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Simultaneous quantification of glutathione, glutathione disulfide and glutathione-S-sulfonate in grape and wine using LC-MS/MS

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ABSTRACT

A fast, sensitive and reproducible method using LC-MS/MS for simultaneous quantification of glutathione (GSH), glutathione disulfide (GSSG) and glutathione-S-sulfonate ($GSSO_3H$) was developed, optimised and applied in analysis of grape juice and wine samples. The results show that only GSH (10–60 mg·L^{-1}) and GSSG (2–11 mg·L^{-1}) are found in grape juice when SO_2 is not added. $GSSO_3H$ was detected in must samples treated with SO_2 but only at a low concentration (<1 mg L^{-1}). In the wine samples, the dominant form of glutathione was $GSSO_3H$ (5–11 mg L^{-1}), followed by GSH (0–5 mg L^{-1}) and GSSG (0–6 mg L^{-1}), underscoring the importance of $GSSO_3H$ quantification. $GSSO_3H$ formation in wine was correlated with the total SO_2 level in the wine. We believe this is the first report on $GSSO_3H$ quantification in wine.

1. Introduction

Glutathione is a thiol-containing tripeptide of glutamic acid, cysteine and glycine found in many organisms. In cells, glutathione generally exists in reduced (GSH) and oxidised forms (GSSG), with a predominance of GSH. In grape berries, GSH represents about 90% of the total glutathione content (Kritzinger et al., 2013a), with the concentration dependent on the grape variety (Cheynier et al., 1989), nitrogen nutrition status of the plant (Choné et al., 2006) and maturity of the grapes (Suklje et al., 2012). The concentration of glutathione in must is highly variable and depends on oenological factors, such as exposure to oxygen, tyrosinase activity or grape skin maceration during pre-fermentation period (Kritzinger et al., 2013a). Generally, GSH concentration decreases during fermentation, whereas the GSSG concentration remains stable or increases (du Toit et al., 2007). Yeast metabolism influences the GSH concentration during alcoholic fermentation, with particular strains able to produce or consume more or less GSH (Lavigne et al. 2007). In wine, the GSH concentration decreases during storage. Recently, Arapitsas et al., 2016 reported that during storage, $GSSO_3H$ was produced in wines containing SO_2. Nikolantonaki et al. (2018) identified sulfonated products of cysteine and glutathione in Chardonnay wine and reported that the level of sulfonated compounds is dependent on the vintage and the addition of GSH after alcoholic fermentation.

GSH is considered a promising molecule to prevent adverse effects of oxidation on the aroma of wine (Nikolantonaki et al. 2014) and to inhibit browning during winemaking and storage (Nikolantonaki et al., 2018; Singleton et al., 1985; Webber et al., 2017; Xu et al., 2019). Because of the positive effect of glutathione on the quality of wine, the International Organisation of Vine and Wine—in resolutions 533–2017 and 534–2017—permits the addition of glutathione to must and wine in the form of glutathione-enriched inactivated yeast. Thus, there is growing interest in quantification of glutathione in must and wine, especially in oenological studies aimed at deepening our understanding of the effects of glutathione addition to wine and optimising these effects.

Several analytical methods for measurements of GSH, GSSG and total glutathione contents in grape juice and wine have been published, including high-performance liquid chromatography (HPLC) with fluorescence detection (Janes et al., 2010; Keller & Menzel, 1985; Marchand & de Revel, 2010; Noctor & Foyer, 1998; Park et al., 2000a; Park et al., 2000b; Webber et al., 2017), HPLC with UV detection (Fracassetti et al., 2011; Fracassetti & Tirelli, 2015; Zacharis et al., 2013) and capillary electrophoresis with laser-induced fluorescence detection methods (Lavigne et al., 2007). All these methods use derivatisation to add a chromophore or fluorescent tag to GSH to enable its detection. In contrast, HPLC with mass spectrometry detection allows sensitive and fast quantification of GSH and GSSG in wine samples without

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derivatisation (du Toit, Lisjak, Stander, & Prevoo, 2007; Ferreira-Lima et al., 2018; Guan, Hoffman, Dwivedi, & Matthees, 2003; Kritzinger, Stander, & Du Toit, 2013b; Roland & Schneider, 2015). Recently, there has been research on quantifying GSH using an electrochemical sensor (Tahernejad-Javazmi et al., 2018), a phosphorescence sensor (Jin et al., 2016) and a fluorescence visual assay (Chen et al., 2018). Keller and Menzel (1985) measured the $GSSO_3H$ level in biological cell samples using HPLC with fluorescence detection. This method allows simultaneous determination of GSH, GSSG, $GSSO_3H$ and $CysSO_3H$. To our knowledge, no quantification method for $GSSO_3H$ in wine has been reported to date.

The aim of our study was to develop a method for simultaneous quantification of the three known forms of glutathione (GSH, GSSG and $GSSO_3H$) in grape juice, must and wine samples. This method fills a gap in the analysis of glutathione, and prevents underestimation of its quantity. The analysis of real samples allows to determine what forms are present during the various stages of winemaking and help to understand the formation of $GSSO_3H$.

2. Materials and methods

2.1. Chemicals

Reduced glutathione, oxidised glutathione, formic acid and sodium disulfite were obtained from Merck (Darmstadt, Germany), L (+)-Ascorbic Acid was obtained from BioChemica AppliChem (Darmstadt, Germany), and ultra-gradient HPLC grade acetonitrile was obtained from J-T Baker (Philipsburg, NJ, U.S.A.).

2.2. Preparation of samples

Grape juice was prepared in the laboratory from intact berries collected in a vineyard with pedicel and crushed using a pneumatic press. Ascorbic acid (400 µL of 25% [m/v] ascorbic acid solution per 40 mL of juice) was immediately added to prevent oxidation.

Must samples were collected after pressuring in the cellar and protected with ascorbic acid (400 µL of 25% [m/v] ascorbic acid solution per 40 mL of must) immediately after collection. The samples were stored at −25 °C until the analysis.

Wines were obtained from the experimental cellar of Agroscope and used without any preparation steps in the analysis, 3–6 months after bottling.

2.3. Preparation of a standard for $GSSO_3H$ quantification

A stock solution of $GSSO_3H$ was prepared by weighing 400 mg of GSSG (0.65 mmol) in a 1 L volumetric flask containing deionised water, adding 600 mg of $Na_2S_2O_5$, and filling the flask with water. The solution was left for one day at room temperature. The reaction mixture was then diluted five times with 5 g L^{-1} of aqueous ascorbic acid. Standards used for the calibration curve of $GSSO_3H$ were prepared from this solution by further dilution with 5 g L^{-1} of aqueous ascorbic acid. The concentration of each standard was calculated using the following equation (Eq. (1)).

$$[GSSO_3H]_1 = 2*\left([GSSG]_0 - [GSSG]_1\right) - [GSH]_1 \quad (1)$$

where 0 was the initial concentration in mol L^{-1} before the reaction with SO_2, calculated using the initial concentration of GSSG in the stock solution with the dilution factor used for the preparation of the calibration standard, and 1 was the concentration measured in the calibration standard.

2.4. LC-MS/MS analysis of glutathione

The method reported by Kritzinger et al., 2013b was adapted in the analysis of GSH, GSSG and $GSSO_3H$. The analysis were performed on an Infinity 1290 UPLC system (Agilent Technologie, Santa Clara, CA, U.S.A.), connected to an Agilent 6460-C Triple Quadrupole LC-MS with an electrospray using Agilent Jet Stream technology and MassHunter software (Agilent Technologie, Santa Clara, CA, U.S.A.). Chromatographic separation was performed on a Poroshell 120 SB-C18 column (150 × 4.6 mm, 2.7 µm; Agilent Technologie, Santa Clara, CA, U.S.A.) using water with 0.1% formic acid as mobile phase A and acetonitrile with 0.1% formic acid at a flow rate of 1 mL min^{-1} as mobile phase B. The elution gradient was as follows: 2% of B from 0 to 0.2 min, 2–10% of B from 0.2 to 4 min, 10–100% of B from 4 to 4.1 min and 100% of B from 4.1 to 5 min. The column was equilibrated for 3 min with 2% of B. The injection volume was 2 µL for wine and 0.4 µL mixed with 1.6 µL of formic acid 5% for grape juice.

Detection was performed by multiple reaction monitoring. The electrospray positive ionization mode (ESI+) was applied using the following source parameters: gas temperature at 300 °C, gas flow at 5 L min^{-1}, nebuliser at 30 psi, sheath gas heater at 250 °C, sheath gas flow at 11 L min^{-1}, capillary voltage at 4,500 V and nozzle voltage at 500 V. The fragmentor voltage and collision energy were optimized with the standards separately and were 119 V and 19 V for GSH, respectively, and 110 V and 7 V for GSSG, respectively. Quantification was performed using the following transitions: 308 m/z → 179 m/z for GSH and $GSSO_3H$ and 613 m/z → 355 m/z for GSSG.

2.5. Validation procedure

The linearity of the LC-MS/MS method was calculated in the range of 1–20 mg L^{-1} for GSH and GSSG and 0.2–8 mg L^{-1} for $GSSO_3H$. The standards were prepared in triplicate at five different levels of concentration in an aqueous solution of ascorbic acid (5 g L^{-1}), the same procedure used for the dilution of the grape juice samples. Calibration curves were established by plotting concentrations against the respective areas.

Accuracy and repeatability were measured by spiking three grape juices (two white grape juices with low and high levels of GSH and one red grape juice) and two wines (white and red) with 20 and 40 mg L^{-1} of GSH and GSSG separately in triplicate. Intermediate reproducibility was determined for GSH and GSSG by analysing frozen grape juice samples in triplicate on three different dates. This parameter was not determined for $GSSO_3H$.

The limit of detection (LOD) and limit of quantification (LOQ) of the LC-MS/MS method were evaluated as the lowest concentration at which the signal-to-noise (S/N) ratio was greater than 3 and 10, respectively, measured in the spiked musts ($n = 3$) and wines ($n = 3$).

3. Results and discussion

3.1. Development and validation of the LC-MS/MS method

3.1.1. Detection conditions for GSH, GSSG and $GSSO_3H$

The LC-MS/MS method with direct injection enabled quantification of the three predominant forms of glutathione in grape juice and wine samples. The method reported earlier by Kritzinger et al., 2013b for quantification of GSH and GSSG was modified in this study for quantification of all three forms of glutathione. First the detection conditions were optimised using standards to obtain the most intense and specific transition in MS-MS mode. The following transitions were retained for the quantification in positive ionisation mode: 308 m/z → 179 m/z for GSH and $GSSO_3H$ and 613 m/z → 355 m/z for GSSG. The chromatographic conditions were then optimised to obtain a good separation of the standards (Fig. 1). The retention times, with the optimised conditions, were 1.8 min, 2.4 min and 3.2 min for $GSSO_3H$, GSH and GSSG, respectively. The total analysis time was 8 min.

Of note, the GSH standard shows a second negligible small peak (GSH2) at 2.6 min in the chromatogram after the main peak (GSH1). In the samples in which oxidation was prevented by addition of ascorbic

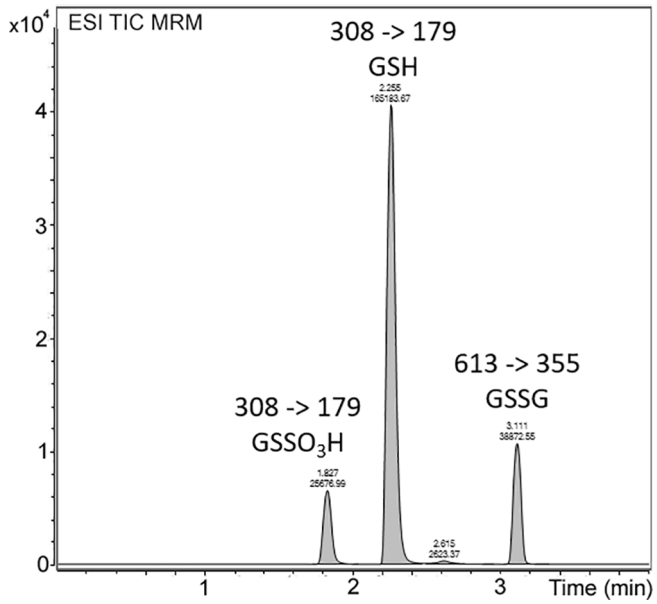

Fig. 1. Total ion chromatogram of glutathione derivative standards ($GSSO_3H$, GSH and GSSG) obtained with LC-MS/MS using ESI ionization in the positive mode and multiple reaction monitoring. Transitions used for the detection, noted on the corresponding peak, are 308 m/z → 179 m/z for GSH and $GSSO_3H$ and 613 m/z → 355 m/z for GSSG.

acid, the area of the GSH2 peak represents less than 2% of the main peak (Fig. 2, top left). However, GSH2 became dominant when the GSH standard was added to the oxidised grape juice (Fig. 2, bottom right). The initial concentration of GSH in the must was lower than 1 mg L^{-1}, the addition of GSH standard (20 mg L^{-1}) generated two peaks, with a peak area ratio of 1:2 (for GSH1:GSH2). However, acidification of the sample with formic acid resulted in a decrease in the peak area of GSH2 and an increase in that of GSH1. These two peaks could possibly reflect two different conformations of GSH, which are separated on the chromatogram. There is a need for further investigation to identify these two forms of glutathione.

To study the effect of acidification on the ratio of the areas of the peaks, 1.6 μL of formic acid solution (with different concentrations from 0 to 10% v/v) was added to 0.4 μL of grape juice sample before the analysis of glutathione, and the peak areas (GSH1 and GSH2) were determined as a function of the formic acid concentration. In this experiment, the initial ratio between the areas of the peaks, GSH1 and GSH2, was near 1:1. As can be seen in Fig. 3, a solution of at least 4% formic acid was necessary to transform more than 90% of GSH into GSH1. The sum of the areas of the two peaks (GSH1 + GSH2) increased with an increase in the formic acid concentration and stabilised when the concentration was higher than 4%. This could be the result of improved ionisation at a lower pH value or an integration error due to the small overlapping peaks.

No double peak was observed in the more acidic grape juice (oxidation prevented) or in the wine after the addition of GSH. To avoid double peaks, the grape juice samples were systematically acidified with 5% formic acid solution (1:4 v/v) before injection into the LC-MS/MS using an injection program.

3.1.2. *Optimization of the preparation of $GSSO_3H$ standard*

Due to the lack of a pure commercial standard, $GSSO_3H$ was synthesised from GSSG and SO_2 (released by $Na_2S_2O_5$) in water, analogous to the reaction of cystine with sulfite proposed for the production of cysteine-S-sulfate by Zecchini et al. (2019). This reaction can occur spontaneously in water, yielding free thiols and sulfate. The thiols can be oxidised to sulfenic acid and further react with SO_2. The formation of the sulfate product depends of the SO_2 concentration and the pH and can be enhanced by UV light.

To maximize the formation of $GSSO_3H$, different ratios of GSSG/SO_2 were tested using three molar concentration ratios (1/2.5, 1/10 and 1/25). As expected, higher SO_2 ratios resulted in increasing $GSSO_3H$ formation. Using a 1/10 GSSG/SO_2 ratio, approximately 50% of the GSSG (9.7 mg L^{-1} from 20 mg L^{-1}) was transformed into GSH and $GSSO_3H$ after 24 h. The 1/25 ratio led to higher conversion of GSSG, and no change was observed in the ratio of GSH/$GSSO_3H$. Finally, a molar ratio of 1/10 was preferred to prepare the $GSSO_3H$ standard for calibration. The concentration of $GSSO_3H$ in the prepared standard was calculated according to the following reactions (Eqs. (2) and (3)).

$$GSSG + SO_2 + H_2O = GSSO_3H + GSH \quad (2)$$

$$GSH + SO_2 \rightarrow GSSO_3H \quad (3)$$

where the concentrations of GSSG and GSH can be determined from the chromatogram of the standard using the calibration curve of each species. In the prepared standard, the molar ratio of $GSSO_3H$:GSH:GSSG was around of 40:33:27, with small intraday variations (<5%). This result confirmed that the second reaction between GSH and SO_2 was very slow as compared to that of the first reaction of GSSG with SO_2, as reported previously (Arapitsas et al., 2016). After dilution with 5 g L^{-1} of ascorbic acid solution (5×), the standard remained stable at 4 °C for several hours. The preparation of the $GSSO_3H$ standard using this method was easy and not time consuming, in contrast to the synthesis described by Eriksson and Rundfelt (1968). Using LC-MS/MS in the analysis permits the use of a stable mixture of glutathione species for quantification of $GSSO_3H$ instead of a pure standard.

3.1.3. *Validation of the LC-MS/MS method*

The linearity of the method was determined first using standard solutions of GSH and GSSG at six concentrations (1, 5, 10, 20, 50 and 100 mg L^{-1}) analysed in triplicate. A linear correlation was found between the concentrations and corresponding GSH peak areas ($R^2 > 0.99$). GSSG concentrations and corresponding peak areas fit a polynomial model ($R^2 > 0.99$), with no linear correlation found even at a reduced concentration range (1–20 mg L^{-1}). $GSSO_3H$ standards were analysed at four concentrations (0.25, 1.89, 3.65 and 6.93 mg L^{-1}). The model with the best fit was a polynomial model. The LOD and LOQ of the method were determined at S/N ratios of 3 and 10, respectively. The LOD was 0.01 mg L^{-1} for GSH, 0.06 mg L^{-1} for GSSG and 0.05 mg L^{-1} for $GSSO_3H$ (Table 1). The LOQ was 0.1 mg L^{-1} for GSH, 0.2 mg L^{-1} for GSSG and 0.2 mg L^{-1} for $GSSO_3H$. The sensitivity of the developed method is inferior to that of the method of Kritzinger et al., 2013b. However, it is sufficient for the determination of glutathione species in wine and grape juice, as the expected concentrations are within the range of the developed method. The measured concentrations were 1–60 mg L^{-1} for GSH 0.1–12 mg L^{-1} for GSSG and 0.2–8 mg L^{-1} for $GSSO_3H$ (Supplementary material 1). The concentration ranges for GSH and GSSG were equivalent to those reported in earlier studies (Fracassetti & Tirelli, 2015; Kritzinger et al., 2013a).

The accuracy of the LC-MS/MS method was evaluated in wine and grape juice matrix. White wine and red wine samples were spiked with known amounts of GSH, GSSG and $GSSO_3H$. In the grape juice samples, accuracy was determined only for GSH and for GSSG. Two white grape varieties with different initial amounts of glutathione (high and low, 58 mg L^{-1} and 8 mg L^{-1}, respectively) and one red variety were used. All tests were performed in triplicate, and the recovery was calculated for each sample by comparing the theoretical spiked amount to the calculated amount. Recoveries were satisfactory, as the values for the five matrices ranged from 95% to 109% (Table 1), except in samples where GSH was added to oxidised grape juice, where the recovery was 71 and 75%. In oxidised juice, highly reactive o-quinones are present and can react with GSH to form adducts, such as GRPs, 2-S-glutathionyl-*trans(or cis)*-caftaric acid (Singleton et al., 1985) and GRP2 (Ferreira-Lima et al.,

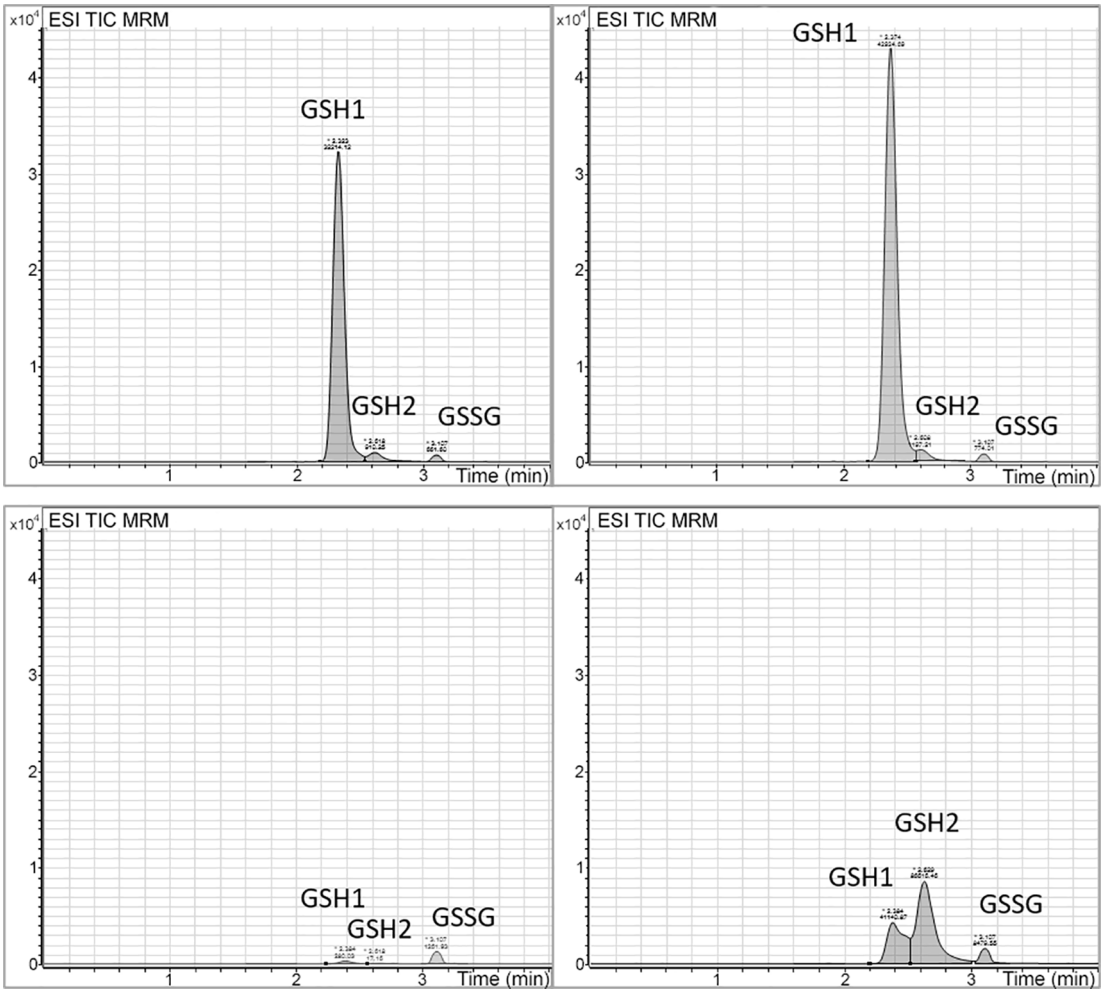

Fig. 2. Chromatographic separation of GSH (GSH1 + GSH2) and GSSG by LC-MS/MS before (left) and after (right) the addition of GSH standard to the grape juice samples in which oxidation was prevented (top) and to the oxidised (bottom) grape juice.

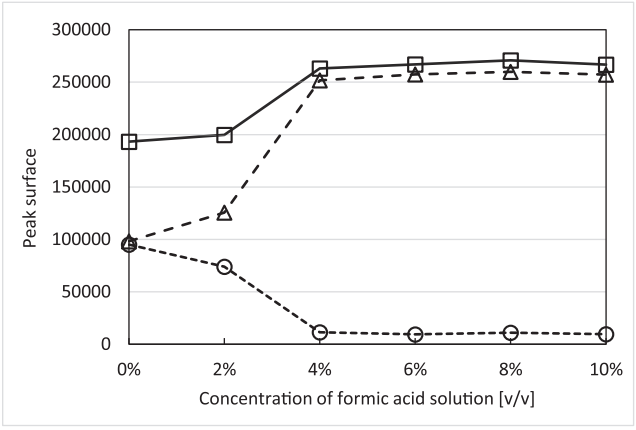

Fig. 3. Peak areas detected in the chromatogram during the LC-MS/MS analysis of oxidised grape juice fortified with GSH as a function of formic acid addition to the samples before injection. o = GSH1, Δ = GSH2, □ = the sum of the area of the two GSH peaks (GSH1 + GSH2).

2016). Consequently, the GSH concentration decreases, resulting in poor recovery in oxidised juice.

The repeatability of the method was measured at three levels of concentrations in grape juice and wine in triplicate, using the same samples as used to evaluate the accuracy of the method. The relative standard deviation (RSD) values ranged from 1% to 10% (Table 1). Intermediate reproducibility was evaluated using grape juice aliquots of white varieties (Chasselas and Doral) and red varieties (Gamay and Gamaret) stored frozen at −20 °C with ascorbic acid additive. The samples were analysed on three separate days immediately after defrosting. The RSD values ranged from 4 to 13.8% for GSH and 1 to 8% for GSSG. Intermediate reproducibility was not determined for $GSSO_3H$.

Table 1
Analytical performance of the LC-MS/MS method.

	LC-MS/MS		
	GSH	GSSG	$GSSO_3H$
Concentration range (mg L^{-1})	1–20	1–20	0.2–8
Regression model	linear	polynomial	polynomial
LOD (mg L^{-1})	0.01	0.06	0.05
LOQ (mg L^{-1})	0.1	0.2	0.2
Recovery	95–108%	96–107%	95–109%
Repeatability (RSD%)	<10%	<5%	<6%
Intermediate reproducibility (RSD%)	<14%	<8%	nd

3.2. *Quantification of predominant glutathione derivatives in grape juice, must and wine*

The concentrations of GSH, GSSG and $GSSO_3H$ in grape juice, must and wine samples (vintage years: 2015–2019) were determined (Supplementary material 1). Grape juice samples were prepared in the

laboratory, and ascorbic acid was added immediately to prevent oxidation. Must and wine samples were obtained from the experimental cellar of Agroscope. They were produced in a pilot-scale winemaking procedure, without using special inert press conditions. The wines were stabilised with a solution of SO_2 (5%) to obtain approximately 50 mg L^{-1} free SO_2 in wine. The results show that the principal form of glutathione in the grape juice sample was GSH, which was found at concentrations between 12 and 61 mg L^{-1}. GSSG, the oxidised form of glutathione, accounted for about 10% of the GSH in the grape juice samples in which oxidation was prevented, with levels of 2–7 mg L^{-1}. Previous studies reported similar values for grape juice produced under reductive conditions, with the grapes were pressed under an inert atmosphere (CO_2 or nitrogen) (du Toit, Lisjak, Stander, & Prevoo, 2007; Fracassetti & Tirelli, 2015; Kritzinger et al., 2013a).

In must samples where pressing was done without protection from oxidation, the GSH level dropped dramatically to<1 mg L^{-1}. Several studies reported the same observation where non-inert press conditions were used (Fracassetti & Tirelli, 2015; Park et al., 2000a; Park et al., 2000b; Pons et al., 2015). These results are not surprising, as phenolic compounds can form highly reactive o-quinones and react with GSH under non-inert conditions. Previous studies reported an increase in the concentration of adducts, such as GRP, which formed during pressing (Cheynier et al., 1993, Maggu et al., 2007). In this study, the GSSG level in must was similar to that in the grape juice samples (4–10 mg L^{-1}).

In the wine samples, which were analysed 3–6 months after bottling, the predominant form of glutathione was $GSSO_3H$, with concentrations as high as 11 mg L^{-1}. In contrast, the GSH concentration remained between 0.2 and 5 mg L^{-1} and that of GSSG remained between 0 and 6 mg L^{-1}. The results presented in Supplementary material 1 show that the total glutathione amount in the wine samples and the individual concentration of GSH, GSSG and $GSSO_3H$ varied widely. As the formation of $GSSO_3H$ is related to the presence of SO_2 in wine (Arapitsas et al., 2016; Nikolantonaki et al., 2018), the relative concentration of $GSSO_3H$ as compared to that of total SO_2 is shown in Fig. 4. These results confirms that the greater the amount of total SO_2, the greater the ratio of $GSSO_3H$ in the wine samples. Thus, the SO_2 content of wine can influence glutathione measurements. This finding demonstrates the importance of quantification of all forms of glutathione in wine. The weak correlation ($R^2 = 0.39$) observed between the relative concentration of $GSSO_3H$ and the amount of total SO_2 in the present study may be explained by the fact that the reaction of glutathione and SO_2 depends on various factors, such as pH, wine composition and light exposure (UV) (Zecchini et al., 2019), which can differ from one wine sample to another.

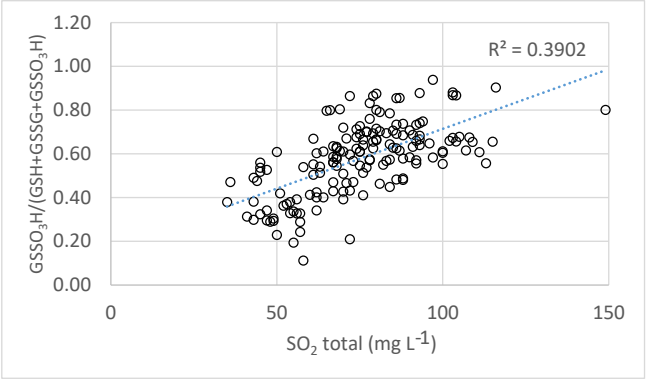

Fig. 4. Concentration of $GSSO_3H$ in relation to the sum of all forms of glutathione quantified in wines (vintage years: 2015–2019) as a function of the total SO_2 concentration in the wines.

3.3. Quantification of predominant glutathione derivatives in wine during storage

The impact of the addition of GSH during alcoholic fermentation (AF) on the concentration of glutathione derivatives in the wine before and after bottling was studied in the experimental cellar of Agroscope in 2015–2017 (unpublished data). Three variants were prepared from the same must of Garanoir: a control variant with no GSH added (A1), a variant with 20 mg L^{-1} of pure GSH standard added on the 1st and 4th days of AF (A2) and a variant with 400 mg L^{-1} of inactivated yeast added on the 1st and 4th days of AF (A3). Glutathione derivatives were quantified in samples taken directly from the tank of the wine variants at the end of AF (EAF) and in wine after 3 months of storage in bottles (Fig. 5). The addition of GSH and the inactivated yeast doubled the total glutathione concentration (GSH + GSSG) in the wine at the EAF, with 4.5 mg L^{-1}, 9.1 mg L^{-1} and 9.0 mg L^{-1} for A1, A2 and A3, respectively (results from 2015). The $GSSO_3H$ concentration was low, ranging from 1.4 to 1.7 mg L^{-1}. After 3 months of storage in the bottles, the total glutathione concentration decreased in all the variants, with levels of 1.1 mg L^{-1}, 2.2 mg L^{-1} and 1.8 mg L^{-1} for A1, A2 and A3, respectively. The most important changes were observed in the concentrations of GSSG, with values ranging from 4.3 to 8.3 mg L^{-1} at the EAF, with these values dropping to 0.5–0.6 mg L^{-1} after 3 months of storage in bottles. The concentration of GSH remained in the same range: 0.2–2.7 mg L^{-1} at the EAF and 0.5–1.6 mg L^{-1} after storage. In contrast, the $GSSO_3H$ concentrations increased to 3.3 mg L^{-1}, 7.6 mg L^{-1} and 6.5 mg L^{-1} for A1, A2 and A3, respectively, as result of the reaction between GSSG and SO_2. This could partly explain the marked decrease in the total glutathione (GSH + GSSG) concentration after bottling and storage. The findings of this study also illustrate that the interval between the addition of SO_2 and the time of the analysis strongly influences the concentrations of $GSSO_3H$, GSH and GSSG in wine. This is significant because studies on wine aging and storage currently focus only on quantification of GSH or total glutathione (GSH + GSSG). Weber et al. (2017) found that one month after the addition of GSH to sparkling wine stored in bottles, the measured concentration of GSH was lower than the added concentration. This decrease could not be only explained by the oxidation of GSH to GSSG, because the total glutathione concentration (GSH + GSSG) after one month was about 1/4 of the added GSH, and this decrease continued after 6 and 12 months. Similar results have been reported in other studies, with both the GSH concentration and total glutathione concentration decreasing during aging (Andujar-Ortiz et al., 2012; Lavigne et al., 2007). Our findings highlight the need for studies to take $GSSO_3H$ concentration into account.

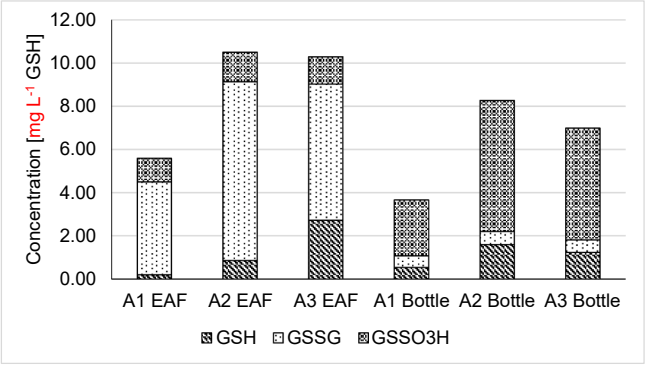

Fig. 5. GSH, GSSG and $GSSO_3H$ concentrations in Garanoir wine subjected to different treatments, measured in 2015 (see lines 158–160 in supplementary material 1) at the end of alcoholic fermentation (EAF) and after 3 months of storage in bottles. The concentrations are given in GSH mg L^{-1} equivalent. A1: control; A2: addition of 2 × 20 mg L^{-1} of GSH during AF; A3: addition of 2 × 400 mg L^{-1} of inactivated yeast during AF; EAF: end of alcoholic fermentation; Bottle: 3 months storage in bottles.

Further investigations are necessary to identify and quantify other glutathione derivatives formed after GSH addition to wine. Nikolantonaki et al. (2018) identified molecular markers of GSH in wine, including $GSSO_3H$ and cysteine-S-sulfate. Bahut et al. (2020) reported that GSH-related compounds formed after reaction with oxidised polyphenols. Van Leeuwen et al. (2020) recently highlighted the existence of glutathionyl polysulfides in wine. As mentioned by these authors, the protective effect of $GSSO_3H$ and glutathionyl polysulfides against oxidative damage of wines remains to be determined.

4. Conclusion

The proposed LC-MS/MS method described in this study is the first to enable simultaneous quantitative determination of GSH, GSSG and $GSSO_3H$ in different samples: grape juice, must after pressuring and during fermentation and wine. Routine analysis revealed good sensitivity and reproducibility of the method. To enhance the accuracy of GSH measurements, grape juice samples should be acidified before injection to transform all GSH into the same conformation. The present study is the first to determine the $GSSO_3H$ level in wine. Our results showed that $GSSO_3H$ represents a significant part of total glutathione in wine samples. Thus, quantification of $GSSO_3H$ in wine samples is recommended to better understand the effect of glutathione added during winemaking or prior bottling on wine quality and aging.

CRediT authorship contribution statement

Ágnes Dienes-Nagy: Investigation, Conceptualization, Writing – original draft, Supervision, Data curation. **Frédéric Vuichard:** Investigation, Methodology, Validation, Writing – original draft. **Sandrine Belcher:** Validation, Writing – review & editing, Data curation. **Marie Blackford:** Writing – review & editing, Validation. **Johannes Rösti:** Investigation, Conceptualization. **Fabrice Lorenzini:** Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132756.

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