INFLUENCE OF NITROGEN ON GLUTATHIONE CONTENT DURING AND AFTER ALCOHOLIC FERMENTATION

Danka Dragojlović[1], Christian Philipp[2], Karin Korntheuer[2], Christian Bader[2], Elsa Patzl-Fischerleitner[2] and Reinhard Eder[2]

1 Institute of Food Technology, University of Novi Sad
RS-21000 Novi Sad, Bulevar cara Lazara 1
2 Federal College and Research Institute for Viticulture and Pomology Klosterneuburg
A-3400 Klosterneuburg, Wiener Straße 74
E-Mail: danka.dragojlovic@fins.uns.ac.rs

Glutathione (γ-L-Glutamyl-L-Cysteinyl Glycine) is a tripeptide of L-glutamate, L-cysteine and glycine and is likely to become more important for the wine industry in the future due to its antioxidative properties. Therefore the aim of this research was to investigate the influence of different sources of nitrogen (organic and inorganic) on the glutathione content during and after alcoholic fermentation. The results showed that the addition of nitrogen into the musts of two different grape varieties caused higher glutathione levels during and after alcoholic fermentation (inorganic: significantly; organic: not significantly).
Keywords: glutathione, nitrogen, alcoholic fermentation, yeast

Einfluss von Stickstoff auf den Glutathiongehalt während und nach der alkoholischen Gärung. Glutathion (γ-L-Glutamyl-L-Cysteinyl-Glycin) ist ein Tripeptid aus L-Glutamat, L-Cystein und Glycin und dürfte aufgrund seiner antioxidativen Eigenschaften in Zukunft für die Weinindustrie wichtiger werden. Ziel dieser Arbeit war es, den Einfluss verschiedener Stickstoffquellen (organisch und anorganisch) auf den Glutathion-Gehalt während und nach der alkoholischen Gärung zu untersuchen. Die Ergebnisse zeigen, dass die Zugabe von Stickstoff zu Mosten zweier verschiedener Rebsorten zu höheren Glutathiongehalten während und nach der alkoholischen Gärung führte (inorganisch: signifikant; organisch: nicht signifikant).
Schlagwörter: Glutahion, Stickstoff, alkoholische Gärung, Hefe

Glutathione (γ-L-Glutamyl-L-Cysteinyl Glycine) is a tripeptide of L-glutamate, L-cysteine and glycine. In the molecule of glutathione, glutamate and cysteine are bonded by γ-peptide bond, which prevents glutathione from being hydrolyzed by most peptidases. It is found that glutathione can exist in both forms, reduced (GSH) and oxidized as glutathione disulfide (GSSG) (Fahey, 2001).

In the must and wine GSH plays a very important role, where it reacts with oxidized phenolic compounds, such as caftaric acid quinones or other oxidation products (Kritzinger et al., 2013; Sonni et al., 2011a, b). GSH concentrations higher than a few milligrams per liter in wine can also effectively protect the varietal thiol compounds, by acting as a competitor for quinone reduction (Dubourdieu and Lavigne, 2004). Similarly, GSH appears to inhibit the formation of atypical ageing characters, for example 2-aminoacetophenone during wine ageing (Dubourdieu and Lavigne, 2004). Protective effect is not exactly known, but assumption is that it is related to the antioxidant properties of GSH (Kritzinger et al., 2013). GSH in the wine is derived either from grapes where it has an important role in plant cells in terms of the antioxidant system, sulphur metabolism and the detoxification of xenobiotics or from yeasts during fermentation (Noctor and Foyer, 1998). GSH is the main sulphur compound in the yeast and depending on the stress conditions, yeasts are able to utilize and to secret it during alcoholic fermentation (Penninckx, 2000; Lavigne et al., 2007; Kritzinger, 2013). It is reported that during alcoholic fermentation GSH concentration either increases or decreases depending on the yeast strains, which have the ability to utilize or to secrete it (Kritzinger et al., 2013). The role of nitrogen supply for the formation of GSH is not yet fully understood and it is described contradictory (Park et al., 2000; Kritzinger et al., 2013). Park et al. (2000) reported that GSH production is directly correlated with both, total nitrogen and assimilable amino acid content of grape juice. Kritzinger et al. (2013), however, reported that there is no significant influence of the nitrogen amount on glutathione concentration.

The aim of this research was to investigate the influence of nitrogen additions on the GSH content during and after alcoholic fermentation and to see if it is possible to increase GSH content in the finished wine by adding a certain amount of nitrogen (in organic or inorganic form) at the beginning of alcoholic fermentation.

MATERIALS

SAMPLES

For studying the influence of nitrogen on the content of GSH, fermentations were carried out under laboratory conditions in pasteurized grape juices, made from different grape varieties ('Grüner Veltliner' and 'Gelber Muskateller'). The composition of the pasteurized grape juices (Table 1) was determined by OenoFossTM (Foss, Hamburg, Germany). Active dry yeast (Oenoferm Klosterneuburg; Erbsloeh, Geisenheim, Germany) was rehydrated as recommended by suppliers and inoculated in pasteurized grape juice. On the third fermentation day, different amounts of diammonium phosphate (DAP), as an inorganic source of nitrogen (35 and 50 mg/l, respectively; Oenofrance, Bordeaux, France) and nutrients for the yeast as a source of organic nitrogen (20 and 60 mg/l, respectively, of Nutricell fullarom (Martin Vialatte, Magenta, France)) were added. These concentrations were chosen due to recommendation of the producers. Fermentation was continued in 1l bottles at 22 ± 2 °C. Every third day of alcoholic fermentation samples were taken in plastic vials (2 ml), purged with nitrogen and immediately frozen under -25 °C. Every second day samples were taken from the refrigerator, thawed and analyzed. Fermentations were conducted in triplicate.

Table 1: Composition of the pasteurized grape juices used in this research

Pasteurized must	°KMW	pH	TA* (g/l)	ON** (mg/l)	NH_4^+ (mg/l)	GSH*** (mg/l)
Grüner Veltliner	20.2	3.36	3.5	221	97	0.21
Muskateller	18.0	3.53	3.7	347	176	n.d.¥

*TA = tartaric acid **ON = organic nitrogen, ***GSH = glutathione in reduced form, ¥n.d. = non-detectable

CHEMICALS AND REAGENTS

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

METHOD

Quantitative analysis of GSH was carried out using the method proposed and validated by Dragojlović et al.(2018). In accordance with this method, each sample was centrifuged 5 min, 7500 rpm (Microcentrifuge, Thermo-Fisher-Scientific, USA), and filtrated through 0.45 µm syringe filter. 0.75 ml of sample were taken for analyzing. Then the sample was 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.

An Agilent 1220 Series HPLC was used for quantification. Glutathione was detected by an Agilent 1100 fluorescence detector: wavelengths excitation 340 nm and emission 450 nm. Using the gradient program for the mobile phases shown in Table 2, derivatives were separated on a column (Nucleoshell RP 18, 2.7 µm, 150 mm × 2 mm; Macherey-Nagel, Düren, Deutschland).

The on-line pre-column derivatization with o-phthalaldehyde and 2-aminoethanol is a modification of a manual analysis described by Mopper and Delmas (1984). The on-line derivatization procedure was as follows: 2 µl of OPA were withdrawn from the vial, the needle was washed with H_2O, 5 µl of sample were withdrawn from the sample vial, the needle was washed again with H_2O. Finally 2 µl of 2-aminoethanol were withdrawn and mixed for exactly 1 min 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.

Table 2. Elution Gradient Time

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

*Sodium acetate ** Methanol

STATISTICAL EVALUATION

The statistical evaluation of the results interpretation was carried out using SPSS-Statistics 22.0 and Excel (Microsoft Office 2016). After a normal distribution and variance homogeneity test, Kruskal Wallis test was used for determining significant differences ($\alpha = 0,05$).

RESULTS AND DISCUSSION

As shown in Figure 1 and 2, at the beginning of the fermentation the content of GSH for all six samples was less than 1 mg/l. On the third day of alcoholic fermentation the inorganic nitrogen (DAP) was added. From this stage on the GSH content started to increase in every sample, but with the different amount of nitrogen the effect of increase was different. In both grape varieties the observed phenomenon was the same: The higher the amount of added DAP, the higher the detected amount of GSH in every stage of alcoholic fermentation and after alcoholic fermentation. For the fermentation of 'Grüner Veltliner' (Fig. 1) the difference was significant beginning with day 9 (day 9: Kruskal Wallis H: 9,692, DF = 2, p = 0,008, significant pairwise comparison: GV-GV max: H = -8,500, p = 0,006; day 12: Kruskal Wallis H: 8,626, DF = 2, p = 0,013, significant pairwise comparison: GV-GV max: H = -7,833, p = 0,013; day 15: Kruskal

Wallis H: 7,887, DF = 2, p = 0,019, significant pairwise comparison: GV-GV max: H = -7,167, p = 0,028; day 18: Kruskal Wallis H: 9,118, DF = 2, p = 0,010, significant pairwise comparison: GV-GV max: H = -8,167, p = 0,009; day 21: Kruskal Wallis H: 9,692, DF = 2, p = 0,008, significant pairwise comparison: GV-GV max: H = -8,500, p = 0,006). A significant difference between GV and GVmax was observed but there was no significant difference between GV and GV min as well as GV max and GV min.

For the fermentation of 'Gelber Muskateller' (Fig. 2) the difference was significant beginning with day 12 (day 12: Kruskal Wallis H: 8,588, DF = 2, p = 0,014, significant pairwise comparison: MU-MU max: H = -8,300, p = 0,010; day 15: Kruskal Wallis H: 10,085, DF = 2, p = 0,006, significant pairwise comparison: MU-MU max: H = -8,667, p = 0,007; day 18: Kruskal Wallis H: 9,916, DF = 2, p = 0,007, significant pairwise comparison: MU-MU max: H = -8,800, p = 0,006; day 21: no significant difference).

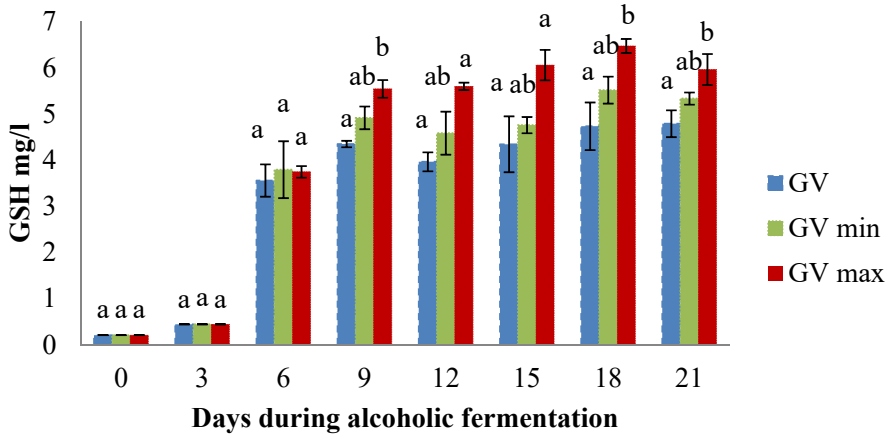

Fig. 1: Amount of GSH during and after alcoholic fermentation in pasteurized must with low, high and without addition of DAP in pasteurized must ('Grüner Veltliner'): GV (Grüner Veltliner), GV min (Grüner Veltliner with minimum addition of DAP), GV max (Grüner Veltliner with maximum addition of DAP). The same letters within the same day indicate that there is no significant difference observed.

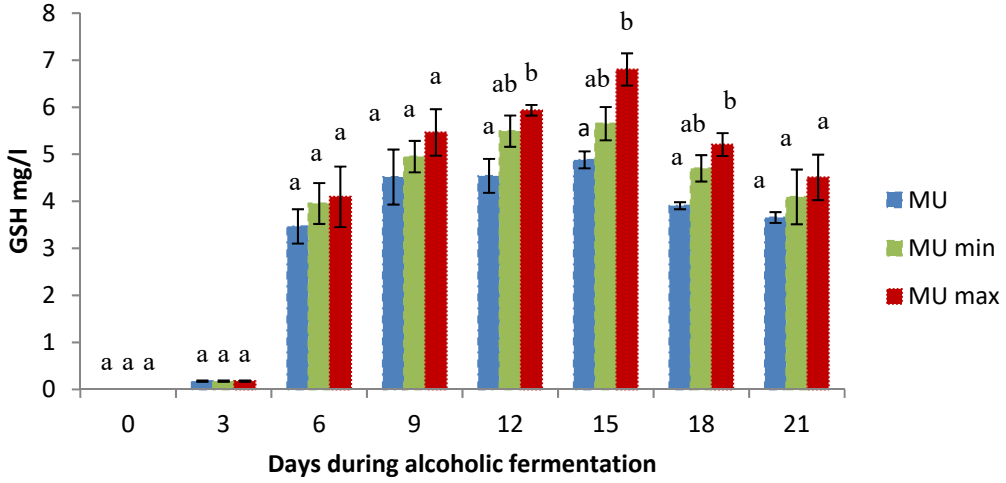

Fig. 2: Amount of GSH during and after alcoholic fermentation in pasteurized must with low, high and without addition of DAP in pasteurized must ('Gelber Muskateller'): MU (Gelber Muskateller), MU min (Gelber Muskateller with minimum addition of DAP), MU max (Gelber Muskateller with maximum addition of DAP). The same letters within the same day indicate that there is no significant difference observed.

This assumption was also confirmed in Figure 3, where the total nitrogen was plotted against the concentration of reduced glutathione in two grape varieties and different amounts of total nitrogen. The amount of GSH did not correlate strongly with the nitrogen amount. Due to the total nitrogen, the GSH amounts in Grüner Veltliner samples were lower than in Gelber Muskateller samples, but the concentration of GSH was higher in Grüner Veltliner. On the other hand the content of GSH was higher in each sample with an addition of nitrogen than in samples without addition. In Figures 1 to 3 it is shown, that addition of DAP led to higher amounts of total nitrogen and GSH.

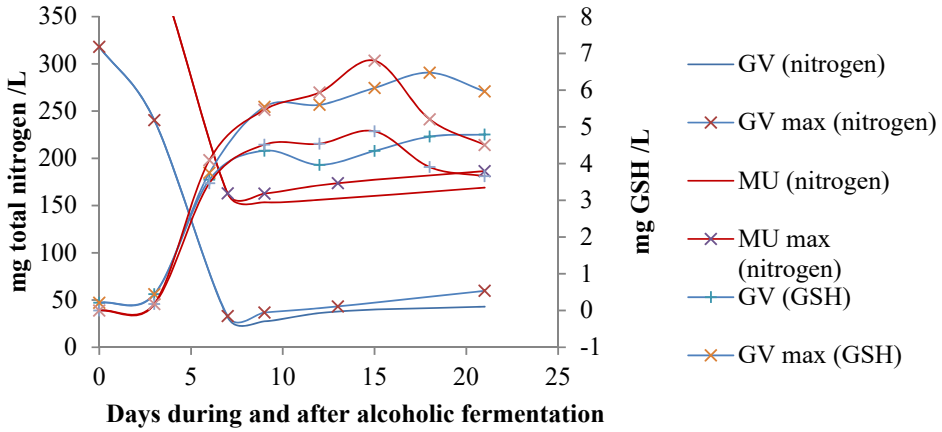

Fig. 3: Influence of total nitrogen amount on GSH concentration: GV (nitrogen) (total nitrogen content in Grüner Veltliner without any addition of DAP), GV max (nitrogen) (total nitrogen content in Grüner Veltliner with a maximum addition of DAP), MU (nitrogen) (total nitrogen content in Gelber Muskateller without any addition of DAP), MU max (nitrogen) (total nitrogen content in Gelber Muskateller with a maximum addition of DAP), GV (GSH) (glutathione content in Grüner Veltliner without any addition of DAP), GV max (GSH) (glutathione content in Grüner Veltliner with a maximum addition of DAP), MU (GSH) (glutathione content in Gelber Muskateller without any addition of DAP), MU max (GSH) (glutathione content in Gelber Muskateller with a maximum addition of DAP)

In Figure 4 to 6 results of the influence of the organic form of nitrogen on the GSH content are shown. It is remarkable that the same trends probably appeared here. The addition of organic form of nitrogen could lead to higher amounts of total nitrogen and higher amounts of GSH. However there was no significant difference in the fermentations observed with the exception of Gelber Muskateller day 9 of fermentation (Kruskal Wallis H: 6,911, DF = 2, p = 0,032, significant pairwise comparison: MU-MU max: H = -7,467, p = 0,026) and Grüner Veltliner day 15 (Kruskal Wallis H: 6,435, DF = 2, p = 0,040, no significant pairwise comparison).

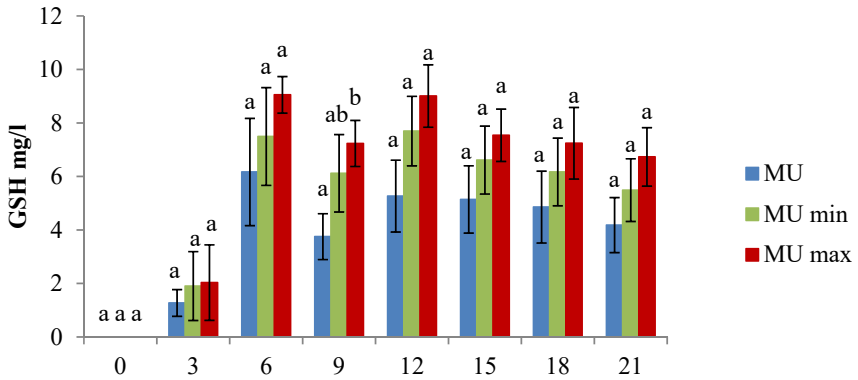

Fig. 4: Amount of GSH during and after alcoholic fermentation in pasteurized must with low, high and without addition of organic nitrogen source (yeast nutrients) in pasteurized must ('Grüner Veltliner'): GV (Grüner Veltliner), GV min (Grüner Veltliner with minimum addition of organic nitrogen source), GV max (Grüner Veltliner with maximum addition of organic nitrogen source)). There is no significant difference between the variants.

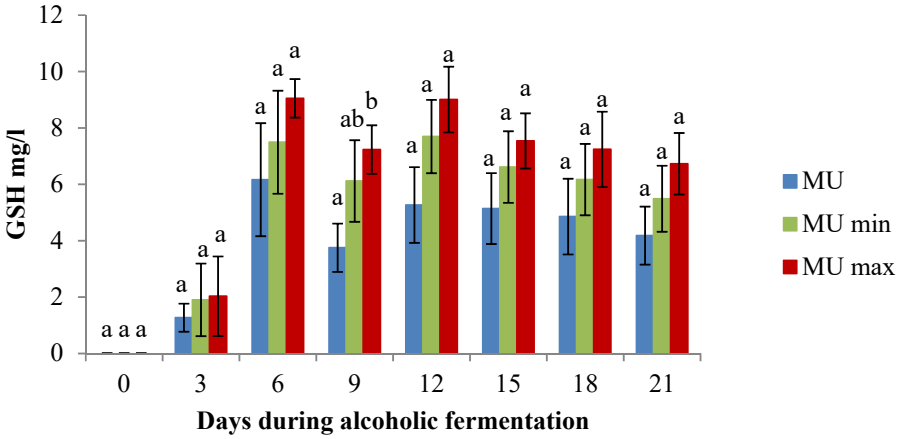

Fig. 5: Amount of GSH during and after alcoholic fermentation in pasteurized must with low, high and without addition of organic nitrogen source (yeast nutrients) in pasteurized must ('Gelber Muskateller'): MU (Gelber Muskateller), MU min (Gelber Muskateller with minimum addition of organic nitrogen source), MU max (Gelber Muskateller with maximum addition of organic nitrogen source)

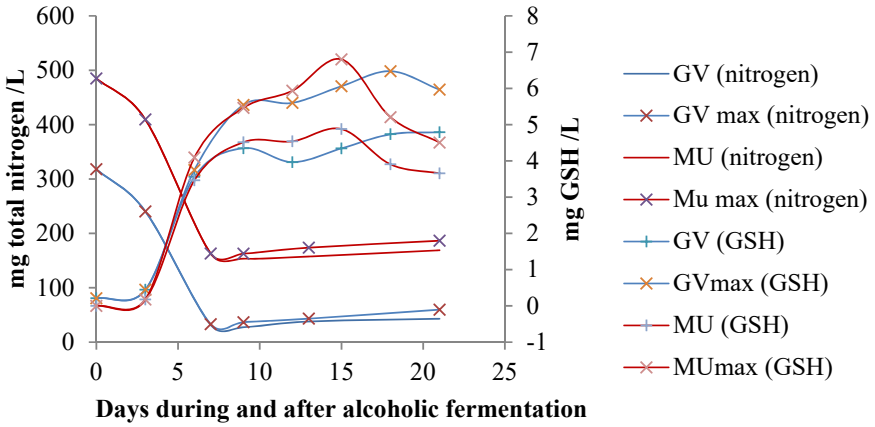

Fig. 6: Influence of total nitrogen amount on GSH concentration: GV (nitrogen) (total nitrogen content in Grüner Veltliner without any addition of organic nitrogen source), GV max (nitrogen) (total nitrogen content in Grüner Veltliner with a maximum addition of organic nitrogen source), MU (nitrogen) (total nitrogen content in Gelber Muskateller without any addition of organic nitrogen source), MU max (nitrogen) (total nitrogen content in Gelber Muskateller with a maximum addition of of organic nitrogen source), GV (GSH) (glutathione content in Grüner Veltliner without any addition of organic nitrogen source), GV max (GSH) (glutathione content in Grüner Veltliner with a maximum addition of organic nitrogen source), MU (GSH) (glutathione content in Gelber Muskateller without any addition of organic nitrogen source), MU max (GSH) (glutathion content in Gelber Muskateller with a maximum addition of organic nitrogen source)

CONCLUSION

In this research it was observed that there was a significant influence of addition of nitrogen (high amounts of DAP: significantly; high amounts of organic nitrogen: not significantly) on the reduced glutathione concentration during and after alcoholic fermentation (Fig. 1 to 3). In the samples with higher amounts of nitrogen, higher amounts of glutathione were found in every phase of alcoholic fermentation and during post-fermentation time. Samples without addition of any nitrogen source, had the lowest glutathione concentration during and after alcoholic fermentation. Based on these results, it can be supposed, that the yeast Oenoferm Klosterneuburg is able to release more glutathione if there is more nitrogen as an available nutrient. These results are in agreement with the results of Park et al. (2000). They reported that GSH production is directly correlated with both, total

nitrogen and assimilable amino acid content of grape juice. However, the results of this study are in opposition to results of Kritzinger et al. (2013), who reported that there is no significant influence of nitrogen amount on glutathione concentration. Yeast strains differ in their nitrogen requirements which may possibly influence the GSH content after alcoholic fermentation (Kritzinger et al., 2013). The results of the present study only apply to the yeast Oenoferm Klosterneuburg, but for this yeast they are very meaningful.

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