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Determination of Glutathione in Austrian Wine Samples: The Effects of Freezing, the Choice of Yeast and Storage

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Abstract

Glutathione (GSH, γ-L-Glutamyl-L-Cysteinyl Glycine) is a tripeptide of L-glutamate, L-cysteine and glycine. GSH in wine is derived from either grapes or yeast, during alcoholic fermentation. The GSH concentration in wine is very variable and depends on the environmental conditions as well as viticultural practices. During winemaking GSH has a significant role in oxidation prevention due to its unique redox and nucleophilic properties. Since GSH is very reactive it is highly important to prepare samples immediately and under inert conditions just prior to the determination of the GSH concentration. Therefore the aim of this research was to implement a method for the quantitative determination of GSH levels in grape juices (musts) made from different Austrian grape varieties and to investigate the influence of yeast on the GSH content in wine after aging. The results of this research have shown that monitoring with nitrogen gas, sulphur dioxide and freezing process at −25 °C led to a good protective effect on the free glutathione amount in wine and grape samples. The GSH concentration in the samples was variable. Levels were ranging from non-detectable to up to 23.10 mg/l, and it showed that grape variety has no impact on GSH concentration in the must. Furthermore the results suggest that the choice of yeast has an impact on GSH content in wine even after 6 and 18 months of aging.

Keywords

glutathione, Austrian grape varieties, HPLC, freezing effect, yeast

1 Introduction

Glutathione (GSH) is a tripeptide of L-glutamate, L-cysteine and glycine. GSH is found in two forms in the cell: reduced (GSH) and oxidized as glutathione disulphide (GSSG) [1]. GSSG is formed during oxidation of GSH, and it can be reduced back to GSH by glutathione reductase [2].

In wine, GSH is derived either from grapes where it fulfills an important role in plant cells in terms of the antioxidant system, sulphur metabolism and the detoxification of xenobiotics or from yeast during fermentation [3]. It is found that, depending on stress conditions, yeasts are able to utilize and secret GSH during alcoholic fermentation, and in addition GSH is the main sulphur compound in the yeast's cell [4-6]. Therefore the GSH concentration in wine is highly variable and depends on the environmental conditions and viticultural practices [7]. In must and wine GSH reacts with oxidized phenolic compounds, such as caftaric acid quinones or other oxidation products [8, 9]. This reaction leads toward the formation of the grape reaction product (GRP). These reactions occur firstly during grape crushing, when phenolic compounds are oxidized by grape polyphenol oxidases but may also occur later in wine when chemical oxidation occurs [8, 10].

GSH has a possible protective function against oxidation. A simple mechanism of protection is based on a reaction in which the reduced glutathione form (GSH) is oxidized to glutathione disulfide (GSSG), thereby releasing protons and electrons used in coupled reactions to preserve molecules against oxidation [11]. Additionally, the level and redox status of GSH in plant can estimate the rate of oxidative stress and eco-toxicological injury [12].

Sulphur dioxide (SO_2) is the most widely used preservative in winemaking, displaying antioxidant, antimicrobial and anti-enzymatic properties [13, 14]. However, health-related concerns have resulted in consumer pressure to

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reduce its use [15]. Using other alternative antioxidants, such as GSH might be able to permit a lower SO_2 dosage in wine. Furthermore, GSH concentration higher than a few milligrams per liter in wine can effectively protect the varietal thiol and aroma compounds such as esters and terpenes, by acting as a competitor for quinone reduction due to its free sulfhydryl (SH) moiety [16]. Similarly, GSH is able to prevent the formation of atypical ageing characters, such as 2-aminoacetophenone [16].

Nevertheless the supplementation of purified glutathione in maximum 20 mg/l in must and wine is allowed in two resolutions of OIV [17, 18]. On one side the use of purified glutathione is forbidden from the European legislation of the European Union, but on the other side it is allowed to add GSH-enriched inactive yeast preparations [6, 10, 19].

Since GSH is a very reactive tripeptide, there are many difficulties for its determination. As GSH concentration decreases, it is of high importance to immediately prepare the samples [20]. There are not many publications regarding the freezing process on GSH concentration in must. Additionally GSH is well known for its benefits against the formation of atypical aging substances. Therefore, the aim of this research was to observe the GSH content in frozen samples of Austrian grape musts and to investigate the yeast influence on GSH content in wines, fermented with different yeasts, after 6 and 18 months of aging.

2 Material and methods

2.1 Samples

This study was taken within two different experiments. To investigate the influence of freezing process on GSH the prestudy was obtained, which included analyzes of 22 fresh and frozen must samples from different vineyards in Austria: Müller Thurgau from 1 vineyard, Sankt Laurent from 1 vineyard, Chardonnay from 8 different vineyards, Zweigelt from 2 different vineyards, Grüner Veltliner from 2 different vineyards, Welschriesling from 3 different vineyards, Blaufränkisch from 1 vineyard, Blauburger from 1 vineyard, Traminer from 1 vineyard, Sauvignon blanc from 1 vineyard, Weissburgunder from 1 vineyard. Subsequently, 51 different must samples were analyzed, from different Austrian vineyard regions. Immediately after crushing the grapes, 50 mg SO_2/l was added to all fresh musts to inhibit GSH oxidation. Samples were frozen under −25 °C for three days, and then analyzed. All samples were analyzed in triplicate.

The second experiment was monitoring the GSH concentration in two different wines which were fermented with different yeasts. The composition of grape juices (musts) is shown in Table 1. The fermentations were carried out in the cellar department in two grape varieties (Welschriesling and Grüner Veltliner). The fermentations of the commercial yeast were conducted in 34 l glass bottles by both varieties. Active dry yeasts (as listed below in Table 2) were rehydrated according to the instructions of the different yeast producers. In addition, 15 g / 34 l yeast nutrition Fermoplus Integrateur (AEB Group, Brescia, Italy) was added. The fermentation temperature was 22 °C in the cellar and fermentation was controlled by analyzing with OenoFossTM (Foss, Hamburg Germany). Fermentations were carried out in triplicate. After the bottling the measurements of reduced glutathione in the variety Grüner Veltliner were done after 18 months and in the variety Welschriesling after 6 months. The bottled wines were stored at 15 °C under the same conditions until they were analyzed. All samples were taken in duplicate from one bottle of each replicate.

2.2 Chemicals and reagents

Deionized water was used, HPLC-grade reagents and solvents were used for the mobile phases. For the mobile phase a preparation of 50 mM sodium acetate (Merck, Germany), pH 5.7 (buffer "A") and methanol (buffer "B") were used. Derivatizing reagents were prepared as followed: 2 mg o-phthalaldehyde (OPA) (Merck, Germany) dissolved in 1 ml methanol (Merck, Germany), 2 µl of 2-aminoethanol dissolved in 1 ml of 0.8 M sodium borate (pH 7.4) (Merck, Germany). Furthermore for the stock standard solutions included reduced glutathione, 98 % purity (105 mg/l) and L-cysteine (Merck, Germany) (209 mg/l), which was prepared in 5 mM sodium acetate buffer containing 0.1 mM EDTA.

2.3 Samples preparation

Each sample was centrifuged for 5 min, 7500 rpm (Micro centrifuge, Thermo-Fisher-Scientific, USA), and filtrated through 0.45 µm syringe filter. For analysis 0.75 ml of sample was taken.

Table 1 Composition of the Grüner Veltliner and Welschriesling grape musts

Grape must	°KMW†	pH	YAN‡ (mg/l)	NH_4^+ (mg/l)	Total acid§ (g/l)†
Grüner Veltliner	18.5	3.3	125	87	4.8
Welschriesling	16.3	3.3	281	195	7.8

† 1° KMW (Klosterneuburger Mostwaage) = 4.86° Oe ‡ YAN = Yeast Assimilable Nitrogen § Total acid is expressed as tartaric acid g/l

Table 2 List of yeasts used

List of yeasts used for Welschriesling grape variety	List of yeasts used for Grüner Veltliner grape variety
SP39 (SP39 Station Oenotechnique de Champagne, SAS Sofralab, Epernay Cedex, France)	Oenoferm Bio (Oenoferm®Bio Selection Klingelberg, Erbslöh Geisenheim AG, Geisenheim, Germany)
SP Organic (SP Organic Station Oenotechnique de Champagne, SAS Sofralab, Epernay Cedex, France)	Oenoferm PinoType (Oenoferm® PinotType, Erbslöh Geisenheim AG, Geisenheim, Germany)
SO. Delight (SO. Delight, Martin Vialatte, SAS Sofralab, Epernay Cedex, France)	Oenoferm Veltliner (Oenoferm®Veltliner, Erbslöh Geisenheim AG, Geisenheim, Germany)
La Persane (La Persane Oenofrance SAS Sofralab, Epernay Cedex, France)	PREZISO Weiß&Fruchtig (PREZISO Weißweinhefe Weiß & Fruchtig, RWA Raiffeisen Ware Austria Aktiengesellschaft, Vienna, Austria)
Zymaflore X16 (Zymaflore® X 16 ,Laffort, Bordeaux Cedex, France)	PREZISO Universal (PREZISO Hefe Universal, RWA Raiffeisen Ware Austria Aktiengesellschaft, Vienna, Austria)
Zymaflore X5 (Zymaflore® X5 ,Laffort Bordeaux Cedex, France)	PREZISO Weiss&Komplex (PREZISO Weissweinhefe Weiss & Komplex, RWA Raiffeisen Ware Austria Aktiengesellschaft, Vienna, Austria),
Actiflore RMS 2 (Actiflore® RMS 2, Laffort Bordeaux Cedex, France)	LittoLevure Elégance (LittoLevure Elégance, Erbslöh Geisenheim AG, Geisenheim, Germany)
Oenoferm Freddo (Oenoferm® Freddo, Erbslöh Geisenheim AG, Geisenheim, Germany)	Fermol Associées (Fermol®Associées, AEB Group, Brescia, Italy)
Oenoferm Riesling (Oenoferm® Riesling, Erbslöh Geisenheim AG, Geisenheim, Germany)	Fermol Iper R(Fermol® Iper R, AEB Group, Brescia, Italy)
Oenoferm X-treme (Oenoferm® X-treme, Erbslöh Geisenheim AG, Geisenheim, Germany)	Fermol Bayanus Lipari (Fermol®Bayanus Lipari, AEB Group, Brescia, Italy)
Oenoferm Klbg (Oenoferm® Klosterneuburg, Erbslöh Geisenheim AG, Geisenheim, Germany)	IOC B 3000 (IOC B3000, Lallemand Specialties GmbH, Vienna, Austria)
PREZISO Universal (PREZISO Hefe Universal, RWA Raiffeisen Ware Austria Aktiengesellschaft, Vienna, Austria)	IOC Revelation Thiols (IOC Revelation Thiols, Lallemand Specialties GmbH, Vienna, Austria),
PREZISO Primeur (PREZISO Weissweinhefe Primeur, RWA Raiffeisen Ware Austria Aktiengesellschaft, Vienna, Austria)	IOC 18 -2007 (IOC18 -2007, Erbslöh Geisenheim AG, Geisenheim, Germany),
PREZISO Weiß & Fruchtig (PREZISO Weißweinhefe Weiß & Fruchtig, RWA Raiffeisen Ware Austria Aktiengesellschaft, Vienna, Austria)	Filtraferm C Fresh (Filtraferm C Fresh, Lallemand Specialties GmbH, Vienna, Austria)
IOC 18 2007 (IOC 18-2007, Erbslöh Geisenheim AG, Geisenheim, Germany)	SIHA White Arome (SIHA®White Arome, EATON, Nettersheim, Germany)
Lalvin QA 23 (Lalvin QA 23, Lallemand Specialties GmbH, Vienna, Austria)	Sihaferm Element (SIHAFERM®Element, EATON, Nettersheim, Germany),
Uvaferm CEG (Uvaferm CEG, Lallemand Specialties GmbH, Vienna, Austria)	SIHA Cryarome (SIHA Cryarome®, EATON, Nettersheim, Germany),
Fermicru LVCB (Fermicru® LVCB, DSM Food Specialties B.V. and DSM Nutritional Products AG,AX Delft, Netherlands)	SIHA Aktivhefe 7 (SIHA®Aktivhefe 7, EATON, Nettersheim, Germany)
Fermicru VB1(Fermicru® VB1, DSM Food Specialties B.V. and DSM Nutritional Products AG,AX Delft, Netherlands),	Uvaferm WAM (Uvaferm WAM, Lallemand Specialties GmbH, Vienna, Austria)
Fermcru LS2 (Fermicru® LS2 , DSM Food Specialties B.V. and DSM Nutritional Products AG, AX Delft, Netherlands)	Fermicru VB1(Fermicru® VB1, DSM Food Specialties B.V. and DSM Nutritional Products AG,AX Delft, Netherlands)
Fermicru AR2 (Fermicru® Ar2, DSM Food Specialties B.V. and DSM Nutritional Products AG, AX Delft Netherlands)	Fermicru AR2 (Fermicru® Ar2, DSM Food Specialties B.V. and DSM Nutritional Products AG, AX Delft, Netherlands),
	Fermicru LVCB (Fermicru® LVCB, DSM Food Specialties B.V. and DSM Nutritional Products AG,AX Delft, Netherlands),
	Zymaflore X5 (Zymaflore® X5 ,Laffort Bordeaux Cedex, France)
	Zymaflore Alpha (Zymaflore®Alpha TD.sacch. Laffort Bordeaux Cedex, France)
	Zymaflore VL 3 (Zymaflore®VL3, Laffort Bordeaux Cedex, France).

The sample was then diluted with 0.75 ml of 5 mM sodium acetate buffer (pH 4) containing 0.1 mM EDTA. All reagents and samples were put into sample vials (1.5 ml) which had been previously purged with nitrogen gas, shortly before sampling. The headspace was also purged with nitrogen gas before sealing the vial

with a Teflon-faced septum. In order to investigate the stability of prepared samples, the prestudy was conducted. Samples were prepared with and without addition of SO_2 and nitrogen purging. Additionally, samples were analyzed after 2, 6, 20 and 48 hours at room temperature, which corresponds to autosampler temperature. Without the addition of SO_2 and nitrogen purging, it was not found any measurable GSH in samples. However, as it is shown in the Fig. 1, after 20 hours of sample's storage, the GSH content intends to decrease. Therefore all prepared samples were in autosampler never longer than 8 hours.

2.4 Instrumentation

An Agilent 1220 Series HPLC was used for quantification. GSH was detected on an Agilent 1100 fluorescence detector: wavelengths excitation 340 nm and emission 450 nm. The gradient program used for the mobile phases is shown in Table 3. Derivatives were separated on a column (Nucleoshell RP 18, 2.7 µm, 150 mm × 2 mm). The online pre-column derivatization with o-phthalaldehyde and 2-aminoethanol (OPA) is a modification of a manual analysis described by Mopper and Delmas [21]. The online derivatization procedure was as follows: 2 µl of OPA was withdrawn from the vial and the needle was afterward washed with H_2O. 5 µl of sample was withdrawn from the sample vial and the needle was washed again with H_2O. Finally, 2 µl of 2-aminoethanol was withdrawn and mixed for exactly 1 minute by moving the reagents and sample volumes back and forth inside the auto-sampler's syringe capillary. The derivatized sample was injected immediately by automatic injector for analysis. For each sample this automatic derivatization procedure was performed just before injection.

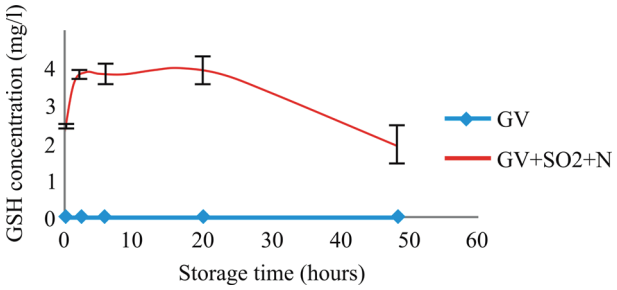

Fig. 1 Influence of storage time of samples on GSH content, at room temperature. Results are shown as mean value (n = 3) ± standard deviation. GV presents Grüner Veltliner grape juice without addition of SO_2 and nitrogen purging, GV + SO_2 + N presents Grüner Veltliner grape juice with addition of SO_2 and nitrogen purging

Table 3 Elution Gradient

Time (min)	Buffer A (%)*	Buffer B (%)**
1	80	20
10	60	40
15	60	40
17	80	20

*Sodium acetate **Methanol

2.5 Statistical analyses

Firstly, the data set was tested for normal distribution using an exploratory data analysis. No outliers were eliminated. In the case of a normal distribution, the mean values of the independent samples were compared using a one-factorial analysis of variance and tested for variance homogeneity. If variance homogeneity was proved, an evaluation was done using the Tukey B test at the significance level of 0.05. If there was no variance homogeneity, the Tunnet-T3 test was also used at the significance level of 0.05. If the respective data set was not distributed normally, a Kruskal-Wallis test was conducted at the significance level of 0.05 and evaluated in the form of pairwise comparisons. The data were statistically analyzed using the Statistica software version 13 (Statsoft Inc., 2013, USA) and SPSS 22.0 (IBM, 2013, USA).

3 Results and discussion

A five-point calibration curve was linear in the range of 0.1 to 26.25 mg/l. Limit of detection (LOD) was 0.15 mg/l and limit of quantification (LOQ) was 0.5 mg/l. In this research glutathione was observed only in the reduced form (GSH). Working area was between 0.1-26.25 mg/l. The retention time of GSH was 4.4 min based on standards.

Results from the study are presented in Table 4, where the differences in GSH content are shown between the fresh samples and in the same samples after three days of freezing at −25 °C. There was a significant statistical difference in most of the samples, thus to handle the same conditions of sample preparation, all samples were frozen, exactly for three days.

3.1 Determination of GSH in different must

The GSH content in must was variable. Levels were ranging from non-detectable to 23.10 mg/l. The results are shown in Table 5. Several factors can influence the GSH concentration in musts, for example tyrosinase activity, environmental conditions and exposure to oxygen [22-24]. A correlation has also been found with the amount of readily assimilable nitrogen in the soil [16]. According to the results of this study, the reduced glutathione amount is not

Table 4 GSH content (mg/l) in fresh and frozen samples

Grape Variety	Fresh samples	Frozen samples
Müller Thurgau1	**20.57 ± 0.09[a]**	**23.09 ± 0.02[b]**
Sankt Laurent	**8.65 ± 0.07[a]**	**9.05 ± 0.07[b]**
Chardonnay1	5.455 ± 0.08[a]	5.70 ± 0.03[a]
Chardonnay2	13.13 ± 0.09[a]	13.89 ± 0.43[a]
Chardonnay3	**23.06 ± 0.05[a]**	**21.70 ± 0.11[b]**
Chardonnay4	14.56 ± 0.09[a]	14.40 ± 0.14[a]
Chardonnay5	**9.35 ± 0.07[a]**	**5.87 ± 0.07[b]**
Chardonnay6	**20.06 ± 0.06[a]**	**15.51 ± 0.13[b]**
Chardonnay7	13.60 ± 0.14[a]	14.76 ± 1.05[a]
Chardonnay8	11.62 ± 0.03[a]	11.56 ± 0.09[a]
Zweigelt1	**15.51 ± 0.25[a]**	**13.90 ± 0.03[b]**
Zweigelt2	**9.4 ± 0.14[a]**	**6.18 ± 0.04[b]**
Grüner Veltliner1	**3.19 ± 0.13[a]**	**0.60 ± 0.14[b]**
Grüner Veltliner2	**9.91 ± 0.15[a]**	**11.15 ± 0.08[b]**
Welschriesling1	17.92 ± 0.17[a]	17.41 ± 0.13[a]
Welschriesling2	**28.01 ± 0.13[a]**	**21.26 ± 0.09[b]**
Welschriesling3	**9.55 ± 0.07[a]**	**11.80 ± 0.29[b]**
Blaufränkisch1	10.58 ± 0.11[a]	10.39 ± 0.09[a]
Blauburger1	**20.05 ± 0.08[a]**	**17.05 ± 0.21[b]**
Traminer1	**9.475 ± 0.11[a]**	**8.07 ± 0.05[b]**
Sauvignon Blanc1	14.30 ± 0.14[a]	14.52 ± 0.11[a]
Weissburgunder1	**7.15 ± 0.07[a]**	**8.40 ± 0.14[b]**

Results are shown as mean value (n = 3) ± standard deviation
*a, b indicates significant difference between value

in correlation with the grape variety. It was discovered that one grape variety contained different glutathione amount. However this is only an assumption and this research demands further investigations.

3.2 Monitoring GSH content in wines fermented with different yeast

21 commercial yeast strains for the grape variety Welschriesling and 25 yeast strains for the variety Grüner Veltliner were used to investigate the influence of the yeast strain on the content of GSH in finished wine. As shown in Fig. 2, concerning the variety Welschriesling after 6 months of aging in the bottle, the content of GSH ranged between 2 and 5 mg/L. These results are in agreement with [6, 25, 26].

The use of different yeasts showed different amounts of GSH in this experiment. Regarding the strain Uvaferm CEG with less GSH and the strains Preziso Weiß & Fruchtig and Fermicru LVCB with higher amount of GSH, statistical differences were found. In common literature it is well described that there is an impact of the yeast strain

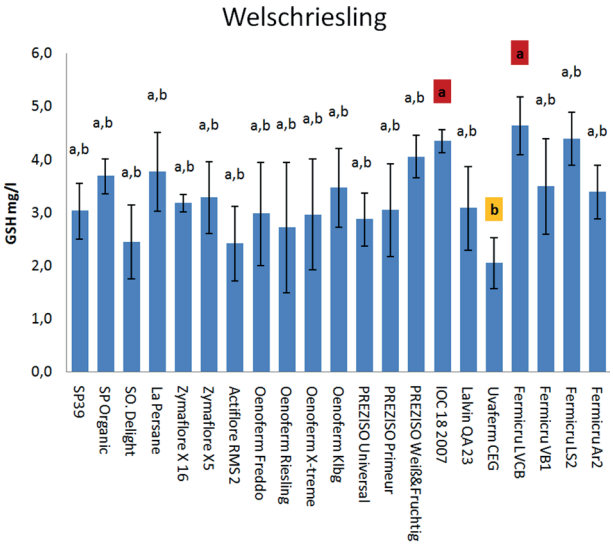

Fig. 2 Average amounts of GSH, variety Welschriesling, vinified with 21 different commercial yeasts, after 6 months aging in the bottle. Results are shown as mean value (n = 6) ± standard deviation
*a, b indicates significant difference between value.

on the final GSH concentration in finished wine [5, 6]. The GSH concentration in Grüner Veltliner wines is shown in Fig. 3. Musts that were fermented with different yeast strains, measured after 18 months of aging, ranged between 0.3 and 1 mg/L. These low concentrations could be explained by the fact that within pH conditions of 3.3 in wine, the autoxidation of GSH is possible and the presence of ortho-quinones leads to the formation of GRP and similar products and hence decreasing the concentration of GSH over the time [19, 27]. Nevertheless there was a statistical difference between the yeast strain Zymaflore VL3

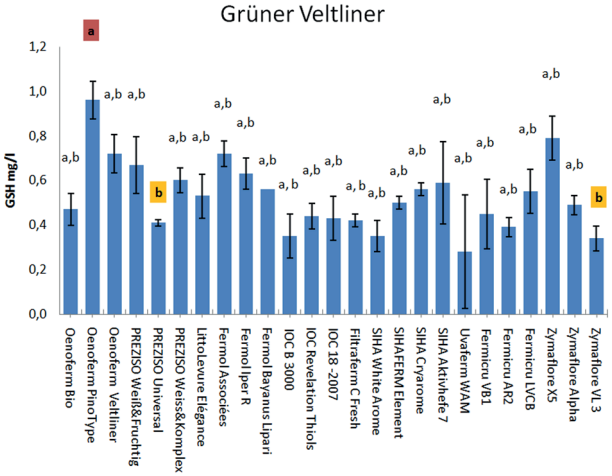

Fig. 3 Average amounts of GSH, variety Grüner Veltliner, vinified with 25 different commercial yeasts, after 18 months aging in the bottle. Results are shown as mean value (n = 6) ± standard deviation
*a, b indicates significant difference between value

Table 5 GSH concentrations in different Austrian grape varieties

Grape variety	Glutathione (mg/l)	Grape variety	Glutathione (mg/l)
Müller Thurgau2	23.10 ± 0.05	Grüner Veltliner9	4.41 ± 0.18
Chardonnay9	5.35 ± 0.46	Grüner Veltliner10	0.36 ± 0.25
Chardonnay10	14.14 ± 0.78	Grüner Veltliner11	2.46 ± 0.35
Chardonnay11	21.82 ± 0.29	Grüner Veltliner12	0.28 ± 0.04
Chardonnay12	14.85 ± 0.49	Grüner Veltliner13	2.43± 0.17
Chardonnay13	5.68 ± 0.33	Grüner Veltliner14	9.60 ± 0.71
Chardonnay14	15.66 ± 0.09	Grüner Veltliner15	1.32 ± 0.10
Chardonnay15	15.76 ± 0.37	Grüner Veltliner16	0.29 ± 0.06
Chardonnay16	11.75 ± 0.37	Grüner Veltliner17	nd
Chardonnay17	5.56 ± 0.08	Welschriesling4	17.15 ± 0.49
Chardonnay18	nd†	Welschriesling5	21.64 ± 0.64
Chardonnay19	0.48 ± 0.20	Welschriesling6	11.85 ± 0.37
Chardonnay20	0.57± 0.10	Welschriesling7	nd
Zweigelt3	14.04 ± 0.23	Welschriesling8	0.59 ± 0.23
Zweigelt4	6.19 ± 0.04	Blaufränkisch2	10.49 ± 0.04
Zweigelt5	nd	Blaufränkisch3	0.37 ± 0.11
Zweigelt6	4.4 ± 0.07	Blaufränkisch 4	1.46 ± 0.11
Zweigelt7	nd	Blauburger2	17.10 ± 0.28
Zweigelt8	nd	Traminer2	7.81 ± 0.31
Zweigelt9	nd	Sauvignon Blanc2	14.05 ± 0.78
Grüner Veltliner3	0.35 ± 0.21	Sauvignon Blanc3	4.03 ± 0.06
Grüner Veltliner4	nd	Weissburgunder2	7.92 ± 0.53
Grüner Veltliner5	11.10 ± 0.02	Gelber Muskateller	nd
Grüner Veltliner6	0.51 ± 0.15	Rheinriesling1	0.48 ± 0.08
Grüner Veltliner7	nd	Rheinriesling2	0.42 ± 0.14
Grüner Veltliner8	0.32 ± 0.03		

Results are shown as mean value (n = 3) ± standard deviation †nd = non-detectable (LOD = 0.15 mg/l)

and Preziso Universal with lower GSH concentrations and Oenoferm PinoType with higher amounts of GSH even after 18 month of aging in the bottle.

4 Conclusion

Glutathione (GSH) is a very reactive tripeptide, therefore inert conditions during sample preparation are required. Monitoring with sulphur dioxide, nitrogen gas, and freezing process at −25 °C leads to a good protective effect on the free glutathione amount in wine and grape samples. The freezing process is suitable for delaying the analysis of samples. Glutathione concentration (GSH) in Austrian grape varieties is very variable, and it is not in correlation with the grape varieties. On the other hand there was an influence of different yeast strains on the content of GSH in finished wine even after 18 months of aging.

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