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Advantages and limitations of a high-throughput analytical method using an automated enzymatic assay to quantify the glutathione in grape juice

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ABSTRACT

Glutathione is a tripeptide, present in plants and other organisms in free (GSH) and dimer (GSSG) forms, known for its antioxidant activity. A high-throughput, economical method was developed to measure the total glutathione (TG) concentration in grape juice, using an enzymatic assay (EA), based on the reaction of thiol with 5,5′-dithio-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase enzyme. This method was automatised to allow high-throughput measurements in the concentration range of 1–100 mg L^{-1}. GSH and GSSG were also quantified separately using the UHPLC-MS/MS method. The two methods (EA and UHPLC-MS/MS) gave comparable results in grape juice (R^2 =0.97), where the concentrations of SO_2 were low (<100 mg L^{-1}). The sample preparation is a critical step in the quantitative analysis of glutathione given the high reactivity of GSH. In this work, ascorbic acid and SO_2, commonly used in oenology, were tested as protecting agents at different concentration. The results given by the EA method could be altered by the reaction of SO_2 and DTNB, however this effect was observed only at high concentration of SO_2 (1 g L^{-1}). Ascorbic acid at a 2.5 mg L^{-1} concentration protected the sample well, without interfering with the analysis, and allowed for storage for up to four months at − 20 °C.

1. Introduction

Biothiols are well-known antioxidants that protect cells from oxidative damage. They are present in many organisms, including vegetables and fruits. One of the most abundant thiols is glutathione, a tripeptide formed from glutamic acid, cysteine and glycine. Glutathione is generally present in reduced (GSH) or oxidised (GSSG) forms, with a predominance of GSH, which in grapes, represents about 90% of the total glutathione (TG) (Kritzinger et al., 2013a). The quantity of glutathione greatly differs among diverse plants (Demirkol et al., 2004; Hu et al., 2020) and also depends on the nitrogen status of the plant (Choné et al., 2006) and its maturity (Suklje et al., 2012).

During winemaking and the storage of wine, GSH is considered a protective molecule against browning (Nikolantonaki et al., 2018; Xu et al., 2019) and oxidation of aroma compounds (Nikolantonaki et al., 2014). For this reason, the initial concentration of glutathione in fruits is an interesting parameter. Due to the high reactivity of GSH, sample preparation is critical for an accurate measurement. Researchers have proposed working at low temperatures (4 °C) and pH to halt enzymatic activities in order to preserve GSH (Demirkol & Cagri-Mehmetoglu, 2008; Rellán-Álvarez et al., 2006). Du Toit et al. (2007) used 1 g L^{-1} SO_2 and 0.5 g L^{-1} ascorbic acid for long term storage of grape juice at − 20 °C. Roland and Schneider (2015) proposed 1 g L^{-1} benzene sulfonic acid with 4.5 g L^{-1} $Na_2S_2O_5$. Derivatisation of the sample also preserves the thiols, and at the same time, allows for the detection of GSH by UV or fluorescence.

Several analytical methods have been proposed to measure GSH, GSSG and TG in fruits. GSH can be quantified after derivatisation by high-performance liquid chromatography (HPLC) with fluorescence detection (Demirkol et al., 2004; Janes et al., 2010; Marchand & de Revel, 2010; Noctor & Foyer, 1998; Park et al., 2000; Webber et al., 2017), by HPLC with UV detection (Fracassetti and Tirelli, 2015; Zacharis et al., 2013) or by capillary electrophoresis (Lavigne et al., 2007). Furthermore, HPLC with dual electrochemical detection was used to measure GSH and GSSG in vegetables (Mills et al., 1997). Notably, HPLC coupled with mass spectrometry allows for the simultaneous detection of GSH and GSSG (du Toit et al., 2007; Ferreira-Lima et al., 2018; Guan et al., 2003; Kritzinger et al., 2013b; Rellán-Álvarez

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et al., 2006; Roland & Schneider, 2015) and even glutathione-S-sulfonate ($GSSO_3H$) (Dienes-Nagy et al., 2022). Recent research reported how chemiluminescence (Rasoulzadeh & Amjadi, 2021) and electrochemical sensors (Tahernejad-Javazmi et al., 2018) can be applied to measure GSH in biological samples, and fluorescence visual assays (Chen et al., 2018), phosphorescence sensors (Jin et al., 2016) or fluorescent nanoprobes (Hu et al., 2020) to detect GSH in food.

The application of an enzymatic assay using 5,5′-dithio-(2-nitrobenzoic acid) (DTNB) for the determination of glutathione in biological samples was proposed by Tietze (1969). This method was then adapted by Adams and Liyanage (1991) to quantify the TG in different tissues of grapevine (leaves, rachis and berries) without the need for HPLC. Nowadays, several companies offer glutathione assay kits to analyse biological samples. Though, to the best of our knowledge no application is reported so far to determine TG in foods. However, these kits have the advantage of being easy to use and require only a spectrophotometer in the laboratory. Rahman et al. (2006) published a protocol for biological samples where they used a 96-well plate to minimise the reagent volumes and reduce the analysis time. Another economic solution is the automatic sequential analyser with spectrophotometric detection (or discrete analyser), a laboratory instrument that allows for the automatization of colorimetric and enzymatic analytical methods. This type of instrument is now widely available and frequently used in small laboratories. It enables the analyses of several important compounds in foods by applying commercial kits or reagents prepared in-house.

In this paper, we propose a high-throughput, fully automatised measurement using an automatic sequential analyser to quantify the TG in grape juice. The protocol was optimised to ensure the specificity of the enzymatic assay and to overcome matrix effects due to the presence of SO_2 in freshly treated grape juices. Indeed, without the adaptation here proposed for enzymatic assay, the reaction between SO_2 and DTNB may significantly influence the result. The optimised method was validated and compared with UHPLC-MS/MS. In addition, the preservation of the highly reactive glutathione during the sample preparation and storage was studied to find out a suitable protective agent adapted to the quantification method.

2. Materials and methods

2.1. Chemicals

Reduced glutathione, oxidised glutathione, glutathione reductase, KH_2PO_4, formic acid and sodium disulfite were purchased from Merck (Darmstadt, Germany). L(+)-ascorbic acid was obtained from Bio-Chemica AppliChem (Darmstadt, Germany), NADPH from Carl Roth GmbH (Karlsruhe, Germany), 5,5′-dithio-(2-nitrobenzoic acid) (DTNB) from Apollo Scientific (Stockport, UK) and ultra-gradient HPLC-grade acetonitrile from J-T Baker (Philipsburg, NJ, USA).

2.2. Preparation and storage of samples

Grape juice was prepared in the laboratory from intact berry collected with the pedicel on a vineyard, crushed by a pneumatic press and immediately protected with an antioxidant additive.

For the storage experiment, four modalities were used: no additive (A), 2.5 g L^{-1} ascorbic acid (B), 2.5 g L^{-1} ascorbic acid and 80 mg L^{-1} SO_2 (C) and 2.5 g L^{-1} ascorbic acid and 1 g L^{-1} SO_2 (D). Two red (Gamay and Gamaret) and three white grape varieties (Chasselas, Doral and Petite Arvine) were used for this experiment. Some samples were analysed immediately after the preparation of the juice with the EA method and with UHPLC-MS/MS; the rest of the samples were frozen and stored at − 20 °C for 1, 5 or 18 weeks.

In further work, the grape juice was protected with 2.5 g L^{-1} ascorbic acid (400 μL 25% (m/v) ascorbic acid solution for 40 mL of juice). The same concentration of ascorbic acid was added to must samples in the cellar. The samples were stored at − 20 °C until the analysis.

2.3. Analysis of glutathione

2.3.1. Enzymatic assay

A kinetic enzymatic recycling assay (Adams et al., 1991), based on the oxidation of GSH by 5,5′-dithio-(2-nitrobenzoic acid) (DTNB), was adapted for wine and must samples to be carried out using an A25 automatic sequential analyser (BioSystem, Barcelona, Spain). Reagent 1 (R1) was constituted of DTNB (60 mg L^{-1}) and glutathione reductase (400 μL/L) in KH_2PO_4 buffer (125 mM) at pH 7 and the reagent 2 (R2) was a solution of NADPH at a concentration of 200 mg L^{-1} in KH_2PO_4 buffer (125 mM) at pH 7. In the first step, 250 μL of R1 and 5 μL of the sample were mixed in a micro-vial; then, 80 μL R2 was added after 120 s, and the absorbance was measured at 405 nm after 150 s (t1) and 210 s (t2). The concentration of total glutathione was calculated from the standard curve of GSH and given in mg L^{-1}.

2.3.2. UHPLC-MS/MS

The analysis was performed on an Infinity 1290 UPLC system (Agilent Technologie, Santa Clara, CA, USA) connected to an Agilent 6460-C Triple Quadrupole LC-MS with an electrospray using Agilent Jet Stream technology and was operated using MassHunter software (Agilent Technologie, Santa Clara, CA, USA), as described by Dienes-Nagy et al. (2022).

2.4. Validation procedure of EA method

The linearity of the enzymatic assay was determined using standard solutions of GSH and GSSG in six concentrations (1, 5, 10, 20, 50 and 100 mg L^{-1}), analysed in triplicate.

The accuracy and repeatability were measured by spiking three grape juices (white grape juices with a low and high level of GSH and one red grape juice) with 20 mg L^{-1} and 40 mg L^{-1} GSH and GSSG (separately) in triplicate. The effect of SO_2 on the accuracy of the method was studied at 0, 50 and 100 mg L^{-1} SO_2 concentrations using grape juice spiked with GSH or GSSG at three levels.

The intermediate reproducibility was determined by analysing frozen grape juice samples in triplicate on three different dates.

The LOQ of the enzymatic assay method was determined, as the concentration were the coefficient of variation of triplicate analyses of standards was lower than 15%. The LOD was defined as the lowest concentration where the value of Δ Abs min^{-1} was 20% higher than the value measured for a water sample (Blanc).

3. Results and discussion

3.1. Development and validation of automated enzymatic assay (EA)

A high-throughput, fully automatised measurement using an automatic sequential analyser was developed and validated for the quantification of glutathione in grape juice. The basis of this assay is the reaction between GSH and DTNB, which results in a chromophore, 2-nitro-5-thiobenzoic acid (TNB). This molecule can be measured at 425 nm. To increase the sensibility and the selectivity of the assay, the disulfide formed by GSH and DTNB (GSTNB) is reduced by glutathione reductase in the presence of NADPH to generate a second mole of the chromophore and regenerate GSH (Fig. 1). The amount of GSH in the mixture is proportional to the reaction rate of GSH with DTNB and GSTNB's reduction by glutathione reductase resulting the chromophore. Since glutathione reductase reduces the GSSG present in the sample, the assay determines the total glutathione (GSH + GSSG) level.

The enzymatic assay was adapted to be carried out using an A25 automatic sequential analyser to allow for automatised measurements. With this instrument, 30 samples can be handled in one hour using optimised reaction conditions. The quantities of reagent and sample required for the analyses are low (250 μL for R1, 80 μL for R2 and 5 μL for the sample), making this an ecological and economical analytical

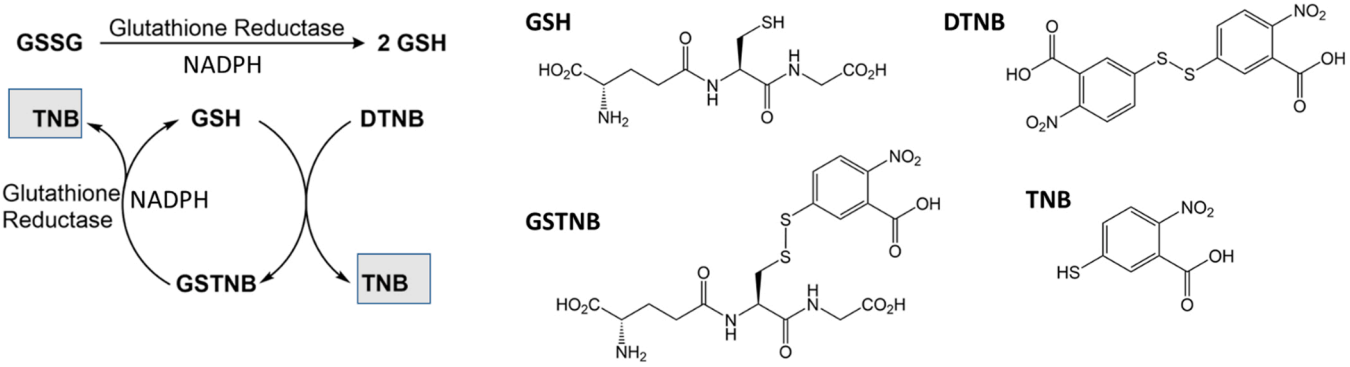

Fig. 1. Reaction scheme of glutathione with DTNB used for TG determination.

method. The measurement range is 1–100 mg L^{-1} total glutathione (TG), corresponding to the concentration reported in grape juice. All reagents were added in excess, DTNB in quantity 23 times and NADPH 13 times greater than the highest concentration of GSH expected in the sample. The absorbance was measured after the addition of the NADPH at 30 s and 90 s to determine the rate of the reaction. This rate was given directly in Δ Abs min^{-1}, which was plotted against the GSH concentration of the calibration solution. A linear correlation was found for both GSH and GSSG standards with $R^2 > 0.99$. At higher concentrations, a small difference was observed between the Δ Abs min^{-1} measured when using GSH or GSSG as the standard. To minimise the error, it was decided to use GSH for the calibration as its concentration is 5–10 times higher in grape juice than that of GSSG (Kritzinger et al., 2013a).

The accuracy of the method was tested by measuring the recovery of GSH and GSSG added to grape juice. The recovery measured in non-protected grape juice was good for GSSG (98–104%) but bad for GSH (12–51%). GSH is known to react with reactive oxygen species in grape juice (Cheynier et al., 1993), and accordingly, should be protected directly after pressing. Ascorbic acid is widely used as an agent for protecting against sample oxidation. Immediate addition of this organic acid to the grape juice at a concentration of 2.5 g L^{-1} resulted in good recovery for both forms of glutathione (101–110%). Validation of the EA method was continued with samples protected with ascorbic acid. The analytical performance of EA, and UHPLC-MS/MS for comparison, are reported in Table 1 (Dienes-Nagy et al., 2022). The EA method was adapted for the analyses of TG in grape juice with a LOQ of 5 mg L^{-1} and LOD of 1 mg L^{-1}, which would be sufficient in the case of a non-oxidised sample. In comparison, the UHPLC-MS/MS method is more sensitive (LOQ of 0.2 mg L^{-1} TG) and thus allows for the quantification of GSH and GSSG separately.

3.2. Optimisation of the grape juice samples' preparation and storage

In winemaking, SO_2 is the most commonly used protective agent against oxidation. Winemakers sometimes add it directly to harvested grapes to prevent quality degradation of the grape juice. SO_2 is known to react with DTNB (Humphrey et al., 1970), one of the reagents used in the EA method. In the here presented method, a high amount of DTNB is added to the sample at the beginning and the absorbance is first measured only after 150 s (t1). This reaction time was determined according to the reaction kinetic of SO_2 and DTNB, measured previously on the same equipment under equivalent reaction conditions for a SO_2 concentration of 200 mg L^{-1} (result not shown). During this initial time DTNB can react with SO_2, if present in the sample, resulting an increase in the absorption measured at t1 but will not influence the kinetic measurement, given by the difference of absorbance at t1 and t2, at 150 s and 210 s respectively. However, in order to ensure accurate results, the quantity of SO_2 in the sample should remain lower than a certain limit, imposed by both the quantity of DTNB and the linear range of absorbance measured by the spectrophotometer. The most restrictive parameter is the available amount of DTNB, equivalent to 440 mg L^{-1} SO_2 in the sample containing 100 mg L^{-1} TG. The absorbance limitation (Abs (max) – Abs (100 mg L^{-1} TG ~ 2) allows about 700 mg L^{-1} SO_2 in the same sample. These calculated values can be influenced by other parameters and substances present in the sample. Therefore, the effect of SO_2 on the TG analysis was tested in the typical concentration range used in oenology by adding 50, 100, 150 or 200 mg L^{-1} SO_2 to grape juice spiked with 30 mg L^{-1} GSH. Adding 50 mg L^{-1} SO_2 to the juice was enough to protect GSH against oxidation (Fig. 2). The recovery, measured immediately after the addition, was 105–108% in the four samples. It can be concluded that up to a 200 mg L^{-1} concentration, SO_2 has no influence on the results of the EA method. However, SO_2 does not protect GSH efficiently in the long term. After three hours of storage at

Table 1
Analytical performance of the EA and UHPLC-MS/MS methods.

	EA	UHPLC-MS/MS	
	TG	GSH	GSSG
Concentration range (mg L^{-1})	5–100	0.1–20	0.2–20
Regression model	linear	linear	polynomial
LOD (mg L^{-1})	1	0.01	0.06
LOQ (mg L^{-1})	5	0.1	0.2
Recovery (%)	84–117	95–108	96–107
Repeatability (RSD%)	< 8	< 10	< 5
Samples per hour	*30*	*7*	*7*

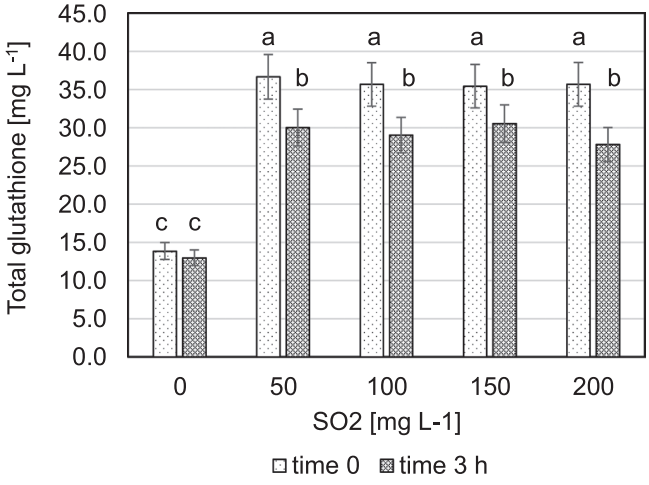

Fig. 2. Total glutathione concentrations in Gamay samples with different quantities of added SO_2. Note: The first measure was taken using the EA method immediately after the sample preparation, and the second three hours later. Different letters indicate significant differences between the samples ($p \leq 0.005$).

room temperature (~23 °C), the TG concentration decreased by about 20%, even with 200 mg L^{-1} SO_2.

Next, with the objective to achieve a longer storage time for the grape juice samples, we compared three variants of protecting-agent combinations: 2.5 g L^{-1} ascorbic acid (B), 2.5 g L^{-1} ascorbic acid and 80 mg L^{-1} SO_2 (C) and 2.5 g L^{-1} ascorbic acid and 1 g L^{-1} SO_2 (D). Ascorbic acid alone (B) resulted in the highest TG concentration measured with the EA method, followed by variant C (Fig. 3a). In contrast, a high concentration of SO_2 (1 g L^{-1}) skewed the TG results obtained with EA as expected. In fact, at that concentration all DTNB should react with the SO_2, but surprisingly in some case low quantity of TG was still detected. Probably the initial time (150 s) was not enough to fully complete the reaction between SO_2 and DTNB, and the remaining DTNB continues to react resulting in a measurable difference of absorbance between t(1) and t(2). However, this artefact is unlikely to occur with real samples, because the usual SO_2 concentrations in a cellar are lower than 200 mg L^{-1}. The extremely high SO_2 concentration in the variant D was chosen to verify if the reaction of SO_2 with glutathione that forms $GSSO_3H$ (Arapatsis et al.,2016) takes place in the sample during storage. Analysis of the sample by UHPLC-MS/MS confirmed that this reaction take place, but in a negligeable amount and the decrease in the TG concentration was due to SO_2 interference with the reagent of the EA method, and not to a reaction between SO_2 and glutathione (Fig. 3b).

In fact, the addition of 1 g L^{-1} SO_2 produced no decrease in the concentration of GSH+GSSG measured by UHPLC-MS/MS even after five weeks' storage. Instead, it was noted that $GSSO_3H$, resulting from the reaction of SO_2 with glutathione, appeared in variants where SO_2 was added to the grape juice (Fig. 3c). However, its concentration was low, even in the variant D containing 1 g L^{-1} SO_2, between 4% and 10% of the TG depending on the grape variety (Fig. 4). The highest concentration was measured in Petite Arvine. In this grape variety, the concentration of GSSG was also the highest, at 15% of the TG in the variant without SO_2 (variant B) (Supplementary Data). In the other grape varieties, GSSG represented only 4–6% of the TG. These results support the hypothesis that the quantity of $GSSO_3H$ depends on the GSSG concentration, and not only on TG (Arapitsas et al., 2016).

After storage for 18 weeks, the concentrations of GSH+GSSG, measured by UHPLC-MS/MS, were stable in the protected samples (B–D). This was contrary to the TG measured with the EA method, which slightly decreased after four weeks despite protection (B and C). The difference between TG measured before storage and at four weeks depended on the grape variety: − 13% Gamay, − 25% Gamaret, − 16% Chasselas, − 19% Doral and + 16% Petite Arvine (Supplementary Data). After five weeks, the concentration remained stable, and it continued to do so until 18 weeks had passed. The addition of 80 mg L^{-1} SO_2 to grape juice already containing ascorbic acid (C) did not improve the stabilisation of TG. For this reason, we recommend using only ascorbic acid (2.5 g L^{-1}) to protect glutathione. The acid should be added immediately after pressing, and the sample should be stored at − 20 °C before analysis with the EA method. The advantage of using ascorbic acid, compared to the protector proposed in the literature (SO_2), is to avoid the protective agent interfering with the EA method and ensure that accurate measurements can be collected.

The analysis of more than 300 samples prepared following these recommendations revealed a good correlation ($R^2 = 0.965$) between the results obtained with the EA and UHPLC-MS/MS methods (Fig. 5). The TG contents in grape juice and must samples of different grape varieties were analysed over four consecutive vintages, and the results confirmed that the EA method can replace the UHPLC-MS/MS and represents a handy, economical alternative for the analyses of TG in grape juice.

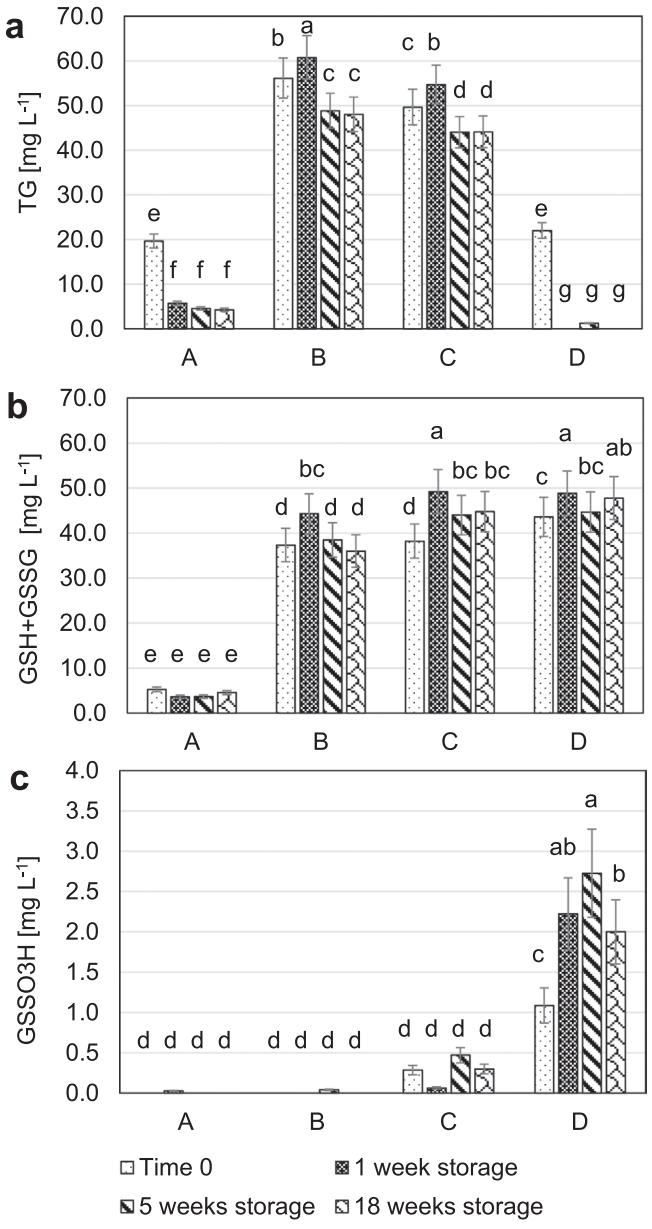

Fig. 3. Total glutathione concentrations measured during the storage of Gamay grape juice using different protecting agents. Note: Variant A is grape juice without a protective agent, B is grape juice with 2.5 g L^{-1} ascorbic acid, C with 2.5 g L^{-1} ascorbic acid and 80 mg L^{-1} SO_2 and D with 2.5 g L^{-1} ascorbic acid and 1 g L^{-1} SO_2. **a**: TG measured using the EA method, **b**: sum of GSH and GSSG measured by UHPLC-MS/MS, **c**: concentration of $GSSO_3H$ measured by UHPLC-MS/MS. Different letters indicate significant differences between the samples ($p \leq 0.001$).

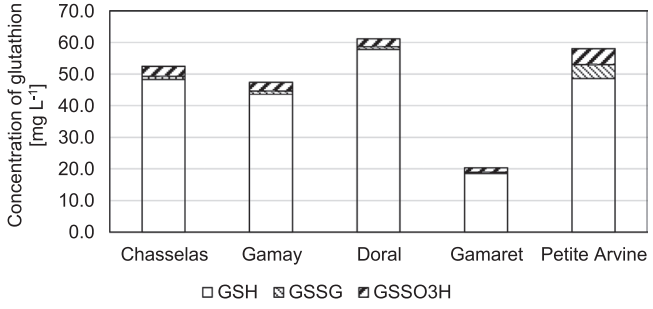

Fig. 4. Glutathione concentrations in juices of different grape varieties after five weeks' storage under condition D (2.5 g L^{-1} ascorbic acid and 1 g L^{-1} SO_2). The concentrations of GSH (□), GSSG (\\\) and $GSSO_3H$ (///) were measured by HPLC-MS/MS.

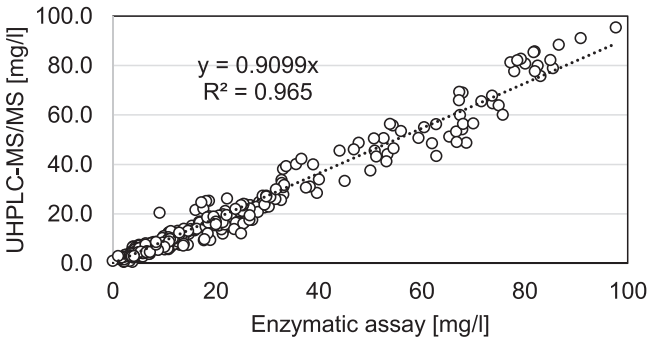

Fig. 5. Correlations between the total glutathione (GSH + GSSG) results obtained with the two methods (EA and UHPLC-MS/MS) using grape juice samples and fermentation study samples in the period of 2015–2018 (more than 300 samples in total).

4. Conclusion

The EA method set out in this paper, carried out using an automatic sequential analyser with spectrophotometric detection, allows for measuring the total glutathione levels of grape juices directly in a wine cellar or small laboratory due to its simplicity and the reasonable price of the instrument. This high-throughput, fully automatised, reproducible method permits the analyses of 30 sample in one hour in the range of 5 – 100 mg L^{-1} TG. Particular attention must be paid to sample preparation given the high reactivity of GSH. Samples should be protected with an antioxidant agent immediately after pressing. The addition of ascorbic acid at a concentration of 2.5 g L^{-1} and freezing at − 20 °C allows for long-term storage (about 18 weeks) of the sample. Yet, the addition of a large amount of SO_2 can skew the results of the EA method as it reacts with the reagents used for the determination of TG. However, we demonstrated that this method is well adapted to the analysis of samples containing low concentration of SO_2, for example grape treated with about 50 mg L^{-1} SO_2 in the cellar. Samples containing higher concentration of SO_2 could instead be analysed using the UHPLC-MS/MS method, which has the added benefit of allowing for the quantification of all glutathione species (GSH, GSSG and $GSSO_3H$) separately. Based on our results, we propose that the enzymatic assay offers a simple, economic and ecological alternative to UHPLC-MS/MS, which can be effectively applied to measure the total glutathione concentrations in grape juices and plant extracts where the dominant form of glutathione is GSH.

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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2023.105440.

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