassium metabisulfite ($K_2S_2O_5$) and polyvinylpolypirrolidone (PVPP) were purchased from Fluka (Switzerland). Glutathione, cysteine (Cys), sodium

fluoride (NaF), ethylenediaminetetraacetic acid (EDTA), caffeic acid, ethanal, ethanol and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis, MO, US). Citric acid was purchased from J. T. Baker (Phillipsburg, NJ, US); HPLC grade methanol was from Panreac (Barcelona, Spain), and HPLC grade water was obtained by a Milli-Q system (Millipore Filter Corp., Bedford, MA, US).

Monitoring of glutathione levels in a triplicate fermentation

Glutathione concentration was monitored in a winemaking process with Chardonnay grape in three batches (6 t each) of hand harvested grape produced in Franciacorta area (Brescia, Italy) in vintage 2010 which were transferred to the winery by 25 kg capacity bins and softly pressed (P < 120 kPa) yielding 2.5 t of must per batch. The pressing was carried out by a 6 t capacity automatic press without removal of its inner air. The musts were transferred into 2.5 t capacity vats and $K_2S_2O_5$ was added (to give 60 mg L^{-1}). The musts were cooled down to 10°C and racked after 14 hours to remove the grape lees. The clear musts were warmed up to 18°C and then hydrated and active commercial dry yeast was added (250 g t^{-1}, IOC, Institut Oenologique de Champagne, Éspernay, France). During AF, temperature was kept at 24 ± 3°C and air was excluded from the must. The dry wines were racked into vats fitted with an air-tight seal and with the headspace flushed with nitrogen to remove air. The vats were air-tight sealed. The wine batches were kept on the yeast lees for 43 days and the yeast lees were weekly re-suspended in the wine by a built-in automatic mixer. The chemical parameters of wines (ethanol, sugars, pH, total and volatile acidity, free and total sulfur dioxide) were determined according to the official methods for wine analyses.

Monitoring of glutathione levels in winemaking

Glutathione concentration was further monitored in 14 different winemaking processes (Table 1) of 4 industrial-scale plants (25 – 40 t) in 2 Italian regions (Lombardia and Tuscany). For the 2009 vintage, must and wine samples were obtained from 4 winemaking processes carried out in 2 different wineries (coded as numbers 3 and 4) with 2 grape cultivars (Chardonnay and Verdicchio) and 4 *S. cerevisiae* strains, at least. For the 2010 vintage, the must and wine samples were obtained from 10 winemaking processes carried out in 4 different wineries (coded as numbers 1, 2, 3 and 4) with 3 grape cultivars (Chardonnay, Verdicchio and Vermentino) and 4 *S. cerevisiae* strains, at least. All the vinifications were carried out following the rational winemaking procedures usually adopted in the winery. These winemaking processes are coded and summarized in Table 1.

Grape sampling was carried out in triplicate by randomly collecting 1 kg of bunches for each sample from different bins (20-50 kg capacity) at the wineries just before the processing.

Must samples were drawn out of the press at the juice collector tank, at the AF tank both before and after clarification, after addition of the yeast inoculum and every 2-3 days during the AF. The AF rate was monitored by the residual content of the reducing sugars. Five to ten wine samples were regularly collected after the completion of the AF for up to 45 days of wine aging on the light lees. In order to prevent sample oxidation, 0.8 g L^{-1} of $K_2S_2O_5$ was added to must and wine samples. Each sample was drawn and analyzed in duplicate.

Preparation of grape, must and wine samples

Samples of pedicel-free grape berries (250-300 g) were randomly collected from the bunches and then vacuum packed into gas-tight plastic bags with 2 mL 0.12 M NaF and

0.5 mL 0.06 M EDTA. Assays were also carried out by adding 2 mM $K_2S_2O_5$ as antioxidant additive instead of NaF and EDTA. The grape berries were hand-crushed inside the bags and the juice was stored at room temperature for 60 minutes in contact with the berry skins. The juice was transferred to a beaker, homogenized under nitrogen flow for 3 minutes, and centrifuged at 5000×*g* for 5 minutes (benchtop centrifuge, Hettich, Tuttlingen, Germany). Two milliliters of clear juice were drawn and derivatized with pBQ (see below). Two bags of each grape sample were analyzed.

Must and white wine samples were centrifuged at 5000×*g* for 5 minutes. Twenty milliliters of red wine were stirred with PVPP (15 g L^{-1}) for 5 minutes and centrifuged (Sorvall, Thermo, Waltham, MA, US) at 5000×*g* for 5 minutes. Two milliliters of supernatant were added to 100 µL 16 mM ethanal and then left at room temperature for 15 minutes before derivatization (see below).

Unknown and standard grape, must and wine samples were analyzed in duplicate.

Preparation of 2-S-glutathionylcaffeic acid

2-S-glutathionylcaffeic acid was prepared in a citric buffer, 50 mM at pH 3.5 where GSH and caffeic acid were added in a molar ratio 4:1, as described by Cilliers and Singleton (1990). The solution contained GSH 360 µM (110 mg L^{-1}) and caffeic acid 90 µM (16 mg L^{-1}). Polyphenol oxidase was obtained as follows: a few grape berries were hand-crushed and the juice was centrifuged at 5000×*g* for 5 minutes at 15°C in a benchtop thermostatted centrifuge. The pellet was dispersed in the buffered GSH/caffeic acid solution and stirred for 5 minutes. Two milliliters of the suspension were derivatized with pBQ as described as follows, filtered through 0.22 µm pore size PTFE membrane (Millipore, Billerica, MA, US) and submitted to the HPLC separation. The

suspension was further stirred for 1 hour at room temperature and PPO was added to complete the formation of the 2-S-glutathionylcaffeic acid.

Derivatization of thiol compounds

Derivatization of GSH and Cys was carried out as described by Fracassetti et al. (2011). Two milliliters of sample were added to 100 µL of 400 µM pBQ dissolved in methanol. After 1 minute mixing, 1 mL of 500 µM 3MPA in 0.3 M citrate buffer at pH 3.5 was added in order to remove any excess of pBQ. The reaction mixture was filtered through a disposable PTFE filter, 0.22 µm pore size, prior to the HPLC separation.

Calibration curves

Calibration curves were obtained by spiking citrate buffer 50 mM at pH 5, white wine and red wine with 5 increasing amounts of GSH up to 50 mg L^{-1}. The grape juice, obtained as described as above, was spiked with GSH concentrations up to 100 mg L^{-1}. Similar Cys amounts were added to the grape juice sample. Determinations were performed in triplicate.

2-S-glutathionylcaftaric acid was quantified as caffeic acid. The calibration curve was obtained with caffeic acid solutions containing known amounts up to 50 mg L^{-1}.

Precision parameters

A red wine sample was spiked with the following concentration of GSH: 1.5 mg L^{-1}, 15.4 mg L^{-1} and 30.7 mg L^{-1}. The standard solutions were submitted to 5 replicated determinations in order to assess the precision parameters of the analytical method.

Recovery

Recovery was calculated by comparing three replicated determinations of samples, spiked and unspiked. For white and red wine, four different concentrations of GSH (1.8 mg L^{-1}, 3.5 mg L^{-1}, 7.0 mg L^{-1} and 17.5 mg L^{-1}) were added. The juice prepared as

described above was spiked with GSH (3.1 mg L^{-1}, 6.2 mg L^{-1}, 15.4 mg L^{-1} and 30.7 mg L^{-1}) and Cys (0.7 mg L^{-1}, 1.3 mg L^{-1}, 2.6 mg L^{-1} and 6.5 mg L^{-1}), as well.

Limits of detection and quantification

The limit of quantification (LOQ) was determined at a signal-to-noise ratio of 10:1 and the limit of detection (LOD) at a signal-to-noise ratio of 3:1. Baseline noise was calculated considering a 3 minutes-long peak-to-peak baseline in two parts of the chromatogram.

High-performance liquid chromatography separation

Reversed phase HPLC separation was performed with a Waters Alliance 2695 (Milford, MA, US) equipped with a photodiode array detector (Waters 2996) and a phenyl-hexyl column (250 x 4.6 mm, 5 µm, 110Å, Phenomenex, Torrence, CA, US). The eluents were (A) water/TFA 0.05% (v/v) and (B) methanol. The separation was carried out by increasing the methanol concentration in the eluent from 10% to 35% in 18 minutes followed by the column washing (4 minutes, 100% methanol) and re-equilibration (15 minutes). The flow rate was 1 mL min^{-1}. Column temperature was 28°C and the injection volume was 50 µL.

Chromatographic data were acquired from 250 nm and 600 nm wavelength and processed at 303 nm by Empower 2 software (Waters).

HPLC/Electrospray Ionization-Mass Spectrometry (ESI-MS)

Mass spectrometry detection of 2-S-glutathionylcaffeic acid and 2-S-glutathionylcaftaric acid was obtained by a LCQ Deca XP spectrometer, controlled by the Excalibur software (Thermo Finnigann Jose, CA, US) operated in positive ion mode. A post column flow splitter was used to introduce 1:15 of the HPLC flow stream into the ESI source. The ESI interface and the ion optics settings were as follows: spray

potential, 5.0 kV; nebulization gas (nitrogen) relative flow value, 10; capillary temperature, 275°C; and cone voltage, 30 V. Full-scan mass spectra were acquired scanning the range 50-800 m/z. Mass accuracy was ensured by calibration with a mixture of caffeine, reserpine, and the tripeptide phosphofructokinase (in methanol:water 1:1, 0.1% acetic acid) infused separately.

Statistical analysis

Statistical analysis was carried out by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US).

Results and discussion

Analytical method development

The analytical approach described by Tirelli et al. (2010) to assess the cysteinyl thiols in yeast cell-wall fractions and by Fracassetti et al. (2011) in juice and white wine, was applied to quantify GSH and Cys in grape, must and red wine, as well. The analytical conditions adopted allowed the separation of GSH and Cys as thio-substituted hydroquinones in grape juice, must and both red and white wines. The addition of NaF and EDTA to the grape samples inhibits phenol oxidation due to the PPO activity since EDTA is a chelating agent and NaF binds the copper ions (Janovitz-Klapp et al. 1990). Experiments were also carried out with 2 mM $K_2S_2O_5$ as antioxidant additive instead of NaF, but the analytical response was lower and poorly repeatable (data not shown).

The extraction yield of GSH from grape berries was monitored in freshly extracted juice stored up to 150 min at room temperature; the highest concentration was detected from 45 min to 75 min. The GSH quantification was not affected by the grape crushing temperature: experiments performed with and without thermostatting the juice at 20°C gave the same analytical response.

The analytical method showed linear response (R^2 > 0.997) for both GSH and Cys concentrations up to 50 mg L^{-1}. The calibration curves for the GSH quantification in citrate buffer and grape juice gave similar response factor (26.0 and 23.4 mAU s L mg^{-1}, respectively). Comparable response factor was also obtained for Cys quantification in grape juice (28.8 mAU s L mg^{-1}). The analytical response of GSH was 20% lower in spiked samples of red and white wines (19.4 and 19.1 mAU s L mg^{-1}, for white wine and red wine, respectively). Such a difference was not found by Fracassetti et al. (2011) since their grape juice was unprotected against air and PPO activity. This condition can decrease the analytical response of the grape juice to the level of the analytical response of the wine where the PPO activity is missing, instead. The lower analytical response observed in ethanol containing samples is probably due to the lower derivatization yield in the presence of ethanol (Fini et al. 2010, Zhao et al. 2011) and it has to be taken into account for the GSH quantification in wine if the derivatization of the external standard is not performed in 12% ethanol solutions.

Ethanal was added to SO_2-containing samples in order to bind the free SO_2 and to prevent the reduction of pBQ to p-hydroquinone. This allows the complete derivatization of Cys and GSH (Fracassetti et al. 2011).

The repeatability was assessed by spiking red wine with known and increasing concentrations of GSH (Table 2). The mean relative standard deviation (RSD) was 3.1% for GSH concentrations ranging from 1.5 to 30 mg L^{-1}.

The recovery was evaluated by GSH addition to either white or red wine as well as by GSH and Cys addition to grape juice. The GSH recovered amounts were 100.1%, 101.5% and 93.9% for grape juice, white wine and red wine, respectively. Similar recovery values were reported in literature for grape juice analyzed by other analytical

approaches but with our analytical conditions GSH recovery obtained in white wine was higher (du Toit et al. 2007, Janěs et al. 2010) or comparable (Marchand and de Revel 2010) to the methods previously reported. The lower recovery obtained with red wine was probably due to residual oxidised phenols not removed by the PVPP treatment. The Cys recovery in grape juice was 102.8%, comparable to the value obtained for GSH.

The LOD values were 0.13 mg L^{-1} and 0.07 mg L^{-1} for GSH and Cys, respectively, and the LOQ values were 0.43 mg L^{-1} and 0.21 mg L^{-1}, respectively. These GSH concentrations are much lower than the amount needed to exert an effective antioxidant activity in must and wine (Lavigne and Dubourdieu 2004).

2-S-glutathionylcaffeic acid was synthesized in order to use its UV absorption spectra as reference for the chromatographic detection of GRP, and to assess its chromatographic peak purity for the grape and wine samples analyzed. The formation of 2-S-glutathionylcaffeic acid from caffeic acid and GSH was confirmed to be fast (Singleton et al. 1985, Riberau-Gayon et al. 2006) and complete since no residual caffeic acid was found; a single S-glutathionylcaffeic acid derivative was detected by MS. The HPLC pattern obtained by the analysis of grape and wine samples showed an interference-free chromatographic peak with a UV absorption spectrum (λ_{max} = 326 nm) comparable to the 2-S-glutathionylcaffeic acid spectrum (Cheynier et al. 1986). The identification of GRP was eventually confirmed by HPLC -MS analysis.

Monitoring of glutathione levels in a triplicate fermentation

The reported analytical conditions were used to assess the GSH concentration during a triplicate fermentation. The GSH concentration detected in grape used for this triplicate fermentation was low (4.0±0.15 mg L^{-1}) and it further decreased in must (1.0±0.046 mg L^{-1}) following to exposition to air while pressing, probably due to the PPO activity.

Each AF occurred regularly in one week and no significant difference was found among the three wines produced, as shown by the chemical parameters analyzed in these wines (Table 3). The GSH was produced by the yeast activity (Figure 1) and the highest GSH concentration (12.4±1.4 mg L^{-1}) was detected at the end of the AF. Then, it decreased after the racking and continued to decrease during aging on the yeast lees (Figure 1).

Monitoring of glutathione levels in winemaking

Further indications of this behavior were obtained by monitoring 14 winemaking processes carried out under industrial-scale conditions. Though only single trials of each of them were performed and very different winemaking conditions were applied (Table 1), similar trends of GSH concentration were observed (Table 4). Low GSH concentrations (< 10 mg L^{-1}) were found in musts even if high GSH concentrations were contained in the grape (up to 84.3 mg L^{-1}) and air-free pressing conditions were applied. Nevertheless, the GRP concentration was lower in the must than in grape (Table 4) if GSH concentration in grape was lower than 35 mg L^{-1}. Such a phenomena can occur when quinones cannot be rapidly reduced by GSH, or other reducing compounds (i.e. sulfur dioxide), as it happens when the GSH concentration is as low as in the grapes evaluated. Under such a condition, quinones can oxidise other compounds in the must like GRP or other phenols (Singleton et al., 1984) owing to their high oxidation potential and reactivity (Danilewicz, 2012), leading to a lower GRP concentration. No release of GSH from the yeast lees could be observed. Our results are in agreement with the data reported by Andujar-Ortiz et al. (2012) and Mattivi et. al (2012) which observed the release of GSH by yeast and a decreasing concentration during the storage on the yeast lees. The highest GSH concentrations were always detected at the end of the AF, then GSH concentration decreased during aging on the

yeast lees. In spite of previous researches (Lavigne et al., 2007; Lavigne and Dubourdieu, 2004), our data give some indications that the yeast lees could not be useful to increase the GSH content in wine. On the contrary, the yeast lees ability of binding the thiol compounds responsible for the reduced defects is known (Lavigne and Dubourdieu, 1996), should be considered and further investigated as also responsible for the GSH loss during the wine aging. Moreover, decrease of GSH concentrations was not observed in must at the beginning of the AF (Figure 1) (Lavigne and Dubourdieu, 2004).

Our analytical approach allowed a reliable evaluation of GSH concentration during the winemaking and it is useful tool for the assessment the winemaking steps affecting GSH concentration in wine. In all the industrial-scale winemaking processes followed, *S. cerevisiae* showed a major role on the GSH content in wine since it produced GSH in concentrations by far higher than the amounts preserved during the extraction of musts.

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Figures' captions

Figure 1: Evolution of GSH concentration (continued line) and progression of alcoholic fermentation expressed as residual sugars (dotted line) in the monitored triplicate fermentation. The bars report the standard deviation of the values (n=3).

Table 1: sample coding of industrial-scale vinifications monitored during 2009 and 2010 vintages. Different numbers and letters in the vinification codes indicate different wineries and grape batches, respectively.

*: commercial yeast strains are referred as indicated by the producer.

Vinification code	Grape	Must exposure to O_2	Vintage	Region	Harvest	Yeast strain*	Must aeration during alcoholic fermentation	Aging on the yeast lees	Notes
1a	Chardonnay	air in press	2010	Lombardy	Early	Unknown	Twelve days after the begin of AF	Yes	Grape stored at 10°C for one day
1b	Chardonnay	N_2 in press	2010	Lombardy	Early	Unknown	No aeration	Yes	Grape stored at 10°C for one day
2a	Vermentino	air in press	2010	Tuscany	At ripening	Indigenous	No aeration	No	--
2b	Vermentino	air in press	2010	Tuscany	At ripening	Indigenous	No aeration	No	--
2c	Vermentino	air in press	2010	Tuscany	At ripening	Indigenous	No aeration	No	Water rinsed grape
3a	Verdicchio	N_2 in press	2010	Lombardy	At ripening	VIN 13	Within 24 hours after the inoculum	Yes	--
3b	Verdicchio	N_2 in press	2010	Lombardy	At ripening	VIN 13	Within 24 hours after the inoculum	Yes	--
4a	Chardonnay	air in press	2010	Lombardy	Early	IOC	No aeration	Yes	--
4b	Chardonnay	air in press	2010	Lombardy	Early	IOC	No aeration	Yes	--
4c	Chardonnay	air in press	2010	Lombardy	Early	CHP	No aeration	Yes	--
3c	Verdicchio	N_2 in press	2009	Lombardy	At ripening	AWRI Fusion	Within 24 hours after the inoculum	Yes	--
3d	Verdicchio	N_2 in press	2009	Lombardy	At ripening	260	Within 24 hours after the inoculum	Yes	--
4d	Chardonnay	air in press	2009	Lombardy	Early	CHP	No aeration	Yes	--
4e	Chardonnay	air in press	2009	Lombardy	Early	IOC	No aeration	Yes	--

Table 2: Precision parameters (n=5) for GSH determination in wine.

Concentration	**Chromatographic Area** *(mAU x sec)*			**SD**	**RSD**
(mg L^{-1})	min	max	average	*(mAU x sec)*	*(%)*
1.5	34.5	39.0	36.8	1.8	5.0
15.4	378	395	386	6.7	1.7
30.7	658	708	686	18.1	2.6

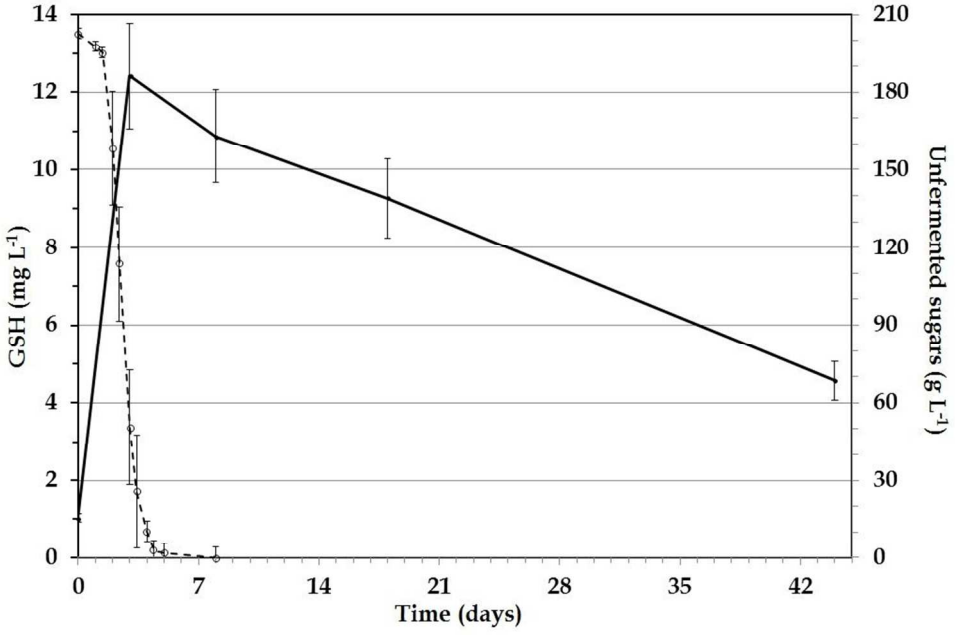
Table 3: Chemical composition of the wines produced in triplicate fermentation.

Parameter	Wine A	Wine B	Wine C
Ethanol (%)	10.9	11.1	11.6
Sugar (g L^{-1})	1.0	1.1	1.0
pH	3.2	3.2	3.2
Total acidity (tartaric acid g L^{-1})	6.5	7.0	6.7
Volatile acidity (acetic acid g L^{-1})	0.65	0.63	0.41
Free sulfur dioxide (mg L^{-1})	< 5	< 5	< 5
Total sulfur dioxide (mg L^{-1})	30	20	31

Table 4: Concentrations of GRP (expressed as caffeic acid equivalents) and GSH detected in grapes, musts and wines at the end of alcoholic fermentation and after aging on the yeast lees (value ± standard deviation); n.a.: not analysed. Days of aging on the lees are reported in brackets. Different vinification numbers indicate different wineries. *: wines maintained 26 days in vat without yeast lees.

Vinification	Vintage	Must exposure to O_2	GRP *(mg L^{-1})*		GSH *(mg L^{-1})*			
			grape	must	grape	must	end of alcoholic fermentation	after in-steel vat storage
1a	2010	air in press	1.5±0.046	9.1±0.28	35.3±1.1	5.3±0.18	27.5±0.88	16.3±0.55 (10)
1b	2010	nitrogen in press	1.5±0.046	4.1±0.13	35.3±1.1	9.4±0.30	14.4±0.46	10.4±0.37 (7)
2a	2010	air in press	11.7±0.36	5.5±0.17	1.4±0.043	0.9±0.028	7.3±0.26	6.2±0.40 (0)*
2b	2010	air in press	11.7±0.36	4.9±0.15	1.4±0.043	0.5±0.018	4.9±0.18	4.9±0.18 (0)*
2c	2010	air in press	11.7±0.36	2.78±0.086	1.4±0.043	3.5±0.096	9.1±0.30	7.4±0.25 (0)*
3a	2010	nitrogen in press	4.6±0.14	12.9±0.40	84.6±2.6	4.0±0.15	29.5±0.94	12.4±0.38 (15)
3b	2010	nitrogen in press	4.6±0.14	13.2±0.41	84.6±2.6	4.3±0.13	35.5±1.1	32.0±1.0 (15)
4a	2010	air in press	1.3±0.040	0.093±0.0029	5.6±0.18	3.9±0.12	15.3±0.52	14.6±0.48 (45)
4b	2010	iperox	1.8±0.056	0.62±0.019	12.1±0.38	3.6±0.11	17.7±0.58	13.5 ±0.43 (42)
4c	2010	air in press	1.8±0.056	0.28±0.0086	12.1±0.38	2.6±0.081	21.9±0.70	21.3±0.75 (41)
3c	2009	nitrogen in press	n.a.	n.a.	n.a.	0.7±0.025	19.3±0.62	16.5±0.53 (10)
3d	2009	nitrogen in press	n.a.	n.a.	n.a.	1.1±0.018	14.2±0.51	13.0±0.43 (8)
4d	2009	air in press	n.a.	n.a.	n.a.	0.8±0.028	10.3±0.40	9.6 ±0.31 (15)
4e	2009	iperox	n.a.	n.a.	n.a.	0.4±0.014	10.9±0.35	4.5±0.14 (32)

Figure 1



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