Winemaking practices affecting glutathione concentrations in white wine

by

Engela Cornelia Kritzinger

Thesis presented in partial fulfilment of the requirements for the degree of

Master of Agricultural Sciences

at

Stellenbosch University

Department of Viticulture and Oenology, Faculty of AgriSciences

Supervisor: Dr WJ du Toit

Co-supervisor: Prof FF Bauer

March 2012

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: U

Copyright © 2012 Stellenbosch University
All rights reserved

Stellenbosch University http://scholar.sun.ac.za

Summary

Glutathione (GSH), a tripeptide consisting of glutamate, cysteine and glycine, is the most ubiquitous non-protein intracellular thiol in a large variety of organisms, including plants, animals and fungi. The thiol moiety of the cysteine residue confers unique redox and nucleophilic properties. In plant cells, GSH fulfils an indispensible role in the antioxidant system, sulphur metabolism and detoxification of xenobiotics.

Upon grape crushing, GSH is extracted into the juice where it exerts several protective effects during the vinification process. In must, it reacts with oxidized phenolic compounds to form the colourless grape reaction product (GRP) which limits must browning to a certain extent. During wine ageing, GSH impedes the decrease of important aroma compounds, including certain esters, terpenes and volatile thiols, while at the same time preventing the development of atypical ageing off-flavours. GSH may also inhibit the yellowing of wine during ageing. It is thus evident that elevated GSH levels in wine, in particular white wine which is more sensitive to oxidation, may be highly valuable for wine quality.

The reductive crushing and pressing of white grape varieties, which limits oxidation and the consequent incorporation of GSH into GRP, promotes higher GSH levels in the juice. The reductive handling of juice also limits the formation of oxidized glutathione (GSSG). However, during alcoholic fermentation and maturation, levels generally decrease as a result of assimilation by the yeast *Saccharomyces cerevisiae,* and inevitable oxidation that takes place during the vinification process. The principal focus of this study was to gain a better understanding of the fate of glutathione during alcoholic fermentation and to establish whether certain oenological applications could result in elevated wine GSH levels. The application studied, included choice of yeast strain, extended lees contact, nitrogen supplementation and supplementation with glutathione enriched inactive dry yeast preparations (GHS-IDYs). In addition, the need for a rapid analytical method for the simultaneous quantification of both GSH and GSSG in must and wine which does not involve derivatization or require extensive sample preparation, led to the development of a novel UPLC-MS/MS method. The method was also employed to determine intracellular GSH and GSSG contents of the yeast *S. cerevisiae* and was studied for the first time in winemaking conditions.

It was shown that the GSH levels fluctuated during alcoholic fermentation, suggesting the uptake and release by yeast. At the end of alcoholic fermentation, levels were generally lower than those initially present in grape juice, but in some cases, concentration increases were also observed. This finding indicates that, in some cases, endogenously-produced GSH may be secreted into must during alcoholic fermentation, contributing to higher GSH levels in wine. Albeit small, significant differences in GSH content could be seen in wines fermented with different yeast strains, implying that yeast strain may to a certain extent influence wine GSH levels. While the effects of lees ageing and nitrogen

Stellenbosch University http://scholar.sun.ac.za

supplementation seem to be insignificant in contributing to higher GSH levels in wine, the supplementation of must with GSH-IDYs could result in increased wine GSH levels, provided the supplementation is done early during fermentation. This study has broadened our knowledge of several oenological factors, influencing GSH levels in wine and provided a new baseline for future research studies.

Stellenbosch University http://scholar.sun.ac.za

Opsomming

Glutatioon (GSH), 'n tripeptied bestaande uit glutamaat, sisteïen en glisien, is die mees algemene nie-proteïenagtige intrasellulêre tiool in 'n wye verskeidenheid organismes, insluitende plante, diere en fungi. Die tioolfunksiegedeelte van die sisteïenresidu verleen unieke redoks- en nukleofiliese eienskappe. GSH vervul 'n onmisbare rol in die antioksidantsisteem, swaelmetabolisme en die ontgiftiging van xenobiotika in plantselle.

Tydens die maal van druiwe word glutatioon in die sap geëkstraeer waar dit verskeie beskermende effekte tydens die wynbereidingsproses uitoefen. GSH reageer met geöksideerde fenoliese verbindings om die kleurlose druifreaksieproduk (DRP) te vorm wat die verbruining van mos in 'n sekere mate beperk. GSH verminder ook die afname van belangrike aromaverbindings tydens wynveroudering, insluitende sekere esters, terpene en vlugtige tiole, terwyl dit terselfdertyd die vorming van atipiese verouderingswangeure belemmer. So ook kan GSH die vergeling van wyn tydens veroudering inhibeer. Dit is dus voor die hand liggend that verhoogde GSH-vlakke in wyn, in die besonder witwyn, wat meer oksidasie-sensitief is, van waarde kan wees vir wynkwaliteit.

Die reduktiewe maal en pers van witdruifvariëteite wat oksidasie en gevolglike inkorporasie van GSH in DRP beperk, bevorder hoër GSH-vlakke in sap. So ook beperk die reduktiewe behandeling van sap die vorming van geoksideerde glutatioon (GSSG). Gedurende alkoholiese gisting en veroudering neem GSH-vlakke egter af as gevolg van assimilasie deur die gis, *Saccaromyces cerevisiae,* asook onvermydelike oksidasie wat gedurende die wynbereidingsproses plaasvind. Die hooffokus van die studie was om 'n beter begrip van die lot van glutatioon tydens alkoholiese gisting te verkry en om vas te stel of sekere wynkundige praktyke verhoogde GSH-vlakke in wyn tot gevolg kan hê. Die studie het gisraskeuse, verlengde gismoerkontak, stikstofaanvulling en aanvulling met glutatioon-verrykte, onaktiewe droëgis ingesluit. Daarbenewens het die behoefte aan 'n vinnige analitiese metode vir die gelyktydige kwantifisering van sowel GSH as GSSG in mos en wyn wat nie derivatisering of uitgebreide monstervoorbereiding vereis nie, gelei tot die ontwikkeling van 'n nuwe UPLC-MS/MS metode. Hierdie metode is ook gebruik om die intrasellulêre GSH- en GSSG-inhoud van die gis *S. cerevisiae* te bepaal wat vir die eerste keer in wynbereiding bestudeer is.

Daar is bewys dat GSH-vlakke tydens alkolholiese gisting fluktueer, wat dui op die opname en vrystelling daarvan deur die gis. Die vlakke aan die einde van alkoholiese gisting was oor die algemeen laer as vlakke aanvanklik teenwoordig in die sap. In sommige gevalle is konsentrasietoenames egter ook waargeneem. Hierdie bevinding dui daarop dat intrasellulêr-vervaardige GSH, in sommige gevalle, in die mos uitgeskei kan word, wat tot hoër GSH-vlakke in wyn lei. Klein, dog beduidende verskille in GSH-inhoud is waargeneem in wyne wat met verskillende gisrasse berei is, wat daarop dui dat gisras in 'n sekere mate die GSH-vlakke in wyn kan beïnvloed. Alhoewel die effek van gismoerveroudering en

Stellenbosch University http://scholar.sun.ac.za

stikstofaanvulling onbeduidend is, kan die aanvulling van mos met glutatioon-verrykte, onaktiewe droëgis tot verhoogde GSH-vlakke in wyn lei, mits die aanvulling vroeg tydens alkoholiese gisting gedoen word. Hierdie studie verbreed ons kennis van verskeie wynkundige praktyke wat GSH-vlakke in wyn beïnvloed en vorm ‘n nuwe basis vir toekomstige navorsingstudies.

Stellenbosch University http://scholar.sun.ac.za

This thesis is dedicated to my parents, Daniel and Antoinette Kritzinger

Stellenbosch University http://scholar.sun.ac.za

Biographical sketch

Engela Cornelia Kritzinger was born on 12 March 1987 in Windhoek, Namibia. She grew up in Stellenbosch where she cultured a love for the winelands and matriculated at Bloemhof Girls' High School in 2005. Engela obtained her BScAgric degree *cum laude* (Oenology Specialized) at Stellenbosch University in 2009. In 2010, she enrolled for a MScAgric in Oenology at the same university.

Stellenbosch University http://scholar.sun.ac.za

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- **The Heavenly Father**, for blessing me with this opportunity;
- **Dr WJ du Toit** (Department of Viticulture and Oenology, Stellenbosch University), who acted as my supervisor and provided continuous guidance, encouragement and support, and for the critical evaluation of this manuscript;
- **Prof FF Bauer** (Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University) who acted as my co-supervisor for his input into this study and for the critical evaluation of this manuscript;
- **Dr MA Stander** (Central Analytical Facility, Stellenbosch University) for developing the new analytical method and sample analyses;
- **Prof M Kidd** (Centre for Statistical Consultation, Stellenbosch University) for assistance with statistical analyses and interpretation;
- **My parents**, Daniel and Antoinette, for their unconditional love, motivation and support;
- **My best friend**, Christo Swartz, for encouragement and showing me what true perseverance is;
- **Marelize van Noordwyk, Anita Smit and Marinda Visagie** for friendship, laughter and support;
- **Fellow colleagues** (Department of Viticulture and Oenology, Institute for Wine Biotechnology, Stellenbosch University), especially Jaco Franken, Michael Bester and Anita Smit for technical assistance and sharing their expertise with me;
- **Cellar staff** (Experimental Cellar, Department of Viticulture and Oenology, Stellenbosch University) for assistance in the cellar;
- **The cellars** (De Grendel, Boschendal, KWV and Hartenberg) for the donation of juice and
- **Lallemand, The National Research Foundation, Winetech, THRIP and Postgraduate Merit Bursary** for financial support.

Stellenbosch University http://scholar.sun.ac.za

Preface

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of South African Journal of Enology and Viticulture.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**
Glutathione: Role in winemaking and *Saccharomyces cerevisiae*

Chapter 3 **Research results**
Influence of yeast strain, extended lees contact and nitrogen supplementation on glutathione concentration in wine

Chapter 4 **Research results**
Novel UPLC-MS/MS method to assess the effect of glutathione-enriched inactive dry yeast preparation or yeast strain on glutathione levels in juice, wine and yeast cells during alcoholic fermentation

Chapter 5 **General discussion and conclusions**

Stellenbosch University http://scholar.sun.ac.za

Contents

CHAPTER 1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 Introduction 1
1.2 Project aims 3
1.3 Literature cited 3

CHAPTER 2. LITERATURE REVIEW

GLUTATHIONE: ROLE IN WINEMAKING AND *SACCHAROMYCES CEREVISIAE*

2.1 Introduction 6
2.2 Glutathione in general 6
2.3 Glutathione in winemaking 7
2.3.1 Glutathione in grapes 7
2.3.2 Glutathione in must and wine 8
2.3.3 Antioxidant activity of glutathione in must and wine: the inhibition of browning 10
2.3.4 Impact of glutathione on aroma 13
2.3.4.1 Esters and terpenes 13
2.3.4.2 Volatile thiols 14
2.3.4.2.1 Protection of volatile thiols 14
2.3.4.2.2 Glutathione as volatile thiol precursor 15
2.3.4.3 Hydrogen sulfide 18
2.3.5 Reduction of atypical ageing character 19
2.3.5.1 Sotolon and 2-aminoacetophenone 19
2.3.6 Glutathione and malolactic fermentation 20
2.3.7 Glutathione-enriched inactive dry yeast preparations 20
2.3.8 Analytical methods for glutathione quantification 22
2.4 Glutathione and *Saccharomyces cerevisiae* 24
2.4.1 Introduction 24
2.4.2 Glutathione synthesis 24
2.4.3 Glutathione transport 24
2.4.4 Glutathione degradation 25
2.4.5 Role of glutathione in *Saccharomyces cerevisiae* during nutritional and oxidative stress 27
2.4.5.1 Sulphur and nitrogen starvation 27
2.4.5.2 Oxidative stress 27
2.4.6 Glutathione: biotechnological production 28
2.5 Conclusion 29
2.6 Literature cited 30

Stellenbosch University http://scholar.sun.ac.za

CHAPTER 3. RESEARCH RESULTS

INFLUENCE OF YEAST STRAIN, EXTENDED LEES CONTACT AND NITROGEN SUPPLEMENTATION ON GLUTATHIONE CONCENTRATION IN WINE

3.1 Introduction 36
3.2 Materials and methods 38
3.2.1 Yeast strains and media 38
3.2.2 Screening of yeast strains in synthetic media 39
3.2.2.1 Fermentation conditions 39
3.2.3 Screening in grape juice to monitor glutathione evolution during alcoholic fermentation 39
3.2.3.1 Fermentation conditions 40
3.2.4 Influence of ageing and lees contact 41
3.2.5 Influence of different yeast assimilable nitrogen contents 42
3.2.6 Influence of oxidative stress 43
3.2.6.1 Fermentation conditions 43
3.2.6.2 H_2O_2 plate essays 43
3.2.7 Sampling procedure, sample preparation and GSH analysis 43
3.2.7.1 Juice and wine sampling 43
3.2.7.2 Extracellular GSH analyses of juice and wine samples 44
3.2.7.3 Intracellular GSH analyses in the oxidative stress experiment 44
3.2.8 Statistical analyses 44
3.3 Results and discussion 45
3.3.1 Preliminary screening of commercial *Saccharomyces cerevisiae* strains for GSH utilization during alcoholic fermentation 45
3.3.2 Screening in grape juice 46
3.3.2.1 GSH evolution during alcoholic fermentation 46
3.3.3 Influence of ageing and extended lees contact on GSH levels in wine 52
3.3.4 Influence of different YAN levels on GSH evolution during fermentation 54
3.3.5 Influence of different intracellular GSH contents on oxidative stress resistance 56
3.4 Conclusions 58
3.5 Acknowledgements 59
3.6 Literature cited 59

CHAPTER 4. RESEARCH RESULTS

NOVEL UPLC-MS/MS METHOD TO ASSESS THE EFFECT OF GLUTATHIONE-ENRICHED INACTIVE DRY YEAST PREPARATION OR YEAST STRAIN ON GLUTATHIONE LEVELS IN JUICE, WINE AND YEAST CELLS DURING ALCOHOLIC FERMENTATION

4.1 Introduction 63
4.2 Materials and methods 65
4.2.1 UPLC-MS/MS method 65
4.2.2 Determination of GSH released from various GSH-IDYs 66
4.2.3 Influence of time, SO_2 and ethanol on GSH release by GSH-IDYs into model solution 66
4.2.4 Influence of GSH-IDY added at different fermentation stages on GSH levels in wine 67
4.2.4.1 Small scale wine production 67
4.2.4.2 Large scale wine production 68

4.2.5 Influence of yeast strain and canopy treatments in vineyard on extra- and intracellular GSH levels during alcoholic fermentation 69
4.2.5.1 Vineyard experimental design 69
4.2.5.2 Fermentation conditions 69
4.2.6 Sampling procedure, sample preparation and GSH analysis 70
4.2.6.1 Model solution, juice and wine sampling 70
4.2.6.2 Sample preparation for extracellular GSH analysis 70
4.2.6.3 Sample preparation for intracellular GSH analysis 71
4.2.7 Statistical analyses 71
4.3 Results and discussion 71
4.3.1 UPLC-MS/MS method 71
4.3.2 Determination of GSH released from various GSH-IDYs 73
4.3.3 Influence of time, SO_2 and ethanol on GSH release by GSH-IDYs into model solution 75
4.3.4 Influence of GSH-IDY added at different fermentation stages on GSH levels in wine 78
4.3.4.1 GSH levels for small scale fermentation 78
4.3.4.2 GSSG levels for small scale fermentation 80
4.3.4.3 GSH levels for large scale fermentations 81
4.3.5 Influence of yeast strain and canopy treatments in vineyard on extra- and intracellular GSH levels during alcoholic fermentation 83
4.3.5.1 Extracellular GSH levels 83
4.3.5.2 Extracellular GSSG levels 85
4.2.5.3 Total, reduced and oxidized intracellular glutathione levels 86
4.4 Conclusions 88
4.5 Acknowledgements 89
4.6 Literature cited 89

CHAPTER 5. GENERAL DISCUSSION AND CONCLUSION

5.1 Concluding remarks and future work 93
5.2 Literature cited 95

Stellenbosch University http://scholar.sun.ac.za

Chapter 1

General introduction and project aims

Stellenbosch University http://scholar.sun.ac.za

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Competition amongst wine producing countries and individual wine producers is bound to intensify due to the persisting gap between wine production and wine consumption, the shift of consumer preferences away from basic commodity wine to quality wine, and economic globalization (Pretorius & Bauer, 2002). In addition to producing quality wines, the ability to meet consumer demands in terms of a healthy product will become an increasingly important economic driver of profitability (Bisson *et al*., 2002). Sauvignon blanc is globally one of the most important cultivars and is the second most widely planted white cultivar after Chardonnay. Wine made from this cultivar, however, is sensitive to oxidation which has detrimental consequences on wine quality, resulting in a loss of characteristic aroma, the development of an atypical ageing flavour character and visual browning. Strategies to improve and preserve wine quality would confer a competitive advantage to the wine producer. Increasing the glutathione (GSH) levels in wine, could assist in obtaining such an advantage considering the quality preserving function this natural antioxidant plays in wine. Apart from limiting oxidative colouration in grape juice and wine (Vaimakis & Roussis, 1996; Dubourdieu & Lavigne, 2004), GSH exerts a protective effect on various impact aroma compounds during wine ageing, such as volatile thiols (Lavigne-Cruège & Dubourdieu, 2002; Dubourdieu & Lavigne, 2004; Ugliano *et al.*, 2011), esters and terpenes (Papadopoulou & Roussis, 2001, 2008; Roussis *et al.*, 2009). It has also been shown that the development of atypical ageing flavour characters, including sotolon and 2-aminoacetophenone, is hampered by the presence of GSH (Dubourdieu & Lavigne, 2004). High levels of this natural antioxidant may also permit the use of lower sulphur dioxide (SO_2) dosages in wine, partially addressing health related concerns regarding the use of SO_2 in wine (Freedman, 1980; Jackson, 2008). The addition of food grade GSH to wine prior to bottling to preserve wine aroma, has been discussed by the International Organization of Vine and Wine (OIV), but to date it is not allowed (Roland *et al*., 2011; Ugliano *et al*., 2011). Even in the case of its approval, cost implications may still render this application not feasible on commercial scale winemaking. Although factors affecting GSH content in grapes (Cheynier *et al*., 1989; Choné *et al*., 2006; Lacroux *et al*., 2008) and grape juice have been elucidated (du Toit *et al.*, 2007; Maggu *et al.*, 2007; Patel *et al.*, 2010), literature on the effect of winemaking practices on GSH levels in wine is scant or contradictory. Similarly, the evolution of GSH during alcoholic fermentation is an unexplored field of study. The identification of oenological practices that may result in elevated wine GSH levels would be of great value to the industry. While work done by Lavigne *et al*. (2007) suggests that the specific wine yeast strain may influence the GSH levels present after alcoholic fermentation, Fracassetti (2010) regarded the influence of yeast strain to be insignificant. GSH can be assimilated by the yeast (Penninckx, 2002), which would lead to reduced wine GSH levels. Differences in

Stellenbosch University http://scholar.sun.ac.za

the GSH assimilation or GSH requirements between yeast strains may hence result in wine GSH content to vary when different strains are used for alcoholic fermentation. Only one report on the effect of lees ageing on wine GSH levels has been published (Lavigne *et al*., 2007) while the effect of bottle ageing has been studied by Penna *et al*. (2001) and Ugliano *et al*. (2011). Those data show that GSH levels normally decrease during bottle ageing, but ageing wines on the lees seems to protect wine GSH levels to a certain extent. When compared to GSH levels initially present in grape juice, the levels in wine have been reported to be either lower (du Toit *et al*., 2007; Patel *et al*., 2010; Coetzee, 2011) or higher (Park *et al*., 2000a,b; Fracassetti, 2010; Andújar-Ortiz *et al*., 2011). Park *et al*. (2000a) reported that wine GSH levels were significantly correlated with both total nitrogen and assimilable amino acid content of the grape juice, but this conclusion was based on different juices with different yeast assimilable nitrogen levels.

A wide range of glutathione enriched inactive dry yeast products (GSH-IDY) are currently available on the market that claims to enhance the sensory stability of wines due to their ability to lead to higher wine GSH levels (Pozo-Bayón *et al*., 2009). However, little to no independent and published research on the influence of GSH-IDY on GSH levels in wine is available. Surprisingly, the single published study by Andújar-Ortiz *et al.* (2011) reported that no significant difference in GSH content was observed between a control and GSH-IDY supplemented wine. Uncertainty also exists as to when these products should be added during alcoholic fermentation.

Over the last few decades several analytical techniques for the analysis of GSH in grape juice and wine have been developed in reaction to the increasing interest in this antioxidant (Park *et al*., 2000b; Lavigne *et al*., 2007; du Toit *et al*., 2007; Marchand & de Revel, 2010; Janes *et al*., 2010; Fracassetti *et al*., 2011). Many of these techniques are, however, time-consuming, requiring derivatization and extensive sample preparation, while the majority of these techniques only allow for the quantification of the reduced form of glutathione. The need thus arises for a rapid method to analyse both reduced (GSH) and oxidized glutathione (GSSG) in a large number of samples without compromising accuracy.

The main focus of this study was thus to obtain a better understanding of the evolution of glutathione during alcoholic fermentation and to ascertain the effects of various oenological factors on its levels in Sauvignon blanc wine. Ultimately, the identification of factors resulting in high GSH levels in wine would be highly beneficial to wine quality and at the same time possibly permit the use of lower SO_2 dosages in wine.

Stellenbosch University http://scholar.sun.ac.za

1.2 PROJECT AIMS

The specific aims of this research project are as follow:

1. to conduct a preliminary screening of commercial yeast strains in terms of GSH concentration present after alcoholic fermentation;

2. to evaluate the evolution of GSH concentration during alcoholic fermentation in Sauvignon blanc juice performed by various commercial wine yeast strains;

3. to determine the effect of lees ageing after alcoholic fermentation on GSH levels in wine over a one year period;

4. to establish the effect of different yeast assimilable nitrogen levels in a chemically defined grape juice medium on GSH concentration after alcoholic fermentation;

5. to determine the influence of a specific GSH-enriched dry yeast preparation on GSH concentrations in finished wines, as well as the optimal addition time of this product during alcoholic fermentation to obtain maximum GSH concentrations after alcoholic fermentation;

6. to monitor the intracellular and extracellular GSH and GSSG evolution during alcoholic fermentation of a grape juice obtained from a vineyard that received different canopy treatments; and

7. the development of a novel UPLC-MS/MS method for the rapid determination of both intracellular and extracellular GSH and GSSG concentrations.

1.3 LITERATURE CITED

Andújar-Ortiz, I. Pozo-Bayón, M. A., Moreno-Arribas, M. V., Martín-Álvarez, P. J. & Rodríguez-Bencomo, J. J., 2011. Reversed-Phase High-Performance Liquid Chromatography–Fluorescence detection for the analysis of glutathione and its precursor γ-glutamyl cysteine in wines and model wines supplemented with oenological inactive dry yeast preparations. Food Anal. Methods. DOI 10.1007/s12161-011-9230-4.

Bisson, L. F., Waterhouse, A. L., Ebeler, S. E., Walker, M. A. & Lapsey, J. T., 2002. The present and the future of the international wine industry. Nature 418, 696-699.

Cheynier, V., Souquet, J. M. & Moutounet, M., 1989. Glutathione content and glutathione to hydroxycinnamic acid ratio in *Vitis vinifera* grapes and musts. Am. J. Enol. Vitic. 40, 320-324.

Choné, X., Lavigne-Cruège, V., Tominaga, T., Van Leeuwen, C., Castagnede, C., Saucier, C. & Dubourdieu, D., 2006. Effect of vine nitrogen status on grape aromatic potential: Flavor precursors (S-cysteine conjugates), glutathione and phenolic content in *Vitis vinifera* L. cv. Sauvignon blanc grape juice. J. Int. Des Sciences De La Vigne Et Du Vin. 40, 1-6.

Coetzee, C. 2011. Oxygen and sulphur dioxide additions to Sauvignon blanc: effect on must and wine composition. MScAgric thesis, Stellenbosch University, Private Bax X1, 7602 Matieland, South Africa.

Dubourdieu, D. & Lavigne, V., 2004. The role of glutathione on the aromatic evolution of dry white wine. Vinidea.net 2, 1–9.

Du Toit, W. J., Lisjak, K., Stander, M. & Prevoo, D., 2007. Using LC-MSMS to assess glutathione levels in South African white grape juices and wines made with different levels of oxygen. J. Agric. Food Chem. 55, 2765-2769.

Fracassetti, D., 2010. Investigation on cysteinyl thiol compounds from yeast affecting wine properties. PdD thesis in Food Science, Technology and Biotechnology, University of Milan, Italy.

Stellenbosch University http://scholar.sun.ac.za

Fracassetti, D., Lawrence, N., Tredoux, A. G. J., Tirelli, A., Nieuwoudt, H. H. & Du Toit, W. J., 2011. Quantification of glutathione, catechin and caffeic acid in grape juice and wine by a novel ultra-performance liquid chromatography method. Food Chem. 128, 1136–1142.

Freedman, B. J.,1980. Sulphur dioxide in foods and beverages: Its use as a preservative and its effect on asthma. Br. J. Dis. Chest 74, 128-134.

Jackson, R. S. Wine Science. Principles and Applications; Academic Press: Burlington, MA, 2008; pp 316 and 697.

Janes, L., Lisjak, K. & Vanzo, A., 2010. Determination of glutathione content in grape juice and wine by high-performance liquid chromatography with fluorescence detection. Anal. Chim. Acta 674, 239-242.

Lacroux, F., Tregoat, O., Van Leeuwen, C., Pons, A., Tominaga, T., Lavigne-Cruege, V. & Dubourdieu, D., 2008. Effect of foliar nitrogen and sulphur application on aromatic expression of *Vitis vinifera* L. cv. Sauvignon blanc. J. Int. Sci. Vigne Vin 42, 125-132.

Lavigne-Cruège, V. & Dubourdieu, D., 2002. Role of glutathione on development of aroma defects in dry white wines. In *13th International Enology Symposium*, Trogus, H., Gafner, J., Sutterlin., Eds. International Association of Enology: Montpellier, France, pp 331-347.

Lavigne, V., Pons, A. & Dubourdieu, D., 2007. Assay of glutathione in must and wines using capillary electrophoresis and laser-induced fluorescence detection - Changes in concentration in dry white wines during alcoholic fermentation and aging. J. Chrom. A 1139, 130-135.

Maggu, M., Winz, R., Kilmartin, P. A., Trought, M. C. T. & Nicolau, L., 2007. Effect of skin contact and pressure on the composition of Sauvignon Blanc must. J. Agric. Food Chem. 55, 10281-10288.

Marchand, S. & de Revel, G., 2010. A HPLC fluorescence-based method for glutathione derivatives quantification in must and wine. Anal. Chim. Acta. 660, 158-163.

Papadopoulou, D. & Roussis, I. G., 2001. Inhibition of the decline of linalool and α-terpineol in muscat wines by glutathione and N-acetyl-cysteine. Int. J. Food Sci. 13, 413-419.

Papadopoulou, D. & Roussis, I. G., 2008. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a model wine medium by glutathione and N-acetyl-cysteine. Int. J. Food Sci. Technol. 43, 1053-1057.

Park, S. K., Boulton, R. B. & Noble, A. C., 2000a. Formation of hydrogen sulfide and glutathione during fermentation of white grape musts. Am. J. Enol. Vitic. 51, 91-97.

Park, S. K. , Boulton, R. B. & Noble, A. C., 2000b. Automated HPLC analysis of glutathione and thiol-containing compounds in grape juice and wine using pre-column derivatization with fluorescence detection. Food Chem. 68, 475-480.

Patel, P., Herbst-Johnstone, M., Lee, S. A., Gardner, R.C., Weaver, R., Nicolau, L. & Kilmartin, P. A., 2010. Influence of juice pressing conditions on polyphenols, antioxidants and varietal aroma of Sauvignon blanc microferments. J. Agric. Food Chem. 58, 7280-7288.

Penna, N. C., Daudt, C. E., Brendel, M. & Henriques, J. A. P., 2001. Evolución de los niveles de glutathiona y ésteres hidroxicinámicos durante la vinificación de Sauvignon Blanc, Chenin Blanc, Niágara y Tannat. Alimentaria 2001, 326, 147–151.

Penninckx M. J., 2002. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. FEMS Yeast Res. 2, 295–305.

Pozo- Bayón, M. A., Andújar-Ortiz, I. & Moreno-Arribas, M. V., 2009. Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. Food Res. Int. 42, 754-761.

Pretorius, I. S., Bauer, F. F., 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends in Biotech. 20, 426-432.

Roland, A., Schneider, R., Razungles, A. & Cavelier, F. 2011. Varietal thiols in wine: Discovery, analysis and applications. Chem. Rev. 111, 7355–7376.

Roussis, I. G., Papadopoulou, D. & Sakarellos-Daitsiotis, M., 2009. Protective effect of thiols on wine aroma volatiles. Open Food Sci. J. 3, 98-102.

Ugliano, M., Kwiatkowski, M. J., Vidal, S., Capone, D., Siebert, T., Dieval, J. B., Aagaard, O. & Waters, E. J., 2011. Evolution of 3-mercatohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. J. Agric. Food Chem. 59, 2564–2572.

Vaimakis, V. & Roussis, I. G., 1996. Must oxygenation together with glutathione addition in the oxidation of white wine. Food Chem. 57, 419-422.

Stellenbosch University http://scholar.sun.ac.za

Chapter 2

Literature review

Glutathione: Role in winemaking and *Saccharomyces cerevisiae*

Stellenbosch University http://scholar.sun.ac.za

2. LITERATURE REVIEW

GLUTATHIONE: ROLE IN WINEMAKING AND *SACCHAROMYCES CEREVISIAE*

2.1 INTRODUCTION

Oxidative spoilage of white wine constitutes a well known problem in the wine industry. White wine, particularly Sauvignon blanc, can be sensitive to oxygen exposure which can lead to a loss in characteristic aroma, the development of atypical ageing characters and undesirable colour changes. Glutathione (GSH), a naturally occuring tripeptide in the grape, plays an integral role in the oxidation of white musts where it traps *ortho*-quinones, formed during oxidation, to limit the amount of browning pigments (Singleton *et al*., 1985; Singleton & Cilliers, 1995; du Toit *et al*., 2006). Furthermore, GSH exerts a protective effect on various aromatic compounds in wine (Dubourdieu & Lavigne, 2004; Papadopoulou & Roussis, 2008; Ugliano *et al*., 2011).

GSH is present in high concentrations up to 10 mM in the yeast, *Saccharomyces cerevisiae* (Penninckx, 2002). This species is also currently employed in the industrial fermentative production of GSH (Li *et al*., 2004). The yeast may influence the GSH concentration in wine due to the metabolism thereof during alcoholic fermentation.

This review has a dual focus. The first section focuses on GSH with regards to its role in winemaking. Special emphasis is given to its occurrence in grapes, must and wine and its role as antioxidant in wine. The effect of GSH on both desirable and undesirable aroma compounds is also outlined. Furthermore, the use of GSH enriched products in winemaking and the various analytical techniques for the quantification of GSH in must and wine are discussed.

The second section of this review focuses on the role of GSH in *S. cerevisiae* with special emphasis given to its role in response to nutritional and environmental stresses.

2.2. GLUTATHIONE IN GENERAL

Glutathione (Y-L-glutamyl-L-cysteinylglycine; GSH) is a tripeptide of L-glutamate, L-cysteine and glycine. It is the most abundant non-protein intracellular thiol (0.2-10 mM) present in most mammalian and many prokaryotic organisms (Anderson, 1998). Its biological significance is mostly related to its free sulfhydryl moiety of the cysteine residue which confers unique redox and nucleophilic properties (Penninckx, 2000). GSH can occur in the cell under the reduced form of GSH, the oxidized form of glutathione disulfide (GSSG) (**Figure 2.1**) as well as mixed disulfides, GS-S-Cys and GS-S-CoA (Fahey *et al*., 2001). Generally speaking, more than 90% of GSH is present in the reduced form in the cell (Li *et al*.,

Stellenbosch University http://scholar.sun.ac.za

2004). GSSG is formed upon oxidation of GSH. At the expense of NADPH, GSSG can be reduced back to GSH by glutathione reductase (Carmel-Harel & Storz, 2000).

The major functions of GSH can be summarized as being an antioxidant, immunity booster and detoxifier (Pastore *et al*., 2003). In living tissues, GSH plays a pivotal role in bioreduction, protection against oxidative stress, xenobiotics and endogenous toxic metabolite detoxification, enzyme activity and sulphur and nitrogen metabolism (Penninckx, 2002). It is thus considered as a powerful, versatile and important self generated-defence molecule (Li *et al*., 2004). GSH is widely used as a pharmaceutical compound and has the potential to be used in food additive and cosmetic industries as well as in sport nutrition (Sies, 19991992, Beutler *et al.*, 1989). However, its supplementation to must during vinification is not allowed by current wine regulation (Ugliano *et al*., 2011).

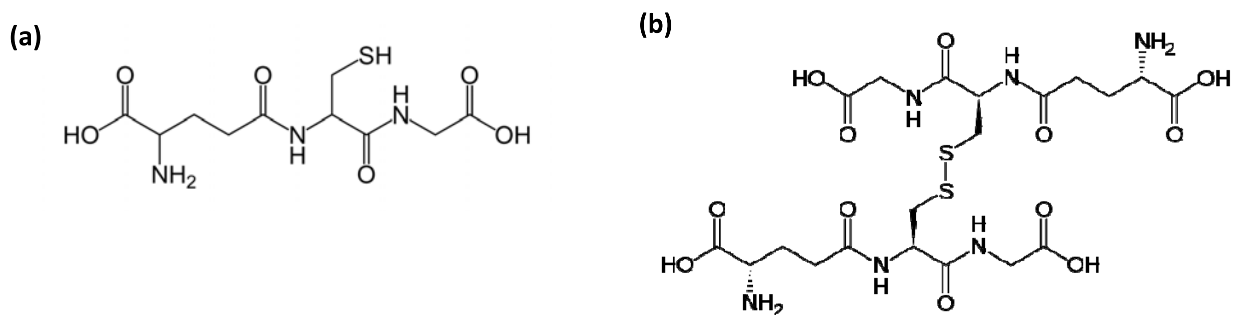


Figure 2.1 Molecular structures of **(a)** glutathione (GSH) and **(b)** glutathione disulfide (GSSG).

2.3 GLUTATHIONE IN WINEMAKING

2.3.1 GLUTATHIONE IN GRAPES

GSH was first quantified in grapes in 1989 (Cheynier *et al*., 1989). These authors conducted a study to analyse the GSH content of both the berries and the respective musts of 28 different *Vitis vinifera* grape varieties. The GSH content varied from 56-372 µmole/kg (17-114 mg/kg) between grape varieties. These great variations among varieties, however, were not strictly dependent on variety alone. Vintage, location and technological practices can all influence the GSH content. GSH is synthesized in the cytosol and chloroplasts of plant cells (Leustek *et al*., 2000). However, the mechanism of GSH accumulation in grapes has not been elucidated (Ribéreau-Gayon *et al*., 2006) and further research in this area is necessary.

GSH has been observed to increase at the onset of ripening of *Vitis vinifera* berries. This increase in GSH content was observed in green, red, seeded and seedless varieties. The strong correlation between accumulated GSH and soluble solids persists until the berries reach 16 °Brix where after the GSH content

Stellenbosch University http://scholar.sun.ac.za

remains stable (Adams & Liyanage, 1993). This result is consistent with findings by Okuda & Yokotsuka (1999). These authors also established that more than 90% of the total glutathione in berries during ripening is in the reduced form (Okuda & Yokotsuka, 1999).

The increase in GSH at the onset of ripening in berries may be ascribed to an increased contribution of phloem components to the berry from sources such as mature leaves. This is supported by another study by Liyanage & Adams (1992) which showed that the increase in berry GSH is accompanied by a decrease in leave GSH content. GSH content in grapes is closely related to the vine nitrogen status estimated as yeast assimilable nitrogen content of the grape juice. A late soil nitrogen fertilization at berry set resulted in juice with a six-fold higher yeast assimilable nitrogen content compared to the control. This juice also contained higher cysteine conjugate precursors and GSH levels (Choné *et al*., 2006). In this study, the GSH content of must originating from nitrogen deficient vines was significanlty lower than must from vines that were fertilized after bloom. Above results were confirmed in another study conducted by Lacroux *et al*. (2008) which evaluated the impact of foliar nitrogen (LEAFN) and foliar nitrogen and sulphur (LEAFNS) applications on wine GSH and wine volatile thiols. Both treatments resulted in juice with higher yeast assimilable nitrogen levels compared to the control. The GSH content of the wine from both treatments was also higher. However, the addition of sulphur to the foliar N spraying did not significantly increase the GSH content of the wine (Lacroux *et al*., 2008).

2.3.2 GLUTATHIONE IN MUST AND WINE

GSH content in must is highly variable. Levels ranging from 0.001-100 mg/L have been reported in grape must (Cheynier *et al*., 1989; Park *et al*., 2000a). GSH levels in South African juice ranged from 1.1 to 71 mg/L, which correlates well with values reported by other authors (Maggu *et al*., 2007; du Toit *et al.*, 2007; Janes *et al*., 2010; Fracassetti *et al.*, 2011). Several factors can influence the GSH concentration in must, ie. exposure to oxygen, tyrosinase activity, grape skin maceration during the pre-fermentation period and pressing (du Toit *et al*., 2007; Maggu *et al*., 2007; Patel *et al*., 2010). Du Toit *et al*. (2007) studied the effect of oxidative and reductive treatments during winemaking on the GSH concentration in South African white grape juice and the resulting wines. The reductive treatment (less than 0.3 mg/L dissolved O_2 pick-up during pressing) resulted in the highest GSH levels in the grape juice and the corresponding wine. The control (1-1.5 mg/L dissolved O_2 added) and especially oxidative treatments (3.5-4 mg/L dissolved O_2 added) displayed significant lower juice and wine GSH levels. During machine harvesting, oxygen uptake is inevitable which results in a decrease in juice GSH content. This is supported by the presence of moderate amounts of S-glutathionyl caftaric acid (9-15 mg/L) that was present in the free run juice of machine harvested Sauvignon blanc grapes (Maggu *et al*., 2007). Free run juice is characterized by higher GSH concentrations compared to higher press fractions (Maggu *et*

Stellenbosch University http://scholar.sun.ac.za

al., 2007; Patel *et al*., 2010). The GSH concentration of three different Sauvignon blanc juices collected after 1 hour of skin contact in a winery trial, had fallen by up to half. Moreover, only one of the three juice pressings obtained at 0.4 atm, had detectable amounts of GSH left (Maggu *et al*., 2007).

Results regarding the evolution of GSH concentration during alcoholic fermentation of grape juice are contradictory. GSH concentrations have been observed to either increase (Park *et al*., 2000a,b; Lavigne *et al.*, 2007; Fracassetti, 2010; Andújar-Ortiz *et al.*, 2011) or decrease during alcoholic fermentation (Lavigne *et al*., 2007; du Toit *et al*., 2007; Patel *et al*., 2010; Coetzee, 2011). Okuda & Yokotsuka (1999) observed an initial increase in GSH concentration in a Koshu ferment where after the GSH content decreased to reach practically zero at the end of alcoholic fermentation. Initial GSSG concentrations in the juice were low, but increased during fermentation and reached a maximum three days after the yeast was added for the Koshu fermentation. In the case of Cabernet Sauvignon, the GSSG concentration was at a maximum one day after yeast addition. On the contrary, Park *et al*. (2000a,b) reported an increase in GSH concentration towards the end of fermentation of eight white grape musts. This increase in GSH was ascribed to the formation thereof by the yeast, *S. cerevisiae* with the subsequent release into the fermenting must. Dubourdieu & Lavigne (2004) observed an almost complete disappearance of GSH from Sauvignon blanc juice at the beginning of fermentation followed by an increase towards the end of fermentation which corroborates with data by Park *et al*. (2000a). The GSH concentration gradually increased after alcoholic fermentation was completed and stabilized 30 days post alcoholic fermentation (Dubourdieu & Lavigne, 2004).

In wines, other authors reported that the GSH concentration was consistently lower than in the corresponding grape juices (du Toit *et al*.,2007; Coetzee, 2011). Janes *et al*. (2010) reported that the average GSH level in 28 young Sauvignon blanc wines was found to be 12.5 mg/L. Yeast strain also seems to influence the final GSH concentration present in wine (Lavigne *et al*., 2007; Rauhut, 2009). Dubourdieu & Lavigne (2004) postulated that yeast metabolises GSH during the active growth phase and releases it upon the end of alcoholic fermentation through yeast autolysis. On the contrary, Cassol & Adams (1994) labelled GSH as well as a glutathione-S conjugate with radioactive 35^S and studied the uptake by yeast in model must over a five hour period. Interestingly, neither of the two compounds was taken up by the yeast in this period.

The GSH concentration decreased during wine ageing (Penna *et al*., 2001; Lavigne *et al*., 2007; Ugliano *et al*., 2011). Yeast lees seems to have a protective effect on GSH levels in wine (Lavigne *et al*., 2007). During barrel ageing of unracked Sauvignon blanc wines, the decrease in GSH concentration was lower compared to racked wines. This was attributed to the reducing properties of yeast lees which impeded the oxidation of GSH. The depletion of GSH in wine was faster if racked wine was aged in new barrels as a result of the greater oxidation effect of new wood (Lavigne *et al*., 2007).

Stellenbosch University http://scholar.sun.ac.za

Variable oxygen exposure during bottle ageing can also influence the GSH concentration in wine (Ugliano *et al*., 2011). Sauvignon blanc wines exposed to lower oxygen levels during bottle ageing, consistently showed higher GSH concentrations compared to wines that were exposed to higher levels of oxygen during storage. This was attributed to the lower oxidation degree in wines with lower oxygen levels.

It is thus clear that the evolution of GSH during winemaking can change drastically and that the concentration can be manipulated by the winemaker by limiting oxidation throughout the vinification and ageing process.

2.3.3 ANTIOXIDANT ACTIVITY OF GLUTATHIONE IN MUST AND WINE: THE INHIBITION OF BROWNING

Browning is an oxidative process occurring during the vinification of wine, that detracts from its sensory properties of appearance, aroma and flavour (Li *et al*., 2008). Phenols, in particular *ortho*-diphenols are responsible for the oxidative browning in wine. Browning can be a result of enzymatic oxidation which almost entirely takes place in grape must, and non enzymatic oxidation, also called chemical oxidation, that occurs predominantly during wine ageing (Oliveira *et al*., 2011). Enzymatic browning in grape must is highly correlated with the hydroxycinnamate content of the juice, presenting the main group of phenolic compounds in white juice (Betes-Saura *et al*., 1996; Li *et al*., 2008). Caftaric (caffeoyl tartaric) acid and coutaric (coumaric tartaric) acid are the the most abundant hydroxycinnamates in grape juice prepared with minimal skin contact (Cheynier *et al*., 1988). In the intact berry, the hydroxycinnamates, predominantly present in the vacuole, are not in contact with grape polyphenoloxidase (PPO), which is located in the cytoplasm, due to different cell membrane systems (Wang, 1990). However, when the berry is crushed in the presence of oxygen, the membrane systems are disrupted and enzymatic oxidation (catalyzed by PPO) converts these hydroxycinnamates to the corresponding reactive electrophilic *ortho*-quinones (Singleton *et al*., 1985; Cilliers & Singleton, 1990). The cresolase activity of grape polyphenoloxidase first converts coutaric acid to caftaric acid before being oxidized further to caftaric acid quinone (Singleton *et al*., 1985). GSH, with its mercapto-group serving as an electron rich nucleophilic centre, substitutes into the electrophilic ring of the caftaric acid quinone (**Figure 2.2**).

Stellenbosch University http://scholar.sun.ac.za

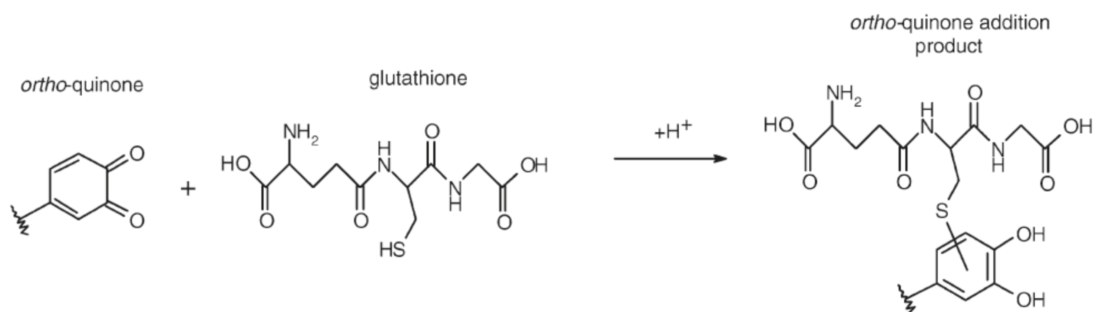


Figure 2.2 Reaction of GSH with *ortho*-quinones (Sonni *et al*., 2011a).

The vicinal dihydroxy ring of the caffeic acid moiety is regenerated by means of proton transfer and the equivalent of an enol-shift (Singleton *et al*., 1985). The product, a thioether known as 2-S-glutathionyl caftaric acid or Grape Reaction Product (GRP) is not a substrate for further oxidation by PPO in spite of its *ortho*-dihydroxyphenol structure (Salgues *et al*., 1986; Singleton & Cilliers, 1995). In this manner, glutathione traps the *ortho*-quinones (**Figure 2.3**) in a colourless form and the formation of brown polymers is limited (Singleton *et al*., 1985). If GSH is depleted in the juice, the caftaric acid quinone, being an oxidant, can oxidize GRP and other flavanols to be reduced back to caftaric acid. Furthermore, the caftaric acid quinone may also polymerize with caftaric acid to form a re-oxidizable phenol (du Toit *et al*., 2006). These polymerization reactions with *ortho*-quinones lead to the browning of grape juice (Salgues *et al*., 1986).

The hydroxycinnamic acid to GSH ratio of a must should hence present a good indication of its oxidation susceptibility with higher ratios leading to darker musts. A ratio of 0.9 to 2.2 characterizes a lightly coloured must. Medium and dark coloured musts are characterized by hydroxycinnamic acid to GSH ratios of 1.1 to 3.6 and 3.8-5.9 , respectively (du Toit *et al*., 2006).

Although resistant to PPO, GRP is, however, a substrate for laccase produced by the fungus, *Botrytis cinerea* and further oxidation with the substitution by GSH in position 5, leads to the formation of 2,5-di-S-glutathionylcaftaric acid (GRP2). It does not seem as if GRP2 can be further oxidized by laccase under winemaking conditions (Boulton *et al*., 1996). The reaction is very sensitive to the presence of oxygen and sulphur dioxide (SO_2). SO_2 inhibits PPO (Dubernet & Ribéreau-Gayon, 1973) which consequently prevents the formation of GRP. In such a case, a high level of free hydroxycinnamates with high browning potential is retained in the must (Oliveira *et al*., 2011).

Stellenbosch University http://scholar.sun.ac.za

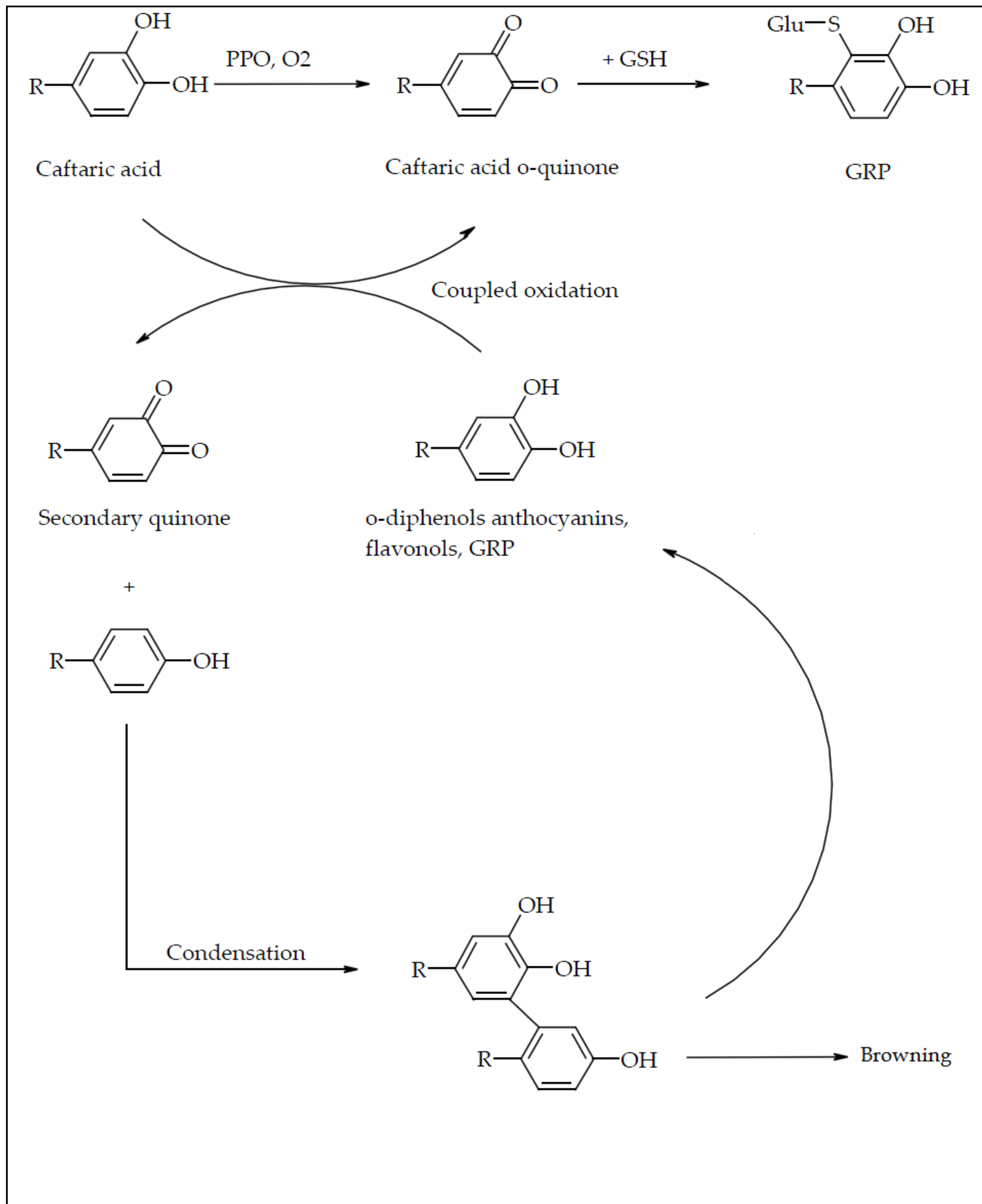


Figure 2.3 Reaction scheme of enzymatic oxidation in white grape juice (Fracassetti, 2010).

GSH added at 10 mg/L to Sauvignon blanc wine at bottling significantly decreased the yellow tint of the wine during a three year ageing period compared to the control wine (Dubourdieu & Lavigne, 2004). This raised interest in the ability of GSH to protect white wine colour during bottle ageing when non-enzymatic oxidation reactions prevail. Non-enzymatic oxidation arises from the oxidation of phenolic compounds and the subsequent polymerization of oxidized products. Polymerization reactions between phenols and other wine constituents, including acetaldehyde and glyoxylic acid may also give rise to

Stellenbosch University http://scholar.sun.ac.za

browning pigments. The phenolic compounds most susceptible to chemical oxidation in wine include caffeic acid and its esters, catechin, epicatechin and gallic acid (Li et al., 2008).

Sonni *et al.* (2011a) demonstrated that sufficient amounts of GSH could inhibit oxidative colouration by delaying the formation of carboxymethine-bridged (+)-catechin dimers formed in the model wine system. Sonni *et al*. (2011b) showed that this delay was due to the ability of GSH to form addition products with carbonyl compounds, such as glyoxylic acid (**Figure 2.4**). Furthermore, these authors proved that the inhibiting effect of GSH on glyoxylic acid-derived dimer formation was independent of temperature (20°C vs 45°C) or the presence of copper and iron. In addition, the authors also reported the formation of another product where GSH reacts with an intermediate compound in the production of the dimer, interrupting the polymerization reactions. This inhibition of carbonyl-derived polymerization reactions hinders the formation of unwanted pigments such as the yellow xanthylium cation which may induce colour changes that are negatively correlated with wine quality. It thus seems as if GSH could exert a protective effect on wine colour during ageing. However, these studies were conducted in model wine solutions under higher temperature and oxygen levels normally present during wine ageing. Further investigation under conditions more relevant to wine ageing is thus needed to produce more conclusive results.

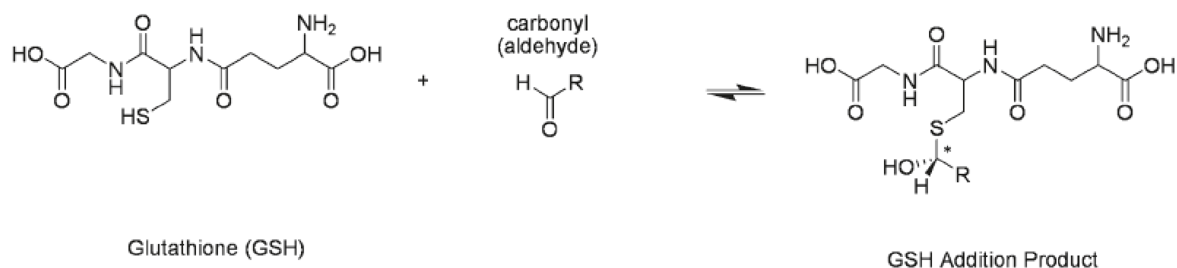


Figure 2.4 Addition reaction of glutathione with carbonyl compounds. (R = CH_3: acetaldehyde, R = COOH or COO^-: glyoxylic acid) (Sonni *et al*., 2011b).

2.3.4 IMPACT OF GLUTATHIONE ON AROMA COMPOUNDS IN MUST AND WINE

2.3.4.1 ESTERS AND TERPENES

Esters of higher alcohols and ethyl esters positively contribute to wine quality by imparting fruity aromas (Etievant, 1991). Terpenes constitute an important aromatic group in several grape varieties and their corresponding wines such as the Muscat wines, Gewürztraminer, Weisser Riesling and Bukettraube. Terpenes, such as linalool, α-terpineol, nerol, geraniol and hotrienol impart floral, rose-

Stellenbosch University http://scholar.sun.ac.za

like, perfumy, coriander and campherous characters in wine (Marais, 1983).

Papadopoulou & Roussis (2008) evaluated the effect of GSH and N-acetylcysteine on the decrease of volatile esters and terpenes in both Debina white wine and a model wine solution during ageing. GSH inhibited the decline of several volatiles such as isoamyl acetate, ethyl hexanoate, ethyl octanoate ethyl decanoate and linalool in Debina wine during storage. Papadopoulou & Roussis (2001) also reported that GSH added at 20 mg/L to dry Muscat wine inhibited the disappearance of linalool and α-terpineol during storage of the wine. In the model wine solution, GSH inhibited the decrease of isoamyl acetate, ethyl hexanoate and linalool in a dose-dependent manner. A low free sulphur dioxide wine (35 mg/L) supplemented with 20 mg/L GSH was more effective in protecting several esters and linalool compared to a wine with 50 mg/L free SO_2 (Roussis *et al*., 2007).

The loss of volatile compounds may be ascribed to oxidation and other chemical reactions taking place. The ester concentration in wine may change due to esterification and hydrolysis reactions taking place (Ramey & Ough, 1980). Furthermore, most terpene alcohols are replaced by terpene oxides whereas linalool may convert to α-terpineol which has a much higher sensory threshold (Jackson, 1994). The protective effect of GSH on some esters and terpenes during wine storage was ascribed to its free sulfhydryl (SH) moiety (Roussis *et al*., 2009) which confers unique redox and nucleophilic properties (Penninckx, 2000).

2.3.4.2 VOLATILE THIOLS

2.3.4.2.1 PROTECTION OF VOLATILE THIOLS

Varietal thiols such as 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexylacetate (3MHA) have been identified as important contributors to the varietal aroma of Sauvignon blanc wine (**Figure 2.5**) (Darriet *et al*., 1995; Tominaga *et al*., 1996; Tominaga *et al*., 1998a; Coetzee & du Toit, 2011). These thiols have also been identified in wines from other cultivars such as Colombard, Riesling, Semillon, Merlot, Cabernet Sauvignon and Grenache (Tominaga *et al*., 2000; Murat *et al*., 2001; Ferreira *et al*., 2002). 4MMP is reminiscent of box tree and black currant with a perception threshold corresponding to 0.8 ng/L in model wine solution (3 ng/L in white and red wine) (Darriet *et al*., 1995; Tominaga *et al*., 1998a). The R and S enantiomers of 3MH is reminiscent of grapefruit and passion fruit, respectively with perception thresholds of 50 and 60 ng/L, respectively. 3MHA also occur in two enantiomeric forms: the R form exhibit passion fruit aromas with a perception threshold of 9 ng/L whereas the S form has a herbaceous odour of boxwood with a much lower perception threshold corresponding to 2.5 ng/L (Tominaga *et al*., 2006).

Stellenbosch University http://scholar.sun.ac.za

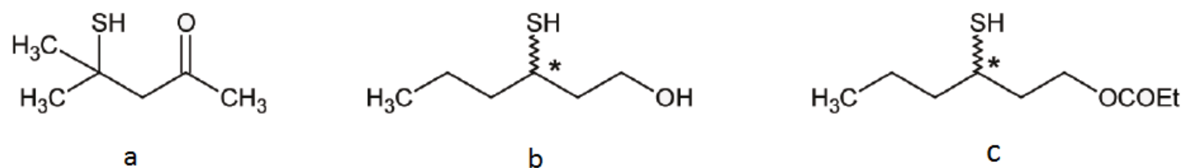


Figure 2.5 Molecular structures of **(a)** 4-mercapto-4-methylpentan-2-one, **(b)** 3-mercaptohexan-1-ol and **(c)** 3-mercaptohexylacetate.

These volatile thiols are particularly susceptible to oxidation during storage (Darriet *et al*., 2002; Blanchard *et al*., 2004), and it has been proposed that GSH plays an important role in the protection of these compounds during bottle ageing (Lavigne-Cruège & Dubourdieu, 2002; Dubourdieu & Lavigne, 2004). The addition of 10 mg/L GSH to Sauvignon blanc wine prior to bottling resulted in significantly higher 3MH levels compared to the control after three years of bottle ageing (Dubourdieu & Lavigne, 2004). This is in agreement with recent findings by Ugliano *et al*. (2011) who reported that the addition of 20 mg/L GSH prior to bottling generally resulted in wines with higher 3MH levels after six months of bottle ageing. As previously mentioned, hydroxycinnamic acids in the wine can be oxidized to *ortho*-quinones in the presence of oxygen. These *ortho*-quinones can easily react with thiols via a Michael addition reaction (Cheynier *et al*., 1986). Furthermore, *ortho*-quinones can also generate peroxides through a series of coupled reactions which are notorious for their oxidative properties against thiols (Wilderandt & Singleton, 1974). The mechanism by which GSH exerts a protective effect on varietal thiols is proposed to be competition based (Fracassetti, 2010). GSH, also being a thiol, may possibly compete with the aromatic thiols to bind to the *ortho*-quinones and consequently limit the loss of varietal aroma (Tirelli *et al*., 2010).

2.3.4.2.2 GLUTATHIONE AS VOLATILE THIOL PRECURSOR

The varietal volatile thiols 4MMP and 3MH are not present in the grapes as free thiols, but are released during alcoholic fermentation from grape-derived, non-volatile precursors (Tominaga *et al*., 1998a; Swiegers *et al*., 2007). Both cysteinylated and glutathionalyted precursors have been identified (Tominaga *et al*., 1998b; Peyrot des Gachons *et al*., 2002a; Subileau *et al*., 2008). S-3-(Hexan-l-ol)-L-cysteine (Cys-3MH) was first identified as the precursor of 3MH in Sauvignon blanc juice (Tominaga *et al*., 1998b). Peyrot des Gachons *et al*. (2002a) then identified S-3-(hexan-1-ol)-glutathione (Glut-3MH) as a pro-precursor of Cys-3MH in Sauvignon blanc juice which was later also confirmed by Thibon *et al*. (2011). 4-(4-methylpentan-2-one)-L-cysteine (Cys-4MMP) and 4-S-glutathionyl-4-methylpentan-2-one (Glut-4MMP) have been identified as potential precursors of 4MMP in Sauvignon blanc juice (Tominaga

Stellenbosch University http://scholar.sun.ac.za

et al., 1995; Fedrizzi *et al*., 2009). Glutathione S-conjugates are involved in detoxification systems of living organisms. The enzyme glutathione S-transferase catalyzes the conjugation of GSH with toxic compounds (Habig *et al*., 1974; Vuilleumier, 1997). Thereafter, γ-glutamyltranspeptidase and carboxypeptidase eliminate the glutamic acid and glycine moieties, respectively, to yield the cysteine S-conjugate (Jakoby *et al*., 1984; Wolf *et al*., 1996). **Figure 2.6** represents a proposed pathway for the biosynthesis of the glutathionylated pro-precursor 3MH and the cysteinylated precursor 3MH in grapevine.

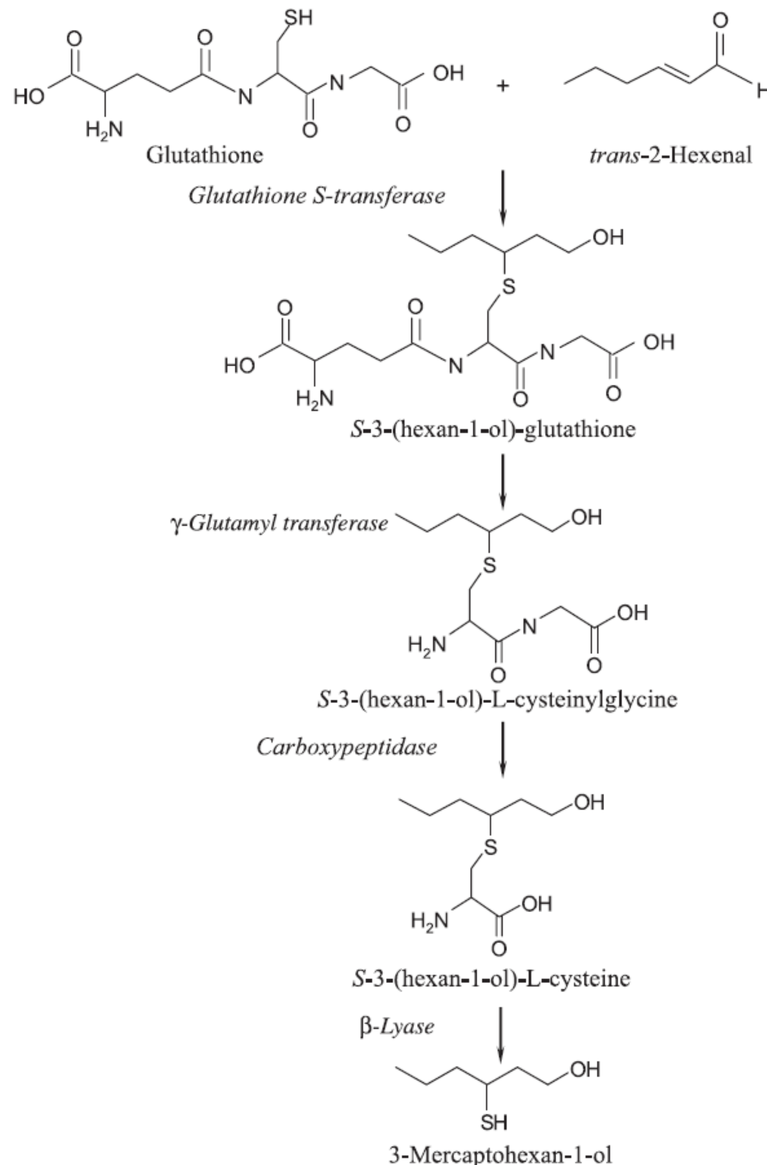


Figure 2.6 Proposed pathway for the biosynthesis of glutathionylated pro-precursor (Glut-3MH) and the cysteinylated precursor (Cys-3MH) in grapevine with the last step taking place during alcoholic fermentation as a result of yeast β-lyase action (Kobayashi *et al*., 2010).

Stellenbosch University http://scholar.sun.ac.za

However, γ-glutamyltranspeptidase has never been identified in grapes (Roland *et al*., 2010a). The detoxification pathways in plants are activated by various stresses including senescence as well as abiotic (oxidation, injury) and biotic factors (pathogens) (Thibon *et al*., 2011). Indeed, Kobayashi *et al*. (2010) reported that Glut-3MH synthesis and the subsequent metabolism to Cys-3MH are enhanced by various environmental stress conditions through the activation of glutathione S-transferases.

Furthermore, Thibon *et al*. (2009) demonstrated that Sauvignon blanc and Semillon must had considerably higher volatile thiol precursor concentrations when the grapes were infected with the fungus, *B. cinerea*. Recent work done by Thibon *et al*. (2011) showed that *B. cinerea* secreted metabolites that stimulated the production of Cys-3MH. The hypothesis states that these metabolites stimulate the formation of (E)-2-hexenal that is conjugated to GSH and then broken down to Cys-3MH.

Subileau *et al*. (2008) suggested that 3MH precursors are transported into the yeast via the *OPT1* regulated GSH-P1 GSH transport system which is discussed at a later stage. It is proposed that a yeast carbon-sulphur lyase enzyme releases the volatile thiol from the cysteine precursor. Tominaga *et al*. (1995) showed that a cell free enzyme extract of *Eubacterium limosum* which displays cysteine-S-conjugate β-lyase activity, could release 4MMP from its precursor Cys-4MMP. When genes encoding putative carbon-sulphur lyases were deleted, a decrease in the amount of 4MMP released, was observed (Howell *et al*., 2005). Furthermore, Swiegers *et al*. (2007) showed that modified wine yeast strains with an over expressed carbon-sulphur lyase gene released 25 times more 4MMP and 3MH in model ferments than control strains. This β-lyase enzyme had no activity towards the GSH precursor, supporting the hypothesised degradation pathway of Glut-3MH to Cys-3MH to form 3MH (Winter *et al*., 2011).

It has been found that neither (E)-2-hexenal nor Cys-3MH accounts for the majority of free 3MH and 3MHA detected in Sauvignon blanc wine (Subileau *et al*., 2008). The authors suggested that Glut-3MH is the main precursor of 3MH since the deletion of *OPT1* gene, which encodes for the main GSH transporter, resulted in lower 3MH formation compared to the wild type strain (Subileau *et al*., 2008). Similarly, Roland *et al*. (2010b) reported that the addition of GSH and (E)-2-hexenal to Sauvignon blanc and Melon B musts, resulted in increased 3MH and 3MHA production. This is contrary to the data of Patel *et al*. (2010) who observed that prefermentation additions of GSH to Sauvignon blanc must resulted in lower 3MH and 3MHA levels in treated wines compared to control wines. The authors explained this repressive effect as competition by GSH for uptake by the yeast of thiol precursors, which again, partly supports the hypothesis of Subileau *et al*. (2008) that the precursors are transported into the yeast by a GSH transport system. Nevertheless, the precursor concentration of both the GSH and cysteine conjugates increase during ripening of the grapes (Roland *et al*., 2010b; Capone *et al*., 2011).

Capone *et al*. (2011) reported a 10 fold increase in cysteine and GSH conjugates between

Stellenbosch University http://scholar.sun.ac.za

preharvest and commercial harvest over a 14 day period for Sauvignon blanc. This was attributed to a loss in membrane integrity at ripeness with the subsequent decompartementalization and mixing of enzymes and precursors. Similarly, the increase in both GSH and (E)-2-hexenal levels during ripening have been reported (Adams & Liyanage, 1993; Kalua & Boss, 2010) and these constituents are inevitable for GSH conjugate formation.

Studies done on the localization of the cysteinylated and glutathionylated precursors in Sauvignon blanc grapes from various regions revealed that the greatest amount of Cys-3MH resided in the skins which is in accordance with other studies (Peyrot des Gachons *et al*. 2002b) whereas the Glut-3MH was fairly evenly distributed between the pulp and the skins. Moreover, 81% of the Glut-4MMP resided in the skins (Roland *et al*., 2011). The same authors reported that variations among precursor distribution and abundance occur for samples from different regions and that these differences might be linked to different GSH concentrations for the various vineyard locations which could participate in Glut-3MH biosynthesis.

Structural damage of the grape berries seems to increase precursor levels. Processing grapes with a stick mixer which results in berry damage, yielded significantly higher Glut-3MH levels compared to free run juice obtained by means of a bag press (Capone *et al*., 2011). Some oxidation is essential for the formation of (E)-2-hexenal. It thus seems that techniques aimed at limiting juice oxidation in effect leads to lower Glut-3MH concentrations.

Roland *et al*. (2010b) studied the effect of grape juice oxidation on cysteine and GSH conjugates in Sauvignon blanc and Melon B musts. Neither the cysteinylated precursors nor the Glut-4MMP levels were affected by the addition of oxygen. Interestingly, a 2.5 fold increase was observed for Glut-3MH in the Sauvignon blanc must after the addition of oxygen compared to the control must without oxygen addition. Consequently, Glut-3MH naturally occurring in grapes, was also formed during prefermentative operations.

2.3.4.3 HYDROGEN SULFIDE

Hydrogen sulfide (H_2S) contributes to the “reductive” off-flavour in wines with an odour which is often described as “rotten egg” or putrefaction. Its detection threshold corresponds to 1.6 µg/L in white wine (Siebert *et al*., 2009). Several factors influence the concentration of H_2S produced during alcoholic fermentation such as the presence of sulphur compounds, yeast strain, fermentation conditions and the nutritional status of the grape juice (Rauhut, 1993; Spiropoulos *et al*., 2000). It is postulated that GSH may also be a potential source of H_2S since cysteine, a constituent amino acid, can be degraded by cysteine desulfhydrase to form H_2S (Tokuyama *et al*. 1973). Preliminary findings by Hallinan *et al*. (1999)

Stellenbosch University http://scholar.sun.ac.za

suggest that GSH may contribute up to 40% of the H_2S liberated by nitrogen starved yeast incubated in the presence of sulphate. When *S. cerevisae* cells were preincubated in a medium containing buthionine-(S,R)-sulphoximine (BSO), a specific inhibitor of GSH synthesis, H_2S liberation was suppressed by 75% and 45% during the first and second hour, respectively, after being transferred to a nitrogen deficient medium.

Recently, it has also been proven that wines treated with GSH prior to bottling accumulated higher H_2S levels during bottle ageing of Sauvignon blanc wine compared to untreated wines. This effect was amplified under low oxygen conditions and the presence of elevated copper(II) concentrations. The authors ascribed the higher H_2S accumulation to the antioxidant capacity of GSH causing reductive conditions which promote H_2S production (Ugliano *et al*., 2011). Further research to elucidate the role of GSH in H_2S production is, however, needed.

2.3.5 REDUCTION OF ATYPICAL AGEING CHARACTER

2.3.5.1 SOTOLON AND 2-AMINOACETOPHENONE

Sotolon (4,5-dimethyl-3-hydroxy-2(5)*H*-furanone) is a volatile compound with an intense curry odour and its perception threshold corresponds to 8 µg/L in wine (Lavigne *et al.*, 2008). This furanone contributes to the aromas of “vins jaunes” (yellow wines) from the Jura and sherries (Dubois *et al*., 1978; Guichard *et al*., 1993) as well as dried fig and rancid nuances in French fortified wines and Port (Cutzach *et al*., 1998; Silva Ferreira *et al*., 2003). The contribution of sotolon to the atypical ageing character of dry white wines has been established (Lavigne-Cruège & Dubourdieu, 2002; Escudero *et al*., 2002; Ferreira *et al*. 2003). 2-Aminoacetophenone (2-AAP) is an atypical ageing off-flavour in wine which confers wet wool, fusel alcohol, naphtalene and furniture polish aromas if present in concentrations that exceeds its sensory threshold of 0.5-1.5 µg/L in wine (Fan *et al*., 2007; Schmarr *et al*., 2007). This compound has been detected in German white wines since the late 1980s (Rapp *et al*., 1993). GSH added at 10 mg/L to Sauvignon blanc wine at bottling has been shown to limit the formation of both these atypical ageing off-odours during three years of storage (**Table 2.1**) (Dubourdieu & Lavigne, 2004).

Stellenbosch University http://scholar.sun.ac.za

Table 2.1 Concentration of sotolon and 2-aminoacetophenone for control and GSH supplemented wines over a three year bottle storage period (Dubourdieu and Lavigne, 2004).

Ageing off-flavour	Wine	Wine + 10 mg/L GSH
Sotolon (μg/L)	9	3
2-Aminoacetophenone (ng/L)	215	125

The sotolon concentration in the control wine exceeded its perception threshold of 8 μg/L in wine, whereas the addition of GSH inhibited its development to levels below its threshold. Although the levels of 2-aminoacetophenone were still lower than the perception threshold, it is evident that GSH suppressed the formation of this compound by almost half. No mechanism was proposed by the authors and further research is required to elucidate the inhibitory effect GSH has on the development of atypical ageing characters in wine.

2.3.6 GLUTATHIONE AND MALOLACTIC FERMENTATION

Reports on GSH in relation to malolactic fermentation (MLF) are scant. Only two studies have been done: one on the effect of GSH on malolactic fermentation (MLF) and one on the influence of MLF on GSH levels in wine. Rauhut *et al*. (2004) observed that the addition of GSH to low pH wines promoted the growth of *Oenococcus oeni* and accelerated the speed of MLF. Marchand & de Revel (2010) evaluated the reduced, oxidized and total glutathione content of five Merlot wines before and after MLF. Their results showed that no tendency could be deducted from the individual GSH or GSSG quantifications although the reduced GSH content generally seemed to either stay constant or decrease during MLF. However, total GSH content decreased significantly during MLF (between 21% and 36% for the five samples). Further investigation regarding the influence of GSH on MLF is, however, required.

2.3.7 GLUTATHIONE-ENRICHED INACTIVE DRY YEAST PREPARATIONS

The use of Inactive Dry Yeast (IDY) preparations in winemaking is currently gaining interest due to its wide range of claimed applications. Commercial inactive yeast preparations are manufactured from thermal inactivation of the yeast, *S. cerevisiae* grown in a highly concentrated sugar medium under aerobic conditions (Pozo-Bayón *et al*., 2009).

Pozo-Bayón *et al*. (2009) classified IDY preparations into four categories: inactive yeast (obtained by thermal inactivation prior to drying), yeast autolysates (intracellular content is partly degraded by an incubation step releasing vacuolar enzymes prior to thermal inactivation), yeast hulls or walls (the

Stellenbosch University http://scholar.sun.ac.za

insoluble component of yeast walls excluding the cytoplasmic content) and yeast extracts (soluble extract after the total degradation of the cytoplasmic content).

Studies to determine the natural thiol content in terms of GSH and free and protein cysteine in oenological dry active yeasts have been done (Tirelli *et al*., 2010). GSH levels ranging from 39 mmol/100g up to 0.92 mmol/100g were reported. The thiol contents of different commercial samples of yeast mannoproteins, hulls, lysates and extracts were also analysed. Different from what was expected, the yeast extracts, being the soluble extract of the cytoplasmic content, and GSH being a cytoplasmic component, did not contain GSH. Indeed, it was the lysate products that displayed the highest GSH content (up to 4.6 mmol/100g) (Tirelli *et al*., 2010).

Traditionally, IDY preparations have been mainly used to improve alcoholic and malolactic fermentation, but their use to enhance the wine sensory characteristics has recently received much attention (Pozo-Bayón *et al*., 2009). Several commercial IDY products (**Table 2.2**) are currently available on the market which claim to preserve wine aroma, extend the shelf life of bottled wines and delay the development of oxidized notes and the yellowing of white wine.

The protective effect of these products is ascribed to the high GSH content with reductive properties. Glutathione-enriched inactive dry yeast preparations (GSH-IDYs) all claim to boost wine GSH content either by the liberation of GSH into the wine, or by allowing the yeast to assimilate GSH precursors during alcoholic fermentation for increased GSH production. However, the required optimum dose, optimal addition time during fermentation and the effects of other winemaking parameters such as temperature, SO_2 and pH on its efficacy remain unknown.

Table 2.2 Examples of some commercially available glutathione enriched inactive dry yeast preparations used in winemaking (Adjusted from Pozo-Bayón *et al*., 2009).

Product[a]	**Product composition**[b]	**Actions in must and wine**[c]
OPTIWHITE®	Inactive yeast with high GSH content	Protect wine colour
BIOAROM®		Preserve freshness and wine aroma
SPRINGAROM®		Increase wine mouthfeel
PROLIE WHITE®		Delay appearance of oxidative flavours
		Decrease bitterness
		Increase tartrate and protein stability
		Enhance alcoholic fermentation
		Favour elimination of astringent polyphenols
		Prevent premature ageing

[a]Products distributed by companies: Lallemand, Laffort, Springer Oenologie and Enartis.

[b]One or more of these components can be included in the composition.

[c]The applications are in agreement to manufacturer's descriptions included in the products.

Stellenbosch University http://scholar.sun.ac.za

Andújar-Ortiz *et al*. (2011) tested the amount of total and reduced GSH released into synthetic wines by commercial GSH-IDYs. In addition, the evolution of these compounds were also monitored over a nine month period in Grenache rosé wine supplemented with a GSH-IDY preparation. Results for the synthetic wine showed that very similar amounts of total and reduced GSH were released into the synthetic wine for the different products. When the preparations were added at a dosage of 0.3 g/L , between 1 and 2 mg/L reduced GSH were released into the synthetic wine 30 minutes after supplementation. Nine days after supplementation, only a slight reduction in reduced GSH concentration was observed for all of the GSH-IDYs.

In the case of the winemaking trial, the total GSH content was at a maximum level directly after alcoholic fermentation for both the control wine and the wine supplemented with the GSH-IDY, corresponding to 8 and 16 mg/L, respectively. Furthermore, the difference in the total GSH content for the control wine and the GSH-IDY wine was higher than expected, taken into account the amount of GSH released by the specific GSH-IDY preparation which was determined in the synthetic wine experiment. Analysis of the nitrogen composition of the wines revealed a much higher amino acid and peptide content for the GSH-IDY wine compared to the control. This led to the hypothesis that the higher nitrogen content in the GSH-IDY wine stimulated the production of reduced GSH by *S. cerevisiae* during alcoholic fermentation. However, no statistical differences in reduced GSH content were observed between the control and the GSH-IDY wine after alcoholic fermentation. The authors explained this phenomenon by a rapid oxidation of reduced GSH released from the GSH-IDY during alcoholic fermentation. Nevertheless, the reduced GSH content of the control wine increased from 0.7 mg/L in the must to 6 mg/L after alcoholic fermentation, suggesting that *S. cerevisiae* is a potential source of GSH. A drastic decrease in GSH concentration during alcoholic fermentation due to oxidation is probably unlikely, due to the reductive environment during this process and this aspect clearly needs further attention.

2.3.8 ANALYTICAL METHODS FOR GLUTATHIONE QUANTIFICATION

As a result of the increasing interest in GSH, different analytical methods have been developed for the determination of reduced, oxidized and total glutathione in grapes, must, wine and inactive dry yeast preparations.

Enzymatic methods based on the reaction of GSH with 5,5'-dithio*bis(*2-nitrobenzoic acid) (DTNB) to yield 2-nitro-5-thiobenzoic acid which allows spectrophotometric absorption at 412 nm have been used for the determination of total GSH in berries, rachis and grapevine leaves (Adams & Liyanage, 1991) as well as white wine (Cassol & Adams, 1995).

Stellenbosch University http://scholar.sun.ac.za

Several liquid chromatography methods as well as a capillary electrophoresis method have been developed for the quantification of GSH and/or GSSG in grape juice and wine which are summarized in **Table 2.3**. Many of these methods are time consuming, requiring extensive sample preparation and only allow for the quantification of GSH with the exception of du Toit *et al*. (2007) and Marchand & de Revel (2010). Clearly an accurate and fast method that does not require extensive sample preparation for GSH and GSSG determination in grape juice and wine would be of great value for further research.

Table 2.3 Methods for the analysis of GSH and/ or GSSG in must and wine.

Method	**Detector**[a]	**Label**[b]	**Matrix**	**Compounds**	**LOD**[c]	**References**
HPLC	DAD	DTNB	Grape juice	Total GSH		Cheynier *et al*., 1989
HPLC	FD	OPA	Grape juice Wine	GSH	1 µg/L	Park *et al*., 2000
HPLC	ESI-MS/MS		Grape juice Wine	GSH, GSSG	0.2 mg/L (GSSG) 0.4 mg/L (GSH)	du Toit *et al*., 2007
CE	LIF	MBB	Grape juice Wine	GSH	0.02 mg/L	Lavigne *et al*., 2007
HPLC	FD	NDA	White wine* Red wine°	GSH,GSSG	0.03 mg/L (GSH)*° 2.45 mg/L* (GSSG) 3.06 mg/L° (GSSG)	Marchand & de Revel, 2010
HPLC	FD	OPA	Grape juice Wine	GSH	0.06 mg/L	Janes *et al*., 2010
UPLC	PAD	pBQ	Grape juice Wine	GSH	0.017 mg/L	Fracassetti *et al*., 2011

[a] DAD: diode array detector; FD: fluorescence director; ESI-MS/MS: electrospray ionization tandem mass spectrometry; PAD: photo array detector

[b] DTNB: 5,5-dithiobis(2-nitrobenzoic acid)/ Ellman's reagent; OPA: *o*-Phtaladehyde; MBB: monobromobimane; NDA: 2,3-naphthalenedialdehyde; pBQ: parabenzoquinone

[c] LOD: limit of detection.

Stellenbosch University http://scholar.sun.ac.za

2.4 GLUTATHIONE AND *SACCHAROMYCES CEREVISIAE*

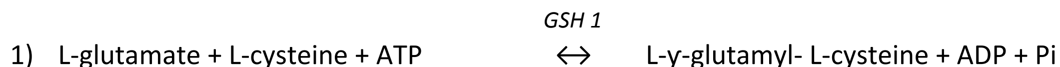
2.4.1 INTRODUCTION

GSH is the most abundant sulphur-containing organic compound in *S. cerevisiae* (Penninckx & Jaspers, 1982). Depending on the growth conditions, GSH may account for 0.5 to 1% of the cell dry weight (Penninckx, 2000) and represents more than 95% of the non-protein thiols in *S. cerevisiae* (Elskens *et al*., 1991). The high concentration of GSH in yeast cells (up to 10 mM), its low redox potential of E'_o = -240 mV for thiol disulfide exchange as well as the fact that GSH is maintained in its reduced state by the NADPH-dependent glutathione disulfide reductase, render this tripeptide a cellular redox buffer (Meister & Anderson, 1983).

GSH is implicated in many stress response mechanisms in *S. cerevisiae* such as sulphur and nitrogen starvation, oxidative stress and the detoxification of heavy metals and xenobiotics. It may also play a role in the maintenance of basic functions of the cell such as cellular structure integrity (Penninckx, 2002).

2.4.2 GLUTATHIONE SYNTHESIS

GSH is synthesized intracellularly in the cytosol by the consecutive actions of y-glutamylcysteine synthetase: y-GCS (Reaction 1) and L- y -glutamylcysteine-glycine y-ligase: GSH synthetase (Reaction 2). These enzymes are respectively encoded by the *GSH1* and *GSH2* genes (Grant & Dawes, 1996).



1) L-glutamate + L-cysteine + ATP $\xleftrightarrow{GSH\ 1}$ L-y-glutamyl- L-cysteine + ADP + Pi

2) L-y-glutamyl-L-cysteine + glycine + ATP $\xleftrightarrow{GSH\ 2}$ GSH + ADP + Pi

The activity of y-GCS is feedback-inhibited by GSH (but not GSSG) to prevent over accumulation of GSH in the cell, which is of physiological significance (Richman & Meister, 1975). Cysteine is usually the limiting substrate in the synthesis of GSH (Meister & Anderson, 1983). GSH synthetase, unlike y-GCS, appears to be an unregulated enzyme (Inoue *et al*., 1998).

2.4.3 GLUTATHIONE TRANSPORT

In addition to intracellular biosynthesis, GSH may also be taken up from the extracellular environment, such as the juice medium. Hgt1p, a high affinity GSH transporter has been identified, cloned and

Stellenbosch University http://scholar.sun.ac.za

characterized in *S. cerevisiae*. The transporter, a 799-amino acid polypeptide, is encoded by the *HGT1* gene. Miyake *et al*. (1998) identified two distinct, kinetically distinguishable GSH transport systems in *S. cerevisiae*, namely GSH-P1 and GSH-P2. GSH-P1 is a high affinity (K_m= 45 µM) , ATP-driven and regulated system, whereas GSH-P2 has a lower affinity (K_m > 2 mM) and is not regulated. Miyake *et al*. (2002) identified *GSH11* as the structural gene encoding GSH-P1. The *GSH11* gene has already been designated as *OPT1* (Hauser *et al*., 2000) and *HGT1* (Bourbouloux *et al*., 2000) and it is now accepted that the Hgt1p and GSH-P1 are homologues and the main GSH transporter in the plasma membrane of *S. cerevisiae*. The high affinity transporter is capable of transporting both GSH and GSH conjugates into the cytoplasm of the cell (Bourbouloux *et al*., 2000). The activity of Hgt1p/GSH-P1 is not only regulated by cysteine (Miyake *et al*., 2002) but also by methionine and GSH (Srikanth *et al*., 2005).

Ycf1p, the yeast orthologue of the mammalian multidrug resistance associated protein (MRP1), acts as a vacuolar pump mediating ATP-dependent transport of GSH and glutathione S-conjugates into the vacuole of *S. cerevisiae*. This vacuolar pump, however, has a very low affinity for GSH (K_m = ±15 mM) and its primary function is the transportation of glutathione S-conjugates (Li *et al*., 1996; Rebbeor *et al*., 1998).

A novel GSH exchanger, Gex1, was recently identified in *S. cerevisiae* and is located at both the vacuolar and plasma membrane (Dhaoui *et al*., 2011). Cells overproducing Gex1, displayed low intracellular GSH contents and high extracellular GSH contents. Furthermore, the overproduction of Gex1 induced acidification of the yeast cytosol, substantiating the possibility that Gex1 is a GSH/proton antiporter. Further research is required to understand the transport of GSH into the extracellular environment.

2.4.4 GLUTATHIONE DEGRADATION

Once GSH is transported into the vacuole of the cell, it is degraded by the vacuolar membrane-bound proteins γ-glutamyltranspeptidase (γ-GT) and L-cysteinyl glycine dipeptidase (Jaspers *et al*., 1985). γ-Glutamyltranspeptidase (γ-GT) encoded by the *CIS2 (ECM38)* gene (Penninckx *et al*., 1980; Mehdi *et al*., 2001) catalyzes the first step in GSH degradation which involves the cleavage of the γ-glutamyl moiety and the release of cysteinylglycine (**Figure 2.7**). The γ-glutamyl moiety is transferred to suitable γ-glutamyl peptides and amino acids whereas cysteinylglycine is further degraded to its constitutive amino acids, cysteine and glycine. The enzyme L-cysteinyl glycine dipeptidase (CGase: L-cysteinyl glycine+ H_2O →L-cysteine + glycine) which catalyzes this action, is associated with the vacuolar membrane of *S. cerevisiae* although the gene encoding for this enzyme, remains unidentified in yeast (Penninckx, 2002).

Stellenbosch University http://scholar.sun.ac.za

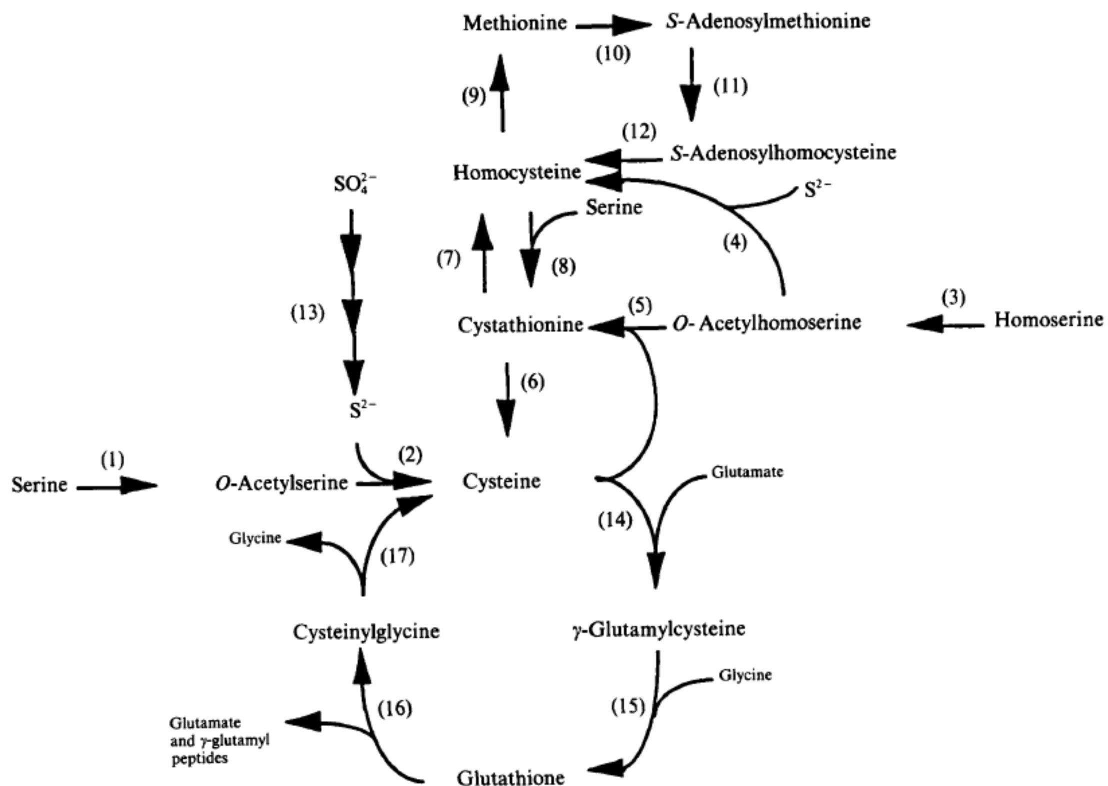


Figure 2.7 A model for the main fluxes of sulphur in *Saccharomyces cerevisiae* (Elskens *et al*., 1991). **(1)** Serine acetyltransferase; **(2)** cysteine synthase; **(3)** homoserine acetyltransferase; **(4)** homocysteine synthase; **(5)** γ-cystathionine synthase; **(6)** γ-cystathionase; **(7)** L-cystathionase; **(8)** L-cystathionine synthase; **(9)** homocysteine methyltransferase; **(10)** S-adenosylmethionine synthetase; **(11)** S-adenosylmethionine demethylase; **(12)** adenosylhomolcysteinase; **(13)** sulphate reducing pathway; **(14)** γ-glutamate-cysteine ligase; **(15)** GSH synthetase; **(16)** γ-glutamyltranspeptidase; **(17)** L-cysteinylglycine dipeptidase.

The biosynthesis of γ-glutamyltranspeptidase was found to be regulated by two different pathways. The enzyme was repressed by ammonium ions as nitrogen source whereas higher cellular enzyme levels were found in the presence of a variety of other nitrogen sources, including amino acids or urea (Penninckx *et al*, 1980; Jaspers *et al*., 1985; Kumar *et al*., 2003). In the case of nitrogen starvation, a strong derepression of γ-glutamyltranspeptidase production was observed (Mehdi & Penninckx, 1997).

Kumar *et al*. (2003) proposed the possibility of an alternative GSH degradation pathway in *S. cerevisiae* independent of γ-GT. This was demonstrated through the disruption in the *EMC38* gene encoding γ-GT enzyme in *met15*Δ strains, which require organic sulphur sources for growth. These cells were able to utilize GSH as sole source of sulphur, supporting the existence of γ-GT-independent pathway for GSH degradation. This pathway is mediated by a novel protein complex involving three new genes, *DUG1*, *DUG2* and *DUG3*, which have been recently characterized in *S. cerevisiae* (Ganguli *et al*., 2007).

Stellenbosch University http://scholar.sun.ac.za

2.4.5 ROLE OF GSH IN *SACCHAROMYCES CEREVISIAE* DURING NUTRITIONAL AND OXIDATIVE STRESS

2.4.5.1 SULPHUR AND NITROGEN STARVATION

S. cerevisiae can use methionine, homocysteine, cysteine or GSH as a sole source of sulphur as a result of a metabolic network in which sulphur atoms are readily exchanged between these compounds (Ono *et al*., 1984, 1988).

GSH is synthesized using cysteine and is degraded to give rise to cysteine. It thus serves as a reservoir of cysteine and sulphur in the cell. When *S. cerevisiae* cells were deprived of external sulphate, GSH was able to serve as an internal sulphur source until it reached a residual concentration of approximately 10% of its normal value. Derepressesion of y-GT synthesis was observed which resulted in an increase in the turnover-rate of GSH. When sufficient sulphate was present as a nutrient, most of the excess sulphur was incorporated into GSH. This was accompanied by a low y-GT activity and an exceptionally slow turnover rate of GSH (Elskens *et al*., 1991).

When *S. cerevisiae* cells were grown in minimal media with $(NH_4)_2SO_4$ as nitrogen source and then transferred to a medium devoid of nitrogen source, the total GSH pool gradually increased in the first two hours from 7-17 nmol/mg dry weight. The GSH pool decreased in the next two hours to attain a stable value of 2.6 nmol/mg dry weight. During growth in the nitrogen containing medium, the cytoplasmic GSH constituted 50% with the remaining 50% occurring in the vacuole. Upon nitrogen starvation, more than 90% of the cellular GSH shifted towards the central vacuole where 90% was consumed (Mehdi & Penninckx, 1997). This is in agreement with Elskens *et al*. (1991) who also reported that only 90% of the GSH pool could serve as an endogenous sulphur source when *S. cerevisiae* cells were deprived of sulphate. It thus seems that *S.cerevisiae* has an inherent mechanism that prevents complete cellular GSH depletion (Mehdi & Penninckx, 1997). It is hypothesised that GSH fulfils the role of a storage compound which is mobilized to the vacuole during starvation. The vacuolar enzymes y-GT and L-Cysteinyl glycine dipeptidase (CGase) then catalyze the hydrolysis of the stored vacuolar GSH to release the constituent amino acids, L-glutamine, L-cysteine and glycine which partly satisfy the yeast's growth requirements in terms of nitrogen (Mehdi & Penninckx, 1997; Penninckx 2002).

2.4.5.2 OXIDATIVE STRESS

All aerobically grown organisms suffer oxidative stress caused by partially reduced forms of molecular oxygen, known as reactive oxygen species (ROS) (Jamieson, 1998). ROS include superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), alkylhydroperoxides (ROOH) and hydroxyl radical ($HO^{\bullet}$) (Wolff *et al*., 1986). These ROS are highly reactive and attack almost all cellular constituents including DNA, proteins

Stellenbosch University http://scholar.sun.ac.za

and lipids. GSH is regarded as an important defence molecule against oxidative damage in S. *cerevisiae.*

GSH can react with ROS non-enzymatically (Anderson, 1998). In this instance, GSH is oxidized to GSSG by direct interaction with free radicals, thereby removing the reactive oxidants from the solution. GSH can also act indirectly as a cofactor for antioxidant enzymes such as GSH peroxidase, glutathione reductase, glutaredoxins and glutathione S-transferases (Grant, 2001).

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides by GSH as reductant: ROOH + 2 GSH → ROH + GSSG + H_2O and was shown to be induced by oxidative conditions in *S. cerevisiae* (Galazzio *et al*., 1987).

In addition to being a radical scavenger, GSH may also be conjugated to toxic electrophiles such as xenobiotics and heavy metals, a reaction catalyzed by cytosolic glutathione S-transferase (GSH + RX →GS-X + RH). The glutathione S-conjugates are then excreted into the central vacuole of the cell by the ATP-dependent GS-X pump (Li *et al.,* 1996; Jamieson, 1998). As mentioned earlier, Ycf1p has been identified as a vacuolar pump mediating transport of glutathione S-conjugates in *S. cerevisiae*. *YCF1*, the gene encoding this transporter, was isolated for its ability to confer cadmium resistance in *S. cerevisiae* (Li *et al*., 1996). Interestingly, the yAP-1 transcription factor transcriptionally activates both the *YCF1* gene and the *GSH1* gene (Wemmie *et al*., 1994; Wu & Moye-Rowley, 1994). The latter encodes y-glutamylcysteine synthetase, the first enzyme involved in GSH biosynthesis. The expression of the *YCF1* gene and the synthesis of one of the precursors for transport by the Ycf1p vacuolar pump are thus co-ordinately regulated (Li *et al*., 1996).

Furthermore, GSH can reversibly bind to protein sulfhydryl groups which render these proteins protected against irreversible oxidative damage (Thomas *et al*., 1995; Cotgreave & Gerdes, 1998).

2.4.6 GLUTATHIONE: BIOTECHNOLOGICAL PRODUCTION

S. cerevisiae is currently being used on industrial scale for the fermentative production of GSH (Li *et al*., 2004). The GSH accumulates intracellularly and the extraction thereof from yeast cells constitutes an important part of the production process (Penninckx, 2002). For this reason, previous studies mainly focused on the optimization of fermentation conditions for intracellular GSH production. The extracellular accumulation of GSH by *S. cerevisiae* has not been studied in detail. Wei *et al*. (2003) made the assumption that GSH, in general, cannot be excreted into the extracellular medium by living cells. However, work done on the recently discovered GSH exchanger in *S. cerevisiae*, Gex1, showed the contrary.

Wei *et al*. (2003) used surfactants [dedecyl sulfate (SDS) and cetyltrimethyl ammonium bromide (CTAB)] to improve the penetration of the cell membrane and the subsequent accumulation of GSH in

Stellenbosch University http://scholar.sun.ac.za

extracellular form. The addition of low concentrations SDS and CTAB (below 0.5 and 0.25 g/L, respectively), resulted in increased extracellular GSH accumulation. Cell growth was drastically inhibited at higher concentrations.

More recently, Rollini *et al*. (2010) studied the effect of various post fermentative chemical and physical treatments on extracellular GSH accumulation in *S. cerevisiae*. The chemicals Triton and Lauroyl sarcosine showed promising results whereas lyophilization also resulted in enhanced extracellular accumulation. Although this application poses an inviting strategy to increase wine GSH content, the addition of surfactants is not allowed by current wine regulation. The extracellular release of GSH by the yeast under wine conditions, remains obscure and necessitates further investigation.

2.5 CONCLUSION

It is evident that GSH plays an integral role as antioxidant in the oxidation of white musts. It scavenges *ortho*-quinones, which limits the formation of browning pigments to a certain extent. GSH exerts a protective effect on several desirable wine aromas, while at the same time, it limits the formation of undesirable ageing off-odours in wine. Furthermore, its role as aroma precursor in the berry has been the focus of many studies which elucidated glutathione's involvement in the varietal thiol production.

GSH content in grape berries may vary according to cultivar, vintage and geographical region. Soil nitrogen deficiencies in vineyard are generally associated with berries containing low GSH levels. The GSH content in grape juice is also dictated by several factors such as oxygen exposure, tyrosinase activity, grape skin maceration during the pre-fermentation period and pressing conditions. Fermentation by *S. cerevisiae* may have an impact on the final GSH concentration in wine due to the metabolism thereof by the yeast. Transporters for both the uptake and secretion of GSH have been identified in *S. cerevisiae* which may result in fluctuating wine GSH concentrations during alcoholic fermentation. It is also suggested that GSH, being an intracellular compound, is released upon yeast autolysis.

Although SO_2 is very efficient in protecting wine against oxidation, certain consumers are sensitive to this antioxidant and an increasing trend, to reduce the use of SO_2, is currently observed. GSH may assist SO_2 in its role as antioxidant, permitting the use of lower SO_2 levels and overcoming possible consumer resistance. The judicious management of GSH during the winemaking process, aimed at retaining high levels in the final wine, may thus be highly valuable for wine quality, especially that of white wine, by limiting oxidation, browning and the loss of aroma.

Clearly further research is required on various aspects of GSH in wine, especially the role that different yeast strains, GSH-IDY additions and oxygen levels play on GSH levels in wine during the vinification process.

Stellenbosch University http://scholar.sun.ac.za

2.6 LITERATURE CITED

Adams, D. O. & Liyanage, C., 1991. Modification of an enzymatic glutathione assay for determination of total glutathione in grapevine tissues. Am J. Enol. Vitic. 42, 137-140.

Adams, D. O. & Liyanage, C., 1993. Glutathione increases in grape berries at the onset of ripening. Am. J. Enol. Vitic. 44, 333-338.

Anderson, M. E., 1998. Glutathione: an overview of biosynthesis and modulation. Chemico-Biological Interactions 112, 1-14.

Andújar-Ortiz, I. Pozo-Bayón, M. A., Moreno-Arribas, M. V., Martín-Álvarez, P. J. & Rodríguez-Bencomo, J. J., 2011. Reversed-Phase High-Performance Liquid Chromatography-Fluorescence detection for the analysis of glutathione and its precursor γ-glutamyl cysteine in wines and model wines supplemented with oenological inactive dry yeast preparations. Food Anal. Methods doi: 10.1007/s12161-011-9230-4.

Betes-Saura, C., Andres-Lacueva, C., & Lamuela-Raventos, R. M., 1996. Phenolics in white free run juices and wines from Penedès by high-performance liquid chromatography: Changes during vinification. J. Agric. Food Chem. 44, 3040–3046.

Beutler, E., 1989. Nutrition and metabolic aspects of glutathione. Annu. Rev. Nutr. 9, 287-302.

Blanchard, L., Darriet, P. & Dubourdieu, D., 2004. Reactivity of 3-mercaptphexanol in red wine: impact of oxygen, phenolic fractions, and sulfur dioxide. Am. J. Enol. Vitic. 55, 115–120.

Boulton, R. B., Singleton, V. L., Bisson, L. F. & Kunkee, R. E., 1996. In: Boulton R. B. (ed). Principles and Practices of Winemaking. Chapman & Hall, New York, NY.

Bourbouloux, A., Shahi, P., Chakladar, A., Delrot, S. & Bachhawat, A. K., 2000. Hgt1p, a high affinity glutathione transporter from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 275, 13259–13265.

Capone, D. L., Sefton, M. A. & Jeffery D. W., 2011. Application of a modified method for 3-Mercaptohexan-1-ol determination to investigate the relationship between free thiol and related conjugates in grape juice and wine. J. Agric. Food Chem. 59, 4649-4658.

Carmel-Harel O. & Storz G., 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Annu. Rev. Microbiol. 54, 439–461.

Cassol, T. & Adams, D. O., 1994. Changes in 35S-labeled glutathione and glutathione conjugates during grape crushing and wine aging. Am. J. Enol. Vitic. 45, 368.

Cassol, T. & Adams, D. O., 1995. Detection of glutathione in white wines using an enzymatic analytical method. Am. J. Enol. Vitic. 46, 410.

Cejudo-Bastante, M. J., Pérez-Coello, M. S. & Hermosín-Gutiérrez, I., 2010. Identification of new derivatives of 2-S-glutathionylcaftaric acid in aged white wines by HPLC-DAD-ESI-MS^n. J. Agric. Food Chem. 58, 11483-11492.

Cheynier, V. F., Trousdale, E. K., Singleton, V. L., Salgues, M. J., & Wylde, R., 1986. Characterization of 2-S-glutathioylcaftaric acid and its hydrolysis in relation to grape wines. J. Agric. Food Chem. 34, 217-221.

Cheynier, V. F. & van Hulst, M. W. J., 1988. Oxidation of trans-caftaric acid and 2-S-glutathionylcaftaric acid in model solutions J. Agric. Food. Chem. 36, 10-15.

Cheynier, V., Souquet, J. M. & Moutounet, M., 1989. Glutathione content and glutathione to hydroxycinnamic acid ratio in *Vitis vinifera* grapes and musts. Am. J. Enol. Vitic. 40, 320-324.

Choné, X., Lavigne-Cruège, V., Tominaga, T., Van Leeuwen, C., Castagnede, C., Saucier, C. & Dubourdieu, D., 2006. Effect of vine nitrogen status on grape aromatic potential: Flavor precursors (S-cysteine conjugates), glutathione and phenolic content in *Vitis vinifera* L. cv. Sauvignon blanc grape juice. J. Int. Sci. Vigne Vin 40, 1-6.

Cilliers, J. J. L. & Singleton, V. L., 1990. Caffeic acid autoxidation and the effects of thiols. J. Agric. Food Chem. 38, 1789-1796.

Coetzee, C. 2011. Oxygen and sulphur dioxide additions to Sauvignon blanc: effect on must and wine composition. MScAgric thesis, Stellenbosch University, Private Bax X1, 7602 Matieland, South Africa.

Coetzee, C. & du Toit, W. J., 2011. A comprehensive review on Sauvignon blanc aroma with a focus on certain positive volatile thiols. Food Res. Int. doi: 10.1016/j.foodres.2011.09.017.

Cotgreave, I. A., & Gerdes, R. G., 1998. Recent trends in glutathione biochemistry glutathione-protein interactions: a molecular link between oxidative stress and cell proliferation? Biochem. Biophys. Res. Commun. 242, 1-9.

Cutzach, I., Chatonnet, P. & Dubourdieu, D., 1998. Rôle du sotolon dans l'arôme des vins doux naturels, influence des conditions d'élevage et de vieillissement. J. Int. Sci. Vigne Vin 32, 223–233.

Stellenbosch University http://scholar.sun.ac.za

Darriet, P., Tominaga, T., Lavigne, V., Boidron, J. & Dubourdieu, D., 1995. Identification of a powerful aromatic compound of *Vitis vinifera* L. var. Sauvignon wines: 4-Mercapto-4-methylpentan-2-one. Flavour Fragr. J. 10, 385-392.

Darriet, P., 2002. Caractérisation des composés volatils associés a la vigne et au vin. Applications technologiques Université Victor Segalen Bordeaux p. 97.

Dhaoui, M., Auchère, F., Blaiseau, P. L., Lesuisse, E., Landoulsi, A., Camadro, J. M., Haguenauer-Tsapis, R. & Belgareh-Touzé, N., 2011. Gex1 is a yeast glutathione exchanger that interferes with pH and redox homeostasis. Mol. Biol. Cell. 22, 2054-2067.

Dubernet, M., & Ribereau-Gayon, P., 1973. Presence et significance dans les moutes et les vins de la tyrosine du raisin. Connaissance Vigne Vin 7, 283–302.

Dubois, P., Rigaud, J. & Dekimpe, J., 1976. Identification de la Diméthyl-4,5 tertrahydrofuranedione-2,3 dans le Vin Jaune du Jura. Lebensm. Wiss. Technol. 9, 366–368.

Dubourdieu, D. & Lavigne, V., 2004. The role of glutathione on the aromatic evolution of dry white wine. Vinidea.net 2, 1–9.

Du Toit, W. J., Marais, J., Pretorius, I. S. & Du Toit, M., 2006. Oxygen in wine: A review. S. Afr. J. Enol. Vitic. 27, 76-94.

Du Toit, W. J., Lisjak, K., Stander, M. & Prevoo, D., 2007. Using LC-MSMS to assess glutathione levels in South African white grape juices and wines made with different levels of oxygen. J. Agric. Food Chem. 55, 2765-2769.

Elskens, M.T., Jaspers, C.J. & Penninckx, M.J., 1991. Glutathione as an endogenous sulfur source in the yeast *Saccharomyces cerevisiae*. J. Gen. Microbiol. 137, 637-644.

Escudero, A., Cacho, J. & Ferreira, V., 2000. Isolation and identification of odorants generated in wine during its isolation: a gas chromatography-olfactometric study. Eur. Food Res. Technol. 211, 105–110.

Etievant, P. X., 1991. Wine. In: Volatile Compounds in Foods and Beverages. Ed. Maarse, H. Marcel Dekker, New York.

Fahey, R.C., 2001. Novel thiols of prokaryotes. Annu. Rev. Microbiol. 55, 533-556.

Fan, W., Tsai, I. M. & Qian, M. C., 2007. Analysis of 2-aminoacetophenone by direct-immersion solid-phase microextraction and gas chromatography-mass spectrometry and its sensory impact in Chardonnay and Pinot gris wines. Food Chem. 105, 1144-1150.

Fedrizzi, B., Pardon, K. H., Sefton, M. A., Elsey, G. M. & Jeffery, D. W., 2009. First identification of 4-S-glutathionyl-4-methylpentan-2-one, a potential precursor of 4-mercapto-4-methylpentan-2-one, in Sauvignon blanc juice. J. Agric. Food Chem. 57, 991-995.

Ferreira, V., Ortin, N., Escudero, A., Lopez, R. & Cacho, J., 2002. Chemical characterization of the aroma of Grenache rose wines: aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. J. Agric. Food Chem. 50, 4048–4054.

Ferreira, A. C. S., Hogg, T. & Guedes de Pinho, P., 2003. Identification of key odorants related to the typical aroma of oxidation-spoiled white wines. J. Agric. Food Chem. 51, 1377–1381.

Fracassetti, D., 2010. Investigation on cysteinyl thiol compounds from yeast affecting wine properties. PhD (Food Science) dissertation, Technology and Biotechnology, University of Milan, Italy.

Fracassetti, D., Lawrence, N., Tredoux, A. G. J., Tirelli, A., Nieuwoudt, H. H. & du Toit, W. J., 2011. Quantification of glutathione, catechin and caffeic acid in grape juice and wine by a novel ultra-performance liquid chromatography method. Food Chem. 128, 1136-1142.

Galiazzo, F., Schiesser, A. & Rotilio, G., 1987. Glutathione peroxidase in yeast. presence of the enzyme and induction by oxidative conditions. Biochem. Biophys. Res. Commun. 147, 1200-1205.

Ganguli, D., Kumar, C. & Bachhawat, A. K., 2007. The alternative pathway of glutathione degradation is mediated by a novel protein complex involving three new genes in *Saccharomyces cerevisiae*. Genetics 175, 1137-1151.

Grant, C.M., MacIver, F.H. & Dawes, I.W., 1996. Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. Curr. Genet. 29, 511-515.

Grant, C. M., 2001. Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. Mol. Microbiol. 39, 533-541.

Guichard, E., Pham, T. T. & Etievant, P., 1993. Quantitative determination of sotolon in wines by high-performance liquid chromatography. Chromatogr. 37, 539–541.

Habig, W. H., Pabst, M. J. & Jakoby, W. B., 1974. Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.

Stellenbosch University http://scholar.sun.ac.za

Hallinan, C. P., Saul, D. J. & Jiranek, V., 1999. Differential utilisation of sulfur compounds for H_2S liberation by nitrogen starved yeasts. Austr. J. Grape Wine Res. 5, 82-90.

Hauser, M., Donhardt, A. M., Barnes, D., Naider, F. & Becker, J. M., 2000. Enkephalins are transported by a novel eukaryotic peptide uptake system. J. Biol. Chem. 275, 3037–3041.

Howell, K. S., Klein, M., Swiegers, J. H., Hayasaka, Y., Elsey, G. M., Fleet, G. H., Hoj, P. B., Pretorius, I. S. & De Barros Lopez, M. A., 2005. Genetic determinants of volatile thiol release by *Saccharomyces cerevisiae* during wine fermentation. Appl. Environ. Microbiol. 71, 5420-5426.

Inoue, Y., Sugiyama, K., Izawa, S. & Kimura, A., 1998. Molecular identification of glutathione synthetase (GSH2) gene from *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 1395, 315-320.

Jackson, S. R., 1994. Wine Science. Principles and Applications. 2nd ed. San Diego: Academic Press.

Jakoby, W. B., Stevens, J., Duffel, M. W. & Weisiger, R. A., 1984. The terminal enzymes of mercapturate formation and the thiomethyl shunt. Rev. Biochem. Toxicol. 6, 97-115.

Jamieson, J. D., 1998. Oxidative stress responses of the yeast *Saccharomyces cerevisiae.* Yeast 14, 1511–1527.

Janes, L., Lisjak, K. & Vanzo, A., 2010. Determination of glutathione content in grape juice and wine by high-performance liquid chromatography with fluorescence detection. Anal. Chim. Acta 674, 239-242.

Jaspers, C. J., Gigot, D. & Penninckx, M. J., 1985. Pathways of glutathione degradation in the yeast *Saccharomyces cerevisiae*. Phytochem. 24, 703-707.

Kalua, C. M. & Boss, P. K., 2010. Comparison of major volatile compounds from Riesling and Cabernet Sauvignon grapes (*Vitis vinifera* L.) from fruitset to harvest. Aust. J. Grape Wine Res. 2010, 16, 337–348.

Kobayashi, H., Takase, H., Kaneko, K., Tanzawa, F., Takata, R., Suzuki, S., 2010. Analysis of S-3-(hexan-1-ol)-glutathione and S-3-(hexan-1-ol)-L-cysteine in *Vitis vinifera* L. cv. Koshu for aromatic wines. Am. J. Enol. Vitic. 61, 176–185.

Kumar, C., Sharma, R. & Bachhawat, A. K., 2003. Utilization of glutathione as an exogenous sulfur source is independent of γ-glutamyl transpeptidase in the yeast *Saccharomyces cerevisiae*: evidence for an alternative gluathione degradation pathway. FEMS Microbiol. Lett. 219 187-194.

Lacroux, F., Tregoat, O., Van Leeuwen, C., Pons, A., Tominaga, T., Lavigne-Cruege, V. & Dubourdieu, D., 2008. Effect of foliar nitrogen and sulphur application on aromatic expression of *Vitis vinifera* L. cv. Sauvignon blanc. J. Int. Sci. Vigne Vin 42, 125-132.

Lavigne-Cruège, V. & Dubourdieu, D., 2002. Role of glutathione on development of aroma defects in dry white wines. In: Trogus, H., Gafner, J. and Sutterlin, A. (eds). 13th International Enology Symposium, International Association of Enology: Montpellier, France, pp 331-347.

Lavigne, V., Pons, A. & Dubourdieu, D., 2007. Assay of glutathione in must and wines using capillary electrophoresis and laser-induced fluorescence detection - Changes in concentration in dry white wines during alcoholic fermentation and aging. J. Chromatogr. A 1139, 130-135.

Lavigne, V., Pons, A., Darriet, P. & Dubourdieu, D., 2008. Changes in the sotolon content of dry white wines during barrel and bottle aging. J. Agric. Food Chem. 56, 2688–2693.

Leustek, T., Martin, M., Bick, J. & Davies, J., 2000. Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Ann. Rev. Plant Physiol. Plant Mol. Biol. 51, 141-165.

Li, Z. S., Szczypka, M., Lu, Y. P., Thiele, D. J. & Rea, P.A., 1996. The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugates pump. J. Biol. Chem. 271, 6509-6517.

Li, Y., Wei, G. & Chen, J., 2004. Glutathione: a review on biotechnological production. Appl. Microbiol. Biotechnol. 66, 233–242.

Liyanage, C. & Adams, D. O., 1992. Glutathione content of grape leaves and berries at the onset of ripening. Am. J. Enol. Vitic. 43, 304.

Maggu, M., Winz, R., Kilmartin, P. A., Trought, M. C. T. & Nicolau, L., 2007. Effect of skin contact and pressure on the composition of Sauvignon Blanc must. J. Agric. Food Chem. 55, 10281-10288.

Marais, J., 1983. Terpenes in the aroma of grapes and wines: A review. S. Afr. J. Enol. Vitic. 4, 49-60.

Marchand, S. & de Revel, G., 2010. A HPLC fluorescence-based method for glutathione derivatives quantification in must and wine. Anal. Chim. Acta 660, 158-163.

Mehdi, K. & Penninckx, M. J., 1997. An important role for glutathione and γ-glutamyltranspeptidase in the supply of growth requirements during nitrogen starvation of the yeast *Saccharomyces cerevisiae.* Microbiol. 143, 1885-1889.

Mehdi, K., Thierie, J. & Penninckx, M. J., 2001. γ-Glutamyltranspeptidase in the yeast *Saccharomyces cerevisiae* and its role in the vacuolar transport and metabolism of glutathione. Biochem. J. 359, 631-637.

Meister, A. & Anderson, M.E., 1983. Glutathione. Annu. Rev. Biochem. 52, 711-760.

Stellenbosch University http://scholar.sun.ac.za

Miyake, T., Hazu, T., Yoshida, S., Kanayama, M., Tomochika, K., Shinoda, S. & Ono, B., 1998. Glutathione transport systems of the budding yeast *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. 62, 1858-1864.

Miyake T., Kanayama, M., Sammoto, H. & Ono, B., 2002. A novel cis-acting cysteine responsive regulatory element of the gene for the high affinity glutathione transporter of *Saccharomyces cerevisiae.* Mol. Genet. Genomics. 266, 1004-1011.

Murat, M. L., Tominaga, T. & Dubourdieu, D., 2001. Assessing the aromatic potential of Cabernet Sauvignon and Merlot musts used to produce rosé wine by assaying the cysteinylated precursor of 3-mercaptohexan-1-ol. J. Agric. Food Chem. 49, 5412-5417.

Okuda T. & Yokotsuka, K., 1999. Levels of glutathione and activities of related enzymes during ripening of Koshu and Cabernet Sauvignon grapes and during winemaking. Am. J. Enol. Vitic. 50, 264-270.

Oliveira, C. M., Ferreira, A. C. S., De Freitas, V. & Silva, A. M. S., 2011. Oxidation mechanisms occurring in wines. Food Res. Int. 44, 1115-1126.

Ono, B., Suruga, T., Yamamoto, M., Yamamoto, S., Murata, K., Kimura, A., Shinoda, S. & Ohmori, S., 1984. Cystathionine accumulation in *Saccharomyces cerevisiae.* J. Bacteriol. 158, 860–865.

Ono, B., Shirahige, Y., Nanjoh, A., Andou, N., Ohue, H. & Ishino-Arao, Y., 1988. Cysteine biosynthesis in *Saccharomyces cerevisiae*; mutation that confers cystathionine γ-synthase deficiency. J. Bacteriol. 170, 5883–5889.

Papadopoulou, D. & Roussis, I. G., 2001. Inhibition of the decline of linalool and α-terpineol in muscat wines by glutathione and N-acetyl-cysteine. Int. J. Food Sci. 13, 413-419.

Papadopoulou, D. & Roussis, I. G., 2008. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a model wine medium by glutathione and N-acetyl-cysteine. Int. J. Food Sci. Technol. 43, 1053-1057.

Park, S. K., Boulton, R. B. & Noble, A. C., 2000a. Formation of hydrogen sulfide and glutathione during fermentation of white grape musts. Am. J. Enol. Vitic. 51, 91-97.

Park, S. K. , Boulton, R. B. & Noble, A. C., 2000b. Automated HPLC analysis of glutathione and thiol-containing compounds in grape juice and wine using pre-column derivatization with fluorescence detection. Food Chem. 68, 475-480.

Pastore A., Federici, G., Bertini, E. & Piemonte, F., 2003. Analysis of glutathione: implication in redox and detoxification. Clin. Chim. Acta 333, 19-39.

Patel, P., Herbst-Johnstone, M., Lee, S. A., Gardner, R.C., Weaver, R., Nicolau, L. & Kilmartin, P. A., 2010. Influence of juice pressing conditions on polyphenols, antioxidants and varietal aroma of Sauvignon blanc microferments. J. Agric. Food Chem. 58, 7280-7288.

Penna, N. C., Daudt, C. E., Brendel, M. & Henriques, J. A. P., 2001. Evolución de los niveles de glutathiona y ésteres hidroxicinámicos durante la vinificación de Sauvignon Blanc, Chenin Blanc, Niágara y Tannat. Alimentaria 2001, 326, 147–151.

Penninckx, M. J., Jaspers, C. J. & Wiame, J. M., 1980. Glutathione metabolism in relation to the amino acid permeation systems in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. 104, 119-123.

Penninckx, M. J. & Jaspers, C.J., 1982. On the role of glutathione in microorganisms. Bull. Inst. Pasteur 80, 291–301.

Penninckx, M. J., 2000. A short review on the role of glutathione in the response of yeasts to nutritional, environmental, and oxidative stresses. Enz. Microbial Techn., 26, 737–742.

Penninckx M. J., 2002. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. FEMS Yeast Res. 2, 295–305.

Peyrot des Gachons, C., Tominaga, T. & Dubourdieu, D., 2002a. Sulfur aroma precursor present in S-glutathione conjugate form: identification of S-3-(hexan-1-ol)-glutathione in must from *Vitis vinifera* L. cv. Sauvignon blanc. J. Agric. Food Chem. 50, 4076-4079.

Peyrot Des Ganchos, C., Tominaga, T. & Dubourdieu, D., 2002b. Localization of S-cysteine conjugates in the berry: Effect of skin contact on aromatic potential of *Vitis vinifera* L. cv. Sauvignon blanc must. Am. J. Enol. Vitic. 53, 144-146.

Pozo- Bayón, M. A., Andújar-Ortiz, I. & Moreno-Arribas, M. V., 2009. Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. Food Res. Int. 42, 754-761.

Ramey, D. D. & Ough, C. S., 1980. Volatile ester hydrolysis or formation during storage of model solutions and wines. J. Agric. Food. Chem. 28, 928-34.

Rapp, A., Versini, G., & Ullemeyer, H., 1993. 2-Aminoacetophenone: The causal agent of the untypical aging flavor (naphthalene note or hybrid note) of wine. Vitis 32, 61–62.

Rauhut, D., 1993. Yeasts – production of sulphur compounds. In: Fleet GH (ed). Wine Microbiology and Biochemistry. Harwood Academic Publishers, Chur, Switzerland, pp 183–223.

Stellenbosch University http://scholar.sun.ac.za

Rauhut, D., Gawron-Scibek, M., Beisert, B., Kondizior, M., Schwarz, R., Kürbel, H., Groβmann, M. & Krieger, S., 2004. Der Einfluss von S-haltigen Aminosäuren und Glutathion auf das Wachstum von *Oenococcus oeni* und die malolaktische Gärung, Weinqualität und biologischer Säureabbau: Zusammenfassung der Berichte des XVIes Entretiens Scientifiques Lallemand, pp 21–23.

Rauhut, D., 2009. Usage and formation of sulphur compounds. In: König, H., Unden, G. A. and Fröhlich, J. (eds). Biology of Microorganisms on Grapes, in Must and in Wine. Springer-Verlag Berlin, Heidelberg, Germany.

Richman, P. G. & Meister, A., 1975. Regulation of gamma-glutamyl-cysteine synthetase by non-allosteric feedback inhibition by glutathione. J. Biol. Chem. 250, 1422-1426.

Rebbeor, J. F., Connolly, G. C., Dumont, M. E. & Ballatori, N., 1998. ATP-dependent transport of reduced glutathione on YCF1, the yeast orthologue of mammalian multidrug resistance associated proteins. J. Biol. Chem. 273, 33449-33454.

Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D., 2006. In: Handbook of Enology, Volume 2. Ed. Ribéreau-Gayon, P., Wiley, Chichester, England.

Roland, A., Schneider, R., Guernevé, C. L., Razungles, A. & Cavelier, F., 2010a. Identification and quantification by LC-MS/MS of a new precursor of 3-mercaptohexan-1-ol (3MH) using stable isotope dilution assay: Elements for understanding the 3MH production in wine. Food Chem. 121, 847–855.

Roland, A., Vialaret, J., Razungles, A., Rigou, P. & Schneider, R., 2010b. Evolution of S-cysteinylated and S-glutathionylated thiol precursors during oxidation of Melon B. and Sauvignon blanc musts. J. Am. Chem. Soc. 58, 4406-4413.

Roland, A., Schneider, R., Charrier, F., Cavelier, F., Rossignol, M. & Razungles, A., 2011. Distribution of varietal thiol precursors in the skin and the pulp of Melon B. and Sauvignon Blanc grapes. Food Chem. 125, 139–144.

Rollini, M., Musatti, A. & Manzoni, M., 2010. Production of glutathione in extracellular form by *Saccharomyces cerevisiae.* Process Biochem. 45, 441–445.

Roussis, I. G., Lambropoulos, I. & Tzimas P., 2007. Protection of volatiles in a wine with low sulfur dioxide by caffeic acid or glutathione. Am. J. Enol. Vitic. 58, 274-278.

Roussis, I. G., Papadopoulou, D. & Sakarellos-Daitsiotis, M., 2009. Protective effect of thiols on wine aroma volatiles. Open Food Sci. J. 3, 98-102.

Salgues, M., Cheynier, V., Gunata, Z., & Wylde, R., 1986. Oxidation of grape juice 2-S glutathionyl caffeoyl tartaric acid by *Botrytis cinerea* laccase and characterization of a new substance. 2,5-Di-S-glutathionyl caffeoyl tartaric acid. J. Food Sci. 51, 1191–1194.

Schmarr, H., Ganß, S., Sang, W. & Potouridis, T., 2007. Analysis of 2 aminoacetophenone in wine using a stable isotope dilution assay and multidimensional gas chromatography–mass spectrometry J. Chrom. A. 1150, 78–84.

Siebert, T., Bramley, B. & Solomon M., 2009. Hydrogen sulfide: aroma detection threshold study in white and red wines. Technol. Rev. 183, 14–16.

Sies, H., 1999. Glutathione and its role in cellular functions. Free Radic. Biol. Med. 27, 916–921.

Silva Ferreira, A. C., Barbe, J. C. & Bertrand, A., 2003. 3-Hydroxy-4,5-dimethyl-25H)-furanone: a key odorant of the typical aroma of oxidative aged port wine. J. Agric. Food Chem. 51, 4356–4363.

Singleton, V. L., Salgues, J., Zaya, J. & Trousdale, E., 1985. Caftaric acid disappearance and conversion to products of enzymatic oxidation in grape must and wine. Am. J. Enol. Vitic. 36, 50-56.

Singleton, V. L., & Cilliers, J. J. L., 1995. Phenolic browning: a perspective from grape and wine. Research Enzymatic Browning and Its Prevention ACS Symposium Series, 600, 23–48.

Singleton, V. L., Salgues, M., Zaya, J. & Trousdale, E., 1985. Caftaric acid disappearance and conversion to products of enzymatic oxidation in grape must and wine. Am J. Enol. Vitic. 36, 50-56.

Sonni F., Clark, A. C., Prenzler, P. D., Riponi, C. & Scollary, G. R., 2011a. Antioxidant action of glutathione and the ascorbic acid/glutathione pair in a model white wine. J. Agric. Food Chem. 59, 3940-3949.

Sonni, F., Moore, E. G., Clark, A. C., Chinnici, F., Riponi, C. & Scollary, G. R., 2011b. Impact of glutathione on the formation of methylmethine- and carboxymethine-bridged (+)-catechin dimers in a model wine system. J. Agric. Food Chem. 59, 7410-7418.

Spiropoulos, A., Tanaka, J., Flerianos, I., Bisson, L. F., 2000. Characterization of hydrogen sulphide formation in commercial and natural wine isolates of *Saccharomyces*. Am. J. Enol. Vitic. 51, 233–248.

Srikanth, C. V., Vats, P., Bourbouloux, A., Delrot, S. & Bachhawat, A. K., 2005. Multiple cis-regulatory elements and the yeast sulphur regulatory network are required for the regulation of the yeast glutathione transporter, Hgt1p. Curr. Genet. 47, 345–358.

Stellenbosch University http://scholar.sun.ac.za

Subileau, M., Schneider, R., Salmon, J. M. & Degryse, E., 2008. New insights on 3-mercaptohexanol (3MH) biogenesis in Sauvignon blanc wines: Cys-3MH and (E)-Hexen-2-al are not the major precursor. J. Agric. Food Chem. 56, 9230-9235.

Swiegers, J. H., Capone, D. L., Pardon, K. H., Elsey, G. M., Sefton, M. A., Francis, I. L. & Pretorius I. S., 2007. Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. Yeast 24, 561-574.

Thibon, C., Dubourdieu, D., Darriet, P. & Tominaga, T., 2009. Impact of noble rot on the aroma precursor of 3-sulfanylhexanol content in *Vitis vinifera* L. cv Sauvignon blanc and Semillon grape juice. Food Chem. 114, 1359-1364.

Thibon, C., Cluzet, S., Merillon, J. M., Darriet, P. & Dubourdieu, D., 2011. 3-Sulfanylhexanol precursor biogenesis in grapevine cells: The stimulating effect of *Botrytis cinerea*. J. Agric. Food Chem. 59, 1344-1351.

Thomas, J. A., Poland, B., & Honzatko, R., 1995. Protein sulfhydryls and their role in the antioxidant function in protein S-thiolation. Arch. Bio- Exp. chem. Biophys. 319, 1–9.

Tirelli, A., Fracassetti, D., & De Noni, I., 2010. Determination of reduced cysteine in oenological cell wall fractions of *Saccharomyces cerevisiae*. J Agric. Food Chem. 58, 4565–4570.

Tokuyama, T., Kuraishi, H., Aida, K. & Uemura T., 1973. Hydrogen sulfide evolution due to a pantothenic acid deficiency in the yeast requiring this vitamin, with special reference to the effect of adenosine triphosphate on yeast cysteine desulfhydrase. J. Gen. Appl. Microbiol. 19, 439–466.

Tominaga, T., Masneuf, I., Dubourdieu D., 1995. A S-cysteine conjugate, precursor of aroma of white Sauvignon. J. Int. Sci. Vigne Vin 29, 227–232.

Tominaga, T., Darriet, P. & Dubourdieu, D., 1996. Identification of 3-mercaptohexyl acetate in Sauvignon wine, a powerful aromatic compound exhibiting box-tree odor. Vitis 35, 207-210.

Tominaga, T., Furrer, A., Henry, R. & Dubourdieu, D., 1998a. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. Flavour Fragr. J. 13, 159-162.

Tominaga, T., Peyrot Des Gachons, C. & Dubourdieu, D., 1998b. A new type of flavour precursors in *Vitis vinifera* L. cv. Sauvignon blanc: S-cysteine conjugates. J. Agric. Food Chem. 46, 5215-5219.

Tominaga, T., Baltenweck-Guyot, R., Des Gachons, C. P. & Dubourdieu, D., 2000. Contribution of volatile thiols to the aromas of white wines made from several *Vitis vinifera* grape varieties. Am. J. Enol. Vitic. 51, 178-181.

Tominaga, T., Niclass, Y., Frérot, E. & Dubourdieu, D., 2006. Stereoisomeric distribution of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate in dry and sweet white wines made from *Vitis vinifera* (Var. Sauvignon blanc and Semillon). J. Agric. Food Chem. 54, 7251-7255.

Ugliano, M., Kwiatkowski, M. J., Vidal, S., Capone, D., Siebert, T., Dieval, J. B., Aagaard, O. & Waters, E. J., 2011. Evolution of 3-mercatohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. J. Agric. Food Chem. 59, 2564–2572.

Vaimakis, V. & Roussis, I. G., 1996. Must oxygenation together with glutathione addition in the oxidation of white wine. Food Chem. 57, 419-422.

Vuilleumier, S., 1997. Bacterial glutathione S-transferases: What are they good for? J. Bacteriol. 179, 1431-1441.

Wang, Z., 1990. Food enzymology. Beijing: China Light Industry Press.

Wei, G., Li, Y., Du, G. & Chen, J., 2003. Effect of surfactants on extracellular accumulation of glutathione by *Saccharomyces cerevisiae*. Process Biochem. 38, 1133–1138.

Wemmie, J. A., Szczypka, M. S., Thiele, D. J., & Moye- Rowley, W. S., 1994. Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP binding cassette transporter-encoding gene YCF1. J. Biol. Chem. 269, 32592-32597.

Wilderandt, H. L. & Singleton, V. L., 1974. The production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging. Am. J. Enol. Vitic. 25, 119-126.

Winter, G., van der Westhuizen, T., Higgins, V. J., Curtin, C. & Ugliano, M., 2011. Contribution of cysteine and glutathione conjugates to the formation of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) during fermentation by *Saccharomyces cerevisiae*. Austr. J. Grape Wine Res. 17, 285-290.

Wolf, A. E., Dietz, K. J. & Schroder, P., 1996. Degradation of glutathione S-conjugates by a carboxypeptidase in the plant vacuole. FEBS Lett. 384, 31-34.

Wolff, S. P., Garner, A. & Dean, R. T., 1986. Free radicals, lipids and protein degradation. TIBS 11, 27–31.

Wu, A. L., & W. S. Moye-Rowley, 1994. GSH1, which encodes gamma-glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. Mol. Cell. Biol. 14, 5832–5839.

Stellenbosch University http://scholar.sun.ac.za

Chapter 3

Influence of yeast strain, extended lees contact and nitrogen supplementation on glutathione concentration in wine

Stellenbosch University http://scholar.sun.ac.za

3. RESEARCH RESULTS

INFLUENCE OF YEAST STRAIN, EXTENDED LEES CONTACT AND NITROGEN SUPPLEMENTATION ON GLUTATHIONE CONCENTRATION IN WINE

ABSTRACT

Glutathione (GSH) is an antioxidant that plays several important roles in wine, including the limitation of browning and atypical ageing off-flavours and the preservation of important varietal aroma compounds. GSH levels in finished wines, however, vary significantly. Here the influence of various oenological factors, including yeast strain, extended lees contact and different yeast assimilable nitrogen contents on the concentrations of GSH in wine or model wine were investigated. Twenty commercial *Saccharomyces cerevisiae* wine yeast strains were evaluated in chemically defined grape juice for differences in GSH content after alcoholic fermentation. Significant differences were observed between strains, with some strains resulting in a seven fold higher wine GSH content. In Sauvignon blanc grape juice with a range of initial GSH concentrations, the levels fluctuated during fermentation. However, after alcoholic fermentation, GSH levels were generally lower than levels initially present in the juice. Strains resulting in the highest GSH levels in synthetic wines, however, did not necessarily display the same tendency in grape juice. While the influence of different YAN levels in chemically defined grape juice on GSH levels after fermentation seemed to be insignificant, GSH generally decreased during ageing, irrespective of the yeast strain or the presence of lees. The data highlight the influence of certain vinification practices on GSH levels in wine, and suggest strategies to increase the level of this compound in commercial wines. Such strategies may help to reduce the use of sulphur dioxide in the industry.

3.1 INTRODUCTION

Oxidation of white wines with the consequent loss of characteristic aroma, the development of atypical ageing flavour character and visual browning constitutes a well-known problem in the wine industry. White wine, in particular Sauvignon blanc, is sensitive to oxygen exposure which may have a detrimental impact on wine quality. Sulphur dioxide (SO_2) is the most widely used preservative in winemaking, displaying antioxidant, antimicrobial and anti-enzymatic properties (Hornsey, 2007). However, although SO_2 is very efficient to protect wine against oxidation, health-related concerns have resulted in consumer pressure to reduce its use (Freedman, 1980; Jackson, 1994). The use of other antioxidants may permit the use of lower SO_2dosages in wine.

Stellenbosch University http://scholar.sun.ac.za

GSH, a naturally occurring tripeptide consisting of L-glutamate, L-cysteine and glycine, is derived from the grapes where it fulfils an indispensable role in plants cells in terms of the antioxidant system, sulphur metabolism and the detoxification of xenobiotics (Noctor & Foyer, 1998). GSH plays a fundamental role during the oxidation of white must and wine where it reacts with oxidized phenolic compounds, such as caftaric acid quinones, to generate 2-S-glutathionyl caftaric acid, also known as the Grape Reaction Product (GRP) or other oxidation products (Singleton *et al.*, 1985; Cheynier *et al.*, 1986; Sonni *et al*. 2011a,b). These reactions occur primarily during grape crushing, when phenolic compounds are enzymatically oxidized by grape polyphenol oxidases, but may also occur later in wine when chemical oxidation takes place (Sonni *et al.*, 2011a; Ugliano *et al.*, 2011). In this manner, GSH traps the *ortho*-quinones in a colourless form and the formation of brown polymers is limited to a certain extent (Singleton *et al.*, 1985).

Furthermore, GSH is known to exert a protective effect on various aroma compounds during wine ageing, which include volatile thiols (Lavigne-Cruège & Dubourdieu, 2002; Dubourdieu & Lavigne, 2004; Ugliano *et al.*, 2011), esters and terpenes (Papadopoulou & Roussis, 2001, 2008; Roussis *et al.*, 2009). Similarly, GSH seems to inhibit the formation of atypical ageing characters, including sotolon and 2-aminoacetophenone, during wine ageing (Dubourdieu & Lavigne, 2004). The mechanism of this protective effect is not exactly known, but is suppose to be related to the antioxidant properties of GSH. More recently, it was also shown that GSH could impede oxidative colouration in model wine solutions (Sonni *et al.*, 2011a,b). Nevertheless, it is clear that GSH exerts several protective effects in both grape juice and wine.

The GSH concentration in grape juice ranges from non-detectable to more than 100 mg/L and is influenced by various factors, including oxygen exposure, tyrosinase activity, grape skin maceration during the pre-fermentation period and pressing conditions (Cheynier *et al.*, 1989; du Toit *et al.*, 2007; Park *et al*., 2000a; Maggu *et al.*, 2007; Patel *et al.*, 2010). During alcoholic fermentation, GSH concentration has been observed to either increase (Park *et al*., 2000a,b; Fracassetti, 2010, Andújar-Ortiz *et al.*, 2011) or decrease (du Toit *et al.*, 2007; Patel *et al*., 2010; Coetzee, 2011). Literature regarding the influence of different yeast strains on GSH concentration in wine as well as the evolution of this compound during alcoholic fermentation, is hence either contradictory or unclear and an extensive study where a large number of commercial wine yeast strains have been screened in terms of final GSH content, has not been undertaken. Yeast could possibly alter the GSH content in wines by utilizing and secreting GSH during fermentation and differences between strains in the quantities assimilated may result in varying wine GSH contents. Lavigne *et al.* (2007) proposed that the amount of GSH present after alcoholic fermentation is dependent on the yeast strain used, since inoculation of the same Sauvignon blanc juice with different strains resulted in different wine GSH contents. Two of the

Stellenbosch University http://scholar.sun.ac.za

three strains tested, resulted in wines with lower GSH contents than the corresponding juices whereas the third strain led to a significant increase in GSH content during alcoholic fermentation. However, in an independent study by Fracassetti (2010), the influence of yeast strain was considered insignificant.

Furthermore, whereas the concentration of GSH decreases during wine ageing (Ugliano *et al.*, 2011; Penna *et al.*, 2001), ageing wines on the yeast lees is presumed to help maintain GSH levels (Lavigne *et al.*, 2007). Park *et al.* (2000a) reported that GSH production is directly correlated with both total nitrogen and assimilable amino acid content of grape juice.

GSH is the most abundant sulphur-containing organic compound in *Saccharomyces cerevisiae* in which it accounts for 0.5 to 1% of the cell dry weight (Elskens *et al.*, 1991). Transporters for both the uptake and secretion of GSH have been characterized in *S. cerevisiae* (Miyake *et al.*, 1998; Bourbouloux *et al.*, 2000; Dhaoui *et al.*, 2011). GSH is regarded as an important defence molecule against oxidative damage in S. *cerevisiae.* It can react non-enzymatically with reactive oxygen species such as hydrogen peroxide (H_2O_2) (Anderson, 1998) to be oxidized to glutathione disulfide (GSSG). GSH can also act indirectly as a cofactor for several antioxidant enzymes such as glutathione peroxidase, glutathione reductase, glutaredoxins and glutathione S-transferases (Grant, 2001).

In view of the positive effects GSH may have on wine quality, in terms of limiting browning and the loss of aroma, the judicious management of this antioxidant, by means of oenological factors such as yeast strain choice, extended lees contact and the manipulation of juice assimilable nitrogen content, would be of great value. This study was undertaken thus to ascertain the influence of commercial wine yeast strain, lees contact and assimilable nitrogen content on GSH concentration in wine. Finally, preliminary results on oxidative stress response in yeast in relation to different intracellular GSH contents, are also reported.

3.2 MATERIALS AND METHODS

3.2.1 Yeast strains and media

The commercial wine yeast strains used in this study are listed in **Table 3.1**. Chemically defined grape juice medium based on a combination of Henschke and Jiranek (1993) and Bely *et al.* (1990) was used for synthetic wine fermentations. The protocol of Henschke and Jiranek (1993) was followed with the exception of the amino acid stock which was based on Bely *et al.* (1990). The yeast assimilable nitrogen content of this medium was 300 mg/L in the form of free alpha amino nitrogen and NH_4Cl. Strains were grown in minimal yeast nitrogen base (YNB) media supplemented with 0.67 % w/v yeast nitrogen base without amino acids (Difco) and 2 % w/v glucose (Merck Chemicals), where indicated. Where required,

Stellenbosch University http://scholar.sun.ac.za

YNB media were solidified by the addition of 2% (w/v) agar. Cells were disrupted in PBS buffer (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl and 2.0 mM KCl, pH 7.4) containing 10 % metaphosphoric acid (Sigma-Aldrich, St. Louis, MO, USA).

3.2.2 SCREENING OF YEAST STRAINS IN SYNTHETIC MEDIA

3.2.2.1 Fermentation conditions

The strains tested in chemically defined grape juice media (CDGJM) are listed in **Table 3.1**. An aliquot of 90 mL CDGJM was divided into 100 mL glass bottles (heat sterilized at 120°C, 20 minutes) and spiked with GSH (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 40 mg/L. Active dry yeasts were rehydrated as recommended by the suppliers and inoculated into the fermentation media to give a cell concentration of 1 x 10^6 cells/mL. The bottles were sealed with sterilized rubber bungs fitted with fermentation locks (CO_2 bubbling outlets filled with water) and fermented at 20°C. Fermentation progress was monitored by weighing the bottles daily. When the accumulated mass loss was stable for three days, completed fermentations were confirmed by the GrapeScan FT 120 (Foss Electric, Denmark) (Nieuwoudt *et al.*, 2004) with results displaying residual sugar concentrations all below 5 g/L. Fermentations were conducted in triplicate. Samples destined for GSH analyses were taken directly before inoculation and after the completion of alcoholic fermentation.

Table 3.1 Commercial *Saccharomyces cerevisiae* wine strains used in this study, listed according to manufacturer.

Strain	Company
VIN7, VIN 13, Alchemy 1, Alchemy 2, NT 116	Anchor
D21, QA23, DV10, Rhône4600, V1116, Ba11, R2, Cross Evolution, EC1118*	Lallemand
CK S102, UCLM S325	Springer Oenologie
X16, X5, VL3	Laffort
ES181, Top Essence	Enartis

* not used in screening experiment, but in grape juice experiment (2011 season).

3.2.3 SCREENING IN GRAPE JUICE TO MONITOR GSH EVOLUTION DURING ALCOHOLIC FERMENTATION

Several strains from the screening experiment conducted in chemically defined grape juice were also used in an additional study in grape juice. Sauvignon blanc juice was obtained after settling from two different cellars (cellar 1 and cellar 2 located in the Durbanville and Franschhoek wine region,

Stellenbosch University http://scholar.sun.ac.za

respectively). The grapes were grown and processed according to standard viticultural and oenological practices. The settled juices were collected in 40 litre stainless steel canisters of which the air was previously displaced by CO_2 gas (Afrox SA). Additional CO_2 gas was blown on the headspace to exclude oxygen and prevent oxidation. Dry ice was also added to the canisters to emit CO_2 gas during transportation to the experimental cellar at Stellenbosch University.

3.2.3.1. Fermentation conditions

General wine analytical parameters (°Brix, pH, and titratable acidity as tartaric acid equivalents) on the juices were measured with a GrapeScan FT 120 instrument (Foss Electric, Denmark) (Nieuwoudt *et al.*, 2004). Free and bound SO_2 were measured using a Metrohm titration unit (Metrohm, Ltd., Switzerland). Ammonia concentration was quantified using the Glutamate Dehydrogenase Enzymatic Bioanalysis UV-method (Roche, Mannheim, Germany). Free α-amino acid nitrogen was determined by the o-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay described by Dukes & Butzke (1998). YAN is expressed as the sum of the α-amino acid nitrogen (FAN) plus ammoniacal nitrogen. All values are presented in **Table 3.2.** The juice was then divided into 4.5 litre glass bottles which had been vigorously sparged with CO_2 gas prior to filling. The GSH content of juice A from cellar 1 was analysed and found to be 20 mg/L (**Table 3.3**). The bottles of juice A were divided in two groups, one of which was spiked with GSH (Sigma-Aldrich, St. Louis, MO, USA) to 80 mg/L, referred to as juice B. The GSH content of juice from cellar 2 was analysed and found to be 10 mg/L and not adjusted. The average GSH contents of the different juices are listed in **Table 3.3**. For the 2011 harvest, settled Sauvignon blanc juice was again obtained from cellar 1. Exactly the same protocol was followed as for the 2010 harvest with the exception that 2 litre in stead of 4.5 litre glass bottles were used as fermentation units. This juice is referred to as juice D.

Table 3.2 Composition of the different Sauvignon blanc juices used in the study.

Juice	Free SO_2 mg/L	Total SO_2 (mg/L)	YAN[a] mg/L	°Brix	pH	TA[b] g/L[c]
A and B	11	38	350	22.6	3.26	5.37
C	28	50	310	22.6	3.32	3.38
D	9	36	320	22.4	3.49	4.8

[a] YAN: yeast assimilable nitrogen (ammonia + α-amino acid nitrogen)
[b] Titratable acidity
[c] Tartaric acid equivalents

Stellenbosch University http://scholar.sun.ac.za

Table 3.3 Average GSH concentration of the different Sauvignon blanc juices.

Juice	Average GSH content (mg/L)	Cellar
A	20	1
B	80	1
C	10	2
D	15	1

The free SO_2 concentration of each treatment was adjusted to 30 mg/L by means of 2.5% SO_2 addition ($K_2S_2O_5$ solution, EVERINTEC, Italy) and the SO_2 levels were confirmed by a Metrohm titration unit (Metrohm, Ltd., Switzerland).

The juice was then inoculated with different commercial preparations of *S. cerevisiae* at 0.3 g/L according to the suppliers' recommendations. The strains used for juices A, B and C were VIN7, QA23, Cross Evolution and VL3. Juice D was inoculated with the aforementioned strains plus three additional strains; R2, X16 and EC1118 (Refer to **Table 3.1**). Each bottle was then sealed with a rubber bung equipped with an airlock to release CO_2 during fermentation and weighed. Fermentation was initiated at room temperature after which the bottles were transferred to a temperature controlled room set at 15°C. FermaidK® yeast nutrient (Lallemand, South Africa) at 0.25 g/L and diammonium phosphate (DAP) at 0.1 g/L were added to the fermenting must after 5 °Brix had fermented out. The bottles were weighed each day to follow the progress of fermentation. When mass loss stopped for three days, GrapeScan FT 120 scan results confirmed that all wines were fermented dry to a residual sugar content < 5 g/L. This experiment was conducted in quadruplet in the 2010 season and triplicate in the 2011 season. Samples destined for GSH analyses were taken five times during fermentation according to the weight loss which corresponded to 0, 25%, 50%, 75% and 100% sugar loss. Juice samples were taken before inoculation after it had been divided into the separate bottles.

3.2.4 INFLUENCE OF AGEING AND LEES CONTACT

The wines fermented with strains Cross Evolution and QA23 in 2010 with an initial GSH concentration of 20 mg/L were used for this experiment. These wines were divided into four separate treatments: wine without lees, wine with lees, wine with lees and added enzyme and model wine with lees. The first treatment was to rack the wine from the yeast lees under CO_2 gas into a 100 mL glass bottle. The yeast lees and wine were then mixed homogenously after which it was divided into three 100 mL bottles. Extralyse enzyme (Laffort Oenologie) was added at a final concentration of 3 g/hL to one bottle. The fourth treatment was centrifuged at 5000 rpm for 5 minutes, the supernatant discarded and model wine medium added to the lees (retentate). The model wine solution consisted of 13% ethanol and 5.3 g/L tartaric acid, and the pH was adjusted to 3.3 with a 5M potassium hydroxide solution (Merck Chemicals).

Stellenbosch University http://scholar.sun.ac.za

The 100 ml bottles were sparged with CO_2 gas to achieve inert atmosphere prior to filling. Once the bottles were filled, additional CO_2 gas was blown in the headspace, the bottles sealed hermitically and stored in a dark room at 20°C. Wine samples for GSH analyses were taken after 3, 6 and 12 months of ageing. The experiment was performed in triplicate.

3.2.5 INFLUENCE OF DIFFERENT YEAST ASSIMILABLE NITROGEN CONTENTS

Chemically defined grape juice medium with the following modifications was used: 110 g glucose and 110 g fructose. The yeast assimilable nitrogen (YAN) content was adjusted to three different levels: 100 mg/L, 250 mg/L and 350 mg/L by adjusting both the free α-amino nitrogen and NH_4Cl content. Another treatment was included where 0.25 g/L FermaidK® yeast nutrient was supplemented to a medium containing 350 mg/L YAN. The codes for the different treatments are listed in **Table 3.4**. An aliquot of 250 mL chemically defined grape juice medium was divided into Erlenmeyer flasks (250 mL, sterilized at 120 °C, 20 minutes) fitted with a side-arm port sealed with a rubber septum for sampling and spiked with GSH to a concentration of 10 mg/L and 40 mg/L. The synthetic juice was inoculated with *S. cerevisiae* strain QA23 (Lallemand, South Africa) according to the supplier's recommendations, the flasks closed with airlocks and fermentation proceeded at 15°C. This experiment was conducted in triplicate. Samples destined for GSH analyses were taken five times during fermentation according to weight loss corresponding to 0, 25%, 50%, 75% and 100% sugar loss.

Table 3.4 Code and description of different YAN and GSH combinations used in chemically defined grape juice medium.

Code	YAN content (mg/L)	GSH content (mg/L)	FermaidK® (g/L)
LL	100	10	0
ML	250	10	0
HL	350	10	0
HLF	350	10	0.25
LH	100	40	0
MH	250	40	0
HH	350	40	0
HHF	350	40	0.25

Stellenbosch University http://scholar.sun.ac.za

3.2.6 INFLUENCE OF OXIDATIVE STRESS

3.2.6.1 Fermentation conditions

The commercial yeast strain *S. cerevisiae* QA23 (Lallemand, South Africa) was used in this study. Cultures of the yeast strain were grown overnight at 30 °C with reciprocal shaking in YNB media with 2% w/v glucose to serve as precultures. YNB media was inoculated to an OD_{600nm}~ 0.1 to serve as a second preculture which was grown at 30°C with reciprocal shaking. Triplicate flasks containing 100 mL freshly prepared YNB media supplemented with 0 mg/L, 20 mg/L and 120 mg/L GSH (Sigma-Aldrich, St. Louis, MO, USA) was inoculated to an OD_{600nm} ~ 0.1 and incubated at 30°C with shaking. Cells were harvested at an OD_{600nm} ~ 1 and washed twice with fresh YNB media.

3.2.6.2 H_2O_2 plate essays

The plates used for the assessment of the effects of various preculture GSH concentrations (0 mg/L, 20 mg/L and 120 mg/L) on oxidative stress resistance were prepared one the day before the cultures were spotted. YNB agar medium with 2 % w/v glucose was left to cool to 50 °C before the addition of H_2O_2 (0 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM and 4 mM). The plateS were stored overnight at 8 °C in the dark. In order to monitor the effect of various intracellular GSH concentrations on the growth and survival against oxidative stress, cultures of the strains grown in different GSH concentrations were spotted in four 10x serial dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) on the plates. The plates were incubated at 30 °C for 3 days and monitored for growth.

3.2.7 SAMPLING PROCEDURE, SAMPLE PREPARATION AND GLUTATHIONE ANALYSIS

3.2.7.1 Juice and wine sampling

Samples destined for GSH analyses were drawn at the stages described in sections 3.2.1.1, 3.2.2.1, 3.2.3 and 3.2.4. This was done by transferring the required volume under CO_2 gas into plastic sampling bottles which had been previously filled with CO_2 gas. Samples in section 3.2.3 and 3.2.4 were drawn with a syringe through the rubber septum under CO_2 backflow. A 1000 mg/L SO_2 ($K_2S_2O_5$, EVERINTEC, Italy) and freshly prepared 500 mg/L ascorbic acid solutions (Sigma-Aldrich, St. Louis, MO, USA) were also added to the sampling bottles prior to sampling. These high SO_2 and ascorbic acid concentrations were used to completely inhibit residual polyphenol oxidase or laccase activity in the samples (du Toit *et al.*, 2007). Additional CO_2 gas was blown into the sampling bottle after the sample was transferred to inhibit any residual oxidation enzyme activity. The samples were then immediately frozen at -20 °C until sample analyses.

Stellenbosch University http://scholar.sun.ac.za

3.2.7.2 Extracellular GSH analyses of juice and wine samples

Samples were thawed on the day of analyses and 4 mL centrifuged (Centrifuge 5415 D, Eppendorf, Hamburg, Germany) at 12 800 rpm for 5 minutes at 20°C. 2 mL of the supernatant was added to 100 µL of 14 mg/L acetaldehyde and left for 15 minutes at room temperature. Derivatization was then carried out as described by Tirelli *et al.* (2010). In brief, 100 µL of 43.2 mg/L *para*-benzoquinone (pBQ) was added to the sample followed by the addition of 1 mL of 53 mg/L 3-mercaptopropionic acid (MPA) to react with the excess pBQ. The reaction mixture was mixed, microfiltered using a 0.22 µm PVDF filter (Millipore) and placed in clear vials prior to injection. GSH in the must and wine was detected and quantified by a novel ultra-performance liquid chromatography method described by Fracassetti *et al.* (2011). Separation was performed using a Waters Acquity UPLC (Milford, MA) equipped with a photo array detector (PAD eλ) (Miford, MA). The column, a BEH-C18 column (1.7 µm x 100 mm x 1.7mm, Waters), was maintained at 25 °C. The mobile phase consisted of water/trifluoroacetic acid (0.05% v/v) and methanol. Detection was carried out at 303 nm for GSH. The injection volume of the derivatized sample was 2 µL.

3.2.7.3 Intracellular GSH analyses in the oxidative stress experiment

Samples for the determination of intracellular GSH content were drawn in the following manner. The growth medium was homogenously mixed, a 2 mL aliquot drawn and centrifuged (Hermle Z233 M-2, Germany) at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet washed with 1 mL of a 1 M EDTA and 20 mM Tris (pH 8.5) solution. The washed cells were resuspended in PBS and disrupted with glass beads (500 µm diameter) for 20 minutes at 8 °C. Cell debris was removed by centrifugation (12 000 rpm, 10 minutes, 12°C). GSH concentrations in the supernatants were determined by the method described in section 4.2.6.3. GSH concentration was expressed as mg GSH per g (as wet weight) cells.

3.2.8 STATISTICAL ANALYSIS

All analyses were done using Statistica V. 10 software (Statsoft Inc., Tulsa, OK). One-way ANOVA's , two-way ANOVA's and mixed model repeated measures ANOVA's were used for statistical analyses. Significant differences were judged on a 5% significance level ($p<0.05$).

Stellenbosch University http://scholar.sun.ac.za

3.3 RESULTS AND DISCUSSION

3.3.1 PRELIMINARY SCREENING OF COMMERCIAL *SACCHAROMYCES CEREVISIAE* STRAINS FOR GSH UTILIZATION DURING ALCOHOLIC FERMENTATION

A screening of 20 commercial yeast strains was conducted in a chemically defined grape juice medium. The medium was supplemented with GSH to 40 mg/L to resemble a natural grape must. This level of GSH is in the the range of GSH concentrations naturally occurring in white grape musts obtained by crushing and pressing under reductive conditions (du Toit *et al.*, 2007; Cheynier *et al.*, 1989). All fermentations achieved dryness (residual sugar level < 5 g/L). The fermentation rates of the different treatments differed slightly with the fastest fermentation completed after 12 days. All alcoholic fermentations were completed after 16 days (results not shown). In order to characterize yeast strains in terms of GSH content in the resulting wines, the GSH concentrations of the different treatments were measured directly after the completion of alcoholic fermentation. The GSH content in the chemically defined grape juice (40 mg/L) decreased considerably during alcoholic fermentation to reach levels ranging from 0.5 mg/L to 3.5 mg/L at the end of fermentation (**Figure 3.1).**

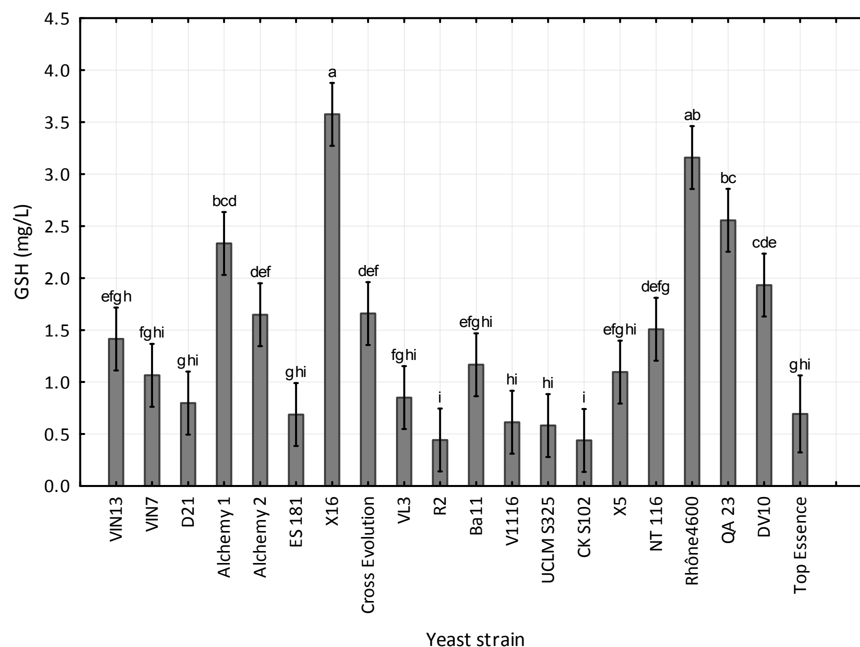


Figure 3.1 Reduced glutathione (GSH) concentration at the end of alcoholic fermentation for 20 different commercial *S. cerevisiae* strains. Vertical bars denote 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

The results obtained are in agreement with results of Marchand & de Revel (2010), Janes *et al*. (2010) and Fracassetti *et al*. (2011) who found GSH contents in similar ranges in several white wines. Since the

Stellenbosch University http://scholar.sun.ac.za

synthetic medium contained no phenolic compounds or polyphenol oxidase, the formation of *ortho*-quinones with the subsequent addition of GSH to form 2-*S*-glutathionyl caftaric acid (GRP), was impossible. There would thus have been no loss in GSH due to its incorporation into GRP. Furthermore, it is also worthwhile to note that oxidation of GSH is pH dependent and that pH values below 7 generally impede auto-oxidation of GSH (Camera & Picardo, 2002). The acidic conditions in this experiment (pH = 3.3) would thus have limited the auto-oxidation of GSH. Moreover, reductive conditions which would further maintain GSH in the reduced form, were soon achieved by alcoholic fermentation. Although the chemically defined grape juice medium contained trace amounts of copper ($CuCl_2$, 15 µg/L) and iron ($FeCl_2$, 30 µg/L), which catalyze oxidation reactions, the amounts were far below levels generally present in natural grape must (0.33 and 1.01 mg/L, respectively) (Sauvage *et al*., 2002). It can thus be assumed that the GSH content present after alcoholic fermentation was a direct result of the yeast interaction with GSH.

Statistically significant differences ($p < 0.05$) in final GSH content were observed among the different treatments. Strains R2 and CK S102 resulted in synthetic wines with the lowest GSH content whereas strains X16 and Rhône4600 displayed a seven fold higher GSH content. It thus seems as if yeast strain may have an impact on the GSH content present after alcoholic fermentation which corroborates with results by Lavigne *et al.* (2007). Furthermore, the data suggest that the subspecies *S. cerevisiae* var. *bayanus* may result in higher GSH concentrations at the end of fermentation compared to *S. cerevisiae* var. *cerevisiae* strains. From **Figure 3.1** it indeed appears that X16, DV10 and QA23 (*S. cerevisiae* var. *bayanus*) resulted in wines with GSH contents on the higher end of the concentration range. However, the other *S. cerevisiae* var. *bayanus* strains, such as R2 and ES 181, and to a lesser extent NT116, were on the lower end of the concentration range. No definite conclusion in this regard could therefore be made.

3.3.2 SCREENING IN GRAPE JUICE

3.3.2.1 GSH evolution during alcoholic fermentation

Based on the data in synthetic must, four yeast strains were selected for further evaluation in real grape juice A, B and C in 2010. In juice D, during 2011, three additional strains were implemented. The fermentation data for the respective juices are shown in **Table 3.5** and **Table 3.6**, respectively. All treatments reached dryness (residual sugar < 5 g/L). Fermentation duration for the 2010 vintage did not differ significantly between the various juices. This implies that supplementation with GSH for juice B in the 2010 season to a concentration of 80 mg/L had no effect on fermentation rate, despite contributing

Stellenbosch University http://scholar.sun.ac.za

to higher nutrient values in the form of amino acids. However, significant differences in fermentation duration were observed for the different strains used (**Table 3.5**). Strains Cross Evolution and QA23 completed alcoholic fermentation significantly faster than VIN7 and VL3. The duration of fermentation for juice D also differed significantly according to the yeast strain used (**Table 3.6**). Strain VIN7 completed alcoholic fermentation last, consistent with findings for juice A, B and C.

The free SO_2 concentrations for the different treatments measured at the end of alcoholic fermentation for the 2010 season are listed in **Table 3.7**. Interestingly, wine B, displayed significantly higher free SO_2 values after completion of alcoholic fermentation compared to wines A and C. This may be ascribed to higher initial GSH content of juice B (80 mg/L), assisting SO_2 in its role as antioxidant and retaining more SO_2 in the free form.

Table 3.5 Duration of fermentation* for the four *Saccharomyces cerevisiae* strains studied in juices A, B and C in 2010.

Yeast strain	Fermentation duration (days)
VIN7	18(1)a
VL3	14(0.51)b
Cross Evolution	12(0.74)c
QA23	12(0.65)c

*Values are means (SD), (n = 12), different letters denote significant differences at p<0.05.

Table 3.6 Duration of fermentation* for the seven *Saccharomyces cerevisiae* strains studied in juice D in 2011.

Yeast strain	Fermentation duration (days)
Vin7	18(0.58)a
VL3	14(0.58)bc
CE	13(0.58)cf
QA23	12(0.58)ef
X16	13(0.58)bcd
EC1118	12(0.58)def
R2	13(0.58)bde

*Values are means(SD) of triplicate analysis; different letters denote significant differences at p<0.05.

Table 3.7 SO_2 concentration (mg/L)* for juice A, B and C of the 2010 season.

	SO_2 (mg/L)		
Yeast strain	**Wine A**	**Wine B**	**Wine C**
Vin7	10.3(1.71)e	17.0(1.41)b	11.0(1.41)e
VL3	10.8(1.26)e	16.5(0.58)b	11.5(0.58)de
CE	13.5(1.00)c	17.0(1.15)b	12.8(0.50)cd
QA23	11.0(0.82)e	20.0(0.82)a	13.0(0.00)c

*Values are means (SD), for quadruplet analysis (n = 4), different letters denote significant differences at p<0.05.

Stellenbosch University http://scholar.sun.ac.za

The GSH evolutions during alcoholic fermentation for juices A, B, C and D are displayed in **Figure 3.2.**

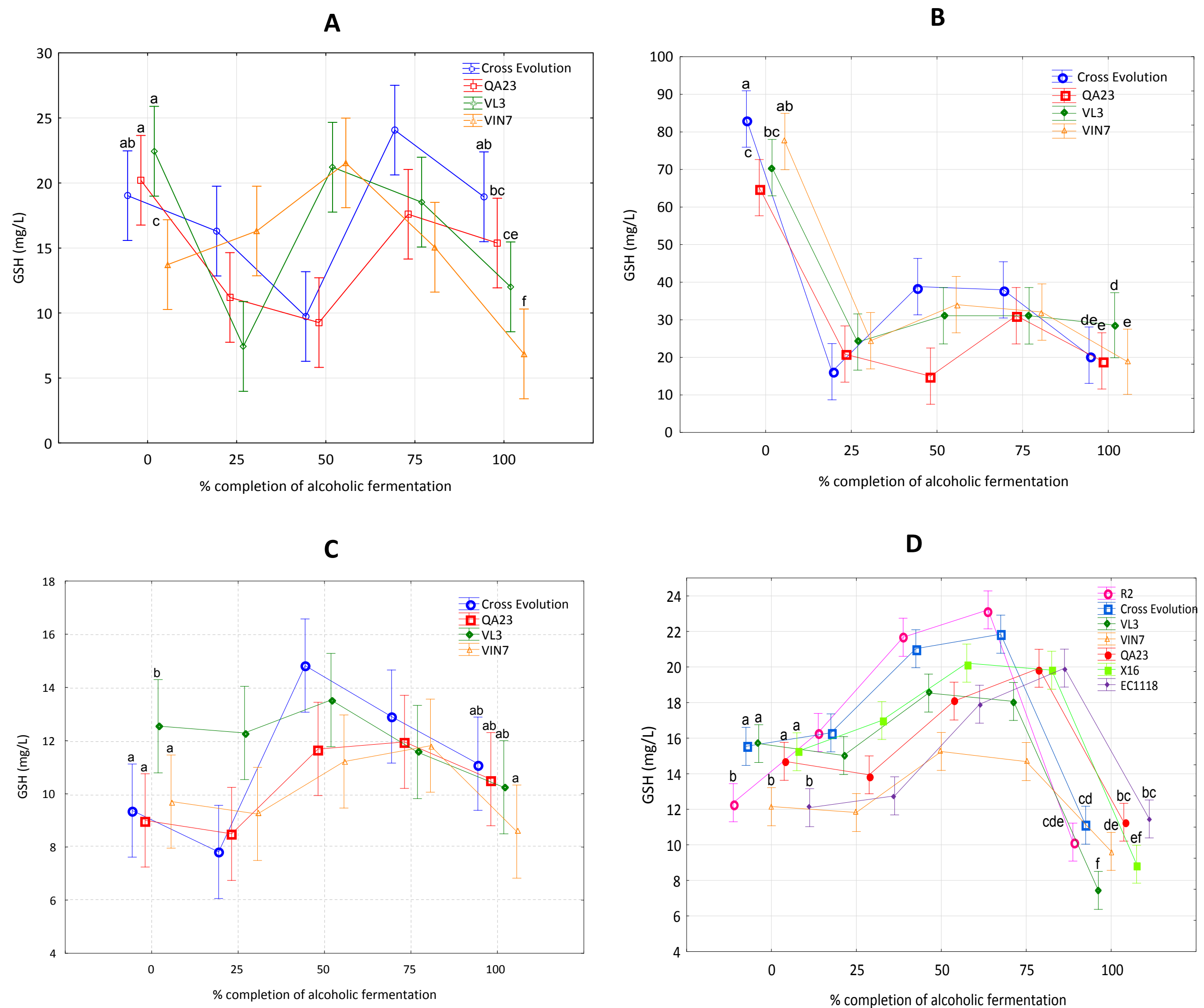


Figure 3.2 Reduced glutathione (GSH) evolution during alcoholic fermentation for different yeast strains for juice A, B, C and D. Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

The evolution of GSH very much differed depending on the initial GSH concentration present in the must and the yeast strain used which highlights the variability in GSH evolution under different conditions. The general observation is that the GSH levels decreased and increased, with the degree and time stage of fluctuation depending on the yeast strain and juice.

At the onset of alcoholic fermentation, the GSH levels either decreased (juices A and B) or stayed relatively constant (juices C and D). The only exception in this regard is the GSH content of juice A fermented with strain VIN7, and juice D fermented with strains R2 and X16 which slightly increased at the onset of fermentation. The GSH content of juice B decreased rapidly by almost 50 mg/L during the first quarter of fermentation, irrespective of the yeast strain.

Stellenbosch University http://scholar.sun.ac.za

In general, the second quarter of fermentation was associated with an increase in GSH content, the only exception here, being juice A fermented with strain Cross Evolution and juices A and B fermented with strain QA23. The GSH content either increased, decreased or stayed constant in the third quarter of fermentation, whereas the last quarter of fermentation was associated with a decrease in GSH content. Interestingly, the degree of decrease for juice D was more severe compared to juices A, B and C. Maximum GSH levels (apart form time zero which coincides with the start of fermentation) were reached at 50% or 75% completion of alcoholic fermentation. Interestingly, in some instances, the GSH concentration increased during alcoholic fermentation to levels higher than initially present in the juice. This was especially observed in juice D. The degree of increase, differed among strains with strain R2 displaying the highest GSH turnover, increasing from 12 mg/L at the start of fermentation to 23 mg/L at 75 % completion of fermentation. Cross Evolution followed more or less the same trend, increasing from 15 mg/L to 22 mg/L whereas VIN7 did not increase as significantly as the other strains. In general, the GSH levels in wines were on par or lower than levels initially present in the grape juice. At the end of alcoholic fermentation, significant differences in GSH content could be observed for the wines fermented with different strains. It should, however, be taken into consideration that, in some instances, the GSH content of certain juices had already been significantly lower at the onset of fermentation. Several measures were taken to assure homogeneity in terms of GSH content between the different treatments; the juice was homogenously mixed prior to being divided into the different bottles. Therefore, all the bottles were also vigorously sparged with CO_2 gas prior to juice division to ensure inert atmosphere. The bottles were also first randomized prior to assigning the different treatments (inoculation with different yeast strain). Furthermore, the GSH samples were taken directly before inoculation, to circumvent the influence the yeast strain may have on the measurement. Despite all the measures taken to assure homogeneity, low, but significant variations in initial GSH content were observed. No possible explanation for this deviation in GSH content can be given at this stage, but low levels of oxidation initially cannot be excluded.

This variance in wine GSH contents between different strains, should hence be interpreted with caution. Strains inoculated in juices with significantly equal GSH contents should thus be compared to one another after alcoholic fermentation. The GSH content of wine A fermented with strain Cross Evolution was significantly higher than wine fermented with strain VL3. Wine B fermented with strain VL3 had a significantly higher GSH content compared to wine fermented with strains VIN7 and QA23. However, no significant differences in GSH content were observed between wine C fermented with Cross Evolution, QA23 and VIN7. Wine D fermented with strain Cross Evolution did not differ significantly in GSH content from wines fermented with strain QA23, but indeed from strains X16 and

Stellenbosch University http://scholar.sun.ac.za

VL3. The GSH content of wine D fermented with strain EC1118 was significantly higher than that fermented with strain VIN7.

As mentioned earlier, the general observation in terms of GSH evolution during alcoholic fermentation is that the levels decreased and increased, depending on the yeast strain, juice and time stage during fermentation. Several hypotheses for the decrease in GSH content can be given. The decrease in GSH in the early stages of fermentation may be due to the incorporation of GSH to form 2-S-glutathionyl caftaric acid (GRP). GSH is known to scavenge *ortho*-quinones formed during the oxidation of phenolic compounds in juice to form GRP. However, low oxygen levels and inhibition of polyphenol oxidase with SO_2 inhibits the formation of GRP to a large extent (Singleton *et al.*, 1985; du Toit *et al.*, 2006). Several measures were taken to prevent the formation of GRP and the subsequent loss in GSH . Not only was the juice treated very reductively, the free SO_2 levels were also adjusted to 30 mg/L free and 60 mg/L total SO_2. Dubernet & Ribéreau-Gayon (1973) reported that the addition of 25 to 75 mg/L SO_2 to clarified juices led to inhibitions of 75% to 97% in polyphenol oxidase activity.

Furthermore, GSH present in the juice can be taken up by the yeast through the ATP-driven, high affinity GSH transporter, Hgt1p (Bourbouloux *et al.*, 2000) where it is implicated in many stress response mechanisms such as sulphur and nitrogen starvation, oxidative stress and the detoxification of heavy metals and xenobiotics (Penninckx, 2002).

GSH can also react with hydrogen peroxide and undergo addition reaction with aldehyde compounds (Sonni *et al.*, 2011a). Acetaldehyde, released by the yeast during alcoholic fermentation, constitutes approximately 90% of the total aldehyde content in wine (Nykanen, 1986) and although it is mostly bound by SO_2, the possibility of GSH incorporation into aldehyde complexes should not be excluded.

In some cases, the GSH concentrations increased to levels exceeding the levels initially present in the must. A possible explanation for this observation is the *de novo* synthesis of GSH by yeast followed by the secretion thereof into the must. Park *et al.* (2000a,b) ascribed the increase in GSH concentration during fermentation to the formation of GSH by *S. cerevisiae.* It has been shown that endogenously produced GSH in the yeast cytocol can be secreted under normal growth conditions, which is then taken up again by the yeast GSH transporter (Perrone *et al.*, 2005). Moreover, the secreted GSH was found to be primarily in the reduced form (GSH). The intracellular GSH may thus cycle with the extracellular GSH present in the medium which might explain the GSH fluctuations observed during alcoholic fermentation in this study. The exact mechanism of GSH export is not known, but a novel GSH exchanger, Gex1, was recently identified in *S. cerevisiae*. This transporter is located at both the vacuolar and plasma membrane and appears to be a GSH/proton antiporter (Dhaoui *et al.*, 2011). This might further explain the influence extracellular pH has on GSH secretion. Growth of *S. cerevisiae* in pH 3.5 led

Stellenbosch University http://scholar.sun.ac.za

to significant GSH secretion whereas growth at pH 6 led to very little or practically no GSH secretion (Perrone *et al.*, 2005). The relative low pH of grape juice and must (pH 3-3.6) poses another possible explanation for the GSH fluctuations observed during fermentation. Furthermore, GSSG can also be reduced back to GSH by the enzymatic action of glutathione reductase (Carmel-Harel & Storz, 2000). Indeed, Okuda and Yokotsuka (1999) showed increased activities of glutathione reductase during winemaking and postulated that the enzyme was derived from the yeast since this enzyme was undetectable in the grape juice. It thus seems as if secreted reductase enzymes and the supply of NADPH by the yeast may further influence the GSH levels in a fermenting media.

Uncertainty remains as to what exactly leads to the strong decrease in GSH concentration in the last quarter of fermentation. The same observation was however also made by Park *et al.* (2000a) in the case of fermenting Palomino grape juice which was not explained by the authors. This might be due to a rapid uptake of GSH by the yeast leading to decreased extracellular levels. Hgt1p, the high affinity yeast GSH transporter, is in fact, maximally induced under sulphur limitation (Srikanth *et al.*, 2005). It is proposed that cysteine is the main repressor molecule of the transporter (Miyake *et al.*, 2002; Srikanth *et al.*, 2005). The depletion of cysteine (although not measured) in the last quarter of fermentation might thus lead to improved uptake of GSH. It is important to note that the hypotheses presented above, are highly speculative as studies on the uptake and release of GSH in wine-like conditions, have not been done yet.

Another possible explanation for the decline in GSH content at the end of fermentation is the oxidation of GSH to GSSG. However, it is highly unlikely that oxygen was responsible for this decrease in GSH content as oxygen was excluded by CO_2 formed during alcoholic fermentation. Okuda & Yokotsuka (1999) ascribed the increase in GSSG levels during alcoholic fermentation to the oxidation of GSH by hydrogen peroxide and other radicals formed during fermentation. The release of higher quantities of radicals at the end of fermentation might have led to decreased GSH levels. Lavigne & Dubourdieu (2004) reported that the yeast assimilable nitrogen content may influence the yeast's ability to release GSH and that a content of 200 mg/L is needed to allow GSH release during fermentation. The yeast assimilable nitrogen contents of all four juices were however far above this limit, excluding the possibility that limiting nitrogen sources could have potentially influenced the data (refer to **Table 3.2**).

However, the trends observed in the small scale fermentations in chemically defined grape juice media were not confirmed in the grape juice fermentations. Some of the strains reacted differently in the two media. Strain X16 which resulted in the highest GSH content and strain R2 which resulted in the lowest GSH content in synthetic wine, showed different trends in wine. Grape juice is a very complex medium in which many compounds interact which could potentially affect the GSH metabolism of the yeast. The different trends observed in the two different media are not uncommon. Several other

Stellenbosch University http://scholar.sun.ac.za

studies conducted in synthetic media showed different sets of results when evaluated in real juice (Guerrini *et al.*, 2002; Kumar *et al.*, 2010) for other compounds.

Nevertheless, it is evident that different GSH evolutions were observed during alcoholic fermentation as a result of the different strains used. This is consistent with finding by Lavigne *et al*. (2007) who also reported differences in GSH concentrations at the end of alcoholic fermentation when the same grape juice was fermented with three different strains. The initial GSH concentration seems to influence the final GSH content in the wine with lower initial levels staying relatively constant during fermentation and higher GSH concentrations leading to decreased levels in wine. It seems as if strains Cross Evolution and QA23 consistently led to slightly higher GSH contents in wine, but this should be further investigated.

3.3.3 INFLUENCE OF AGEING AND EXTENDED LEES CONTACT ON GSH LEVELS IN WINE

The GSH content at the end of alcoholic fermentation for wines fermented with strains QA23 and Cross Evolution with an initial GSH content of 20 mg/L, were not significantly different. These wines were subjected to an ageing period of one year where the wine GSH content was measured non-destructively after 3, 6 and 12 months of ageing. The data obtained are shown in **Table 3.8**. The GSH content of both wines decreased after 3 months of ageing to concentrations below 11.1 mg/L which corroborates with data by Penna *et al.* (2001) who also observed a considerable decline in GSH content after 3 months of bottle ageing. No significant differences were observed between treatments after 3 months of ageing for wines fermented with QA23. However, wines fermented with Cross Evolution, showed a significant difference in GSH content between wine aged without lees and wine aged in the presence of lees.

Stellenbosch University http://scholar.sun.ac.za

Table 3.8 GSH concentration for different treatments over a 12 month ageing period.

		GSH* (mg/L)	
Treatment	Ageing months	Cross Evolution	QA23
Without lees	3	10.9(1.66)a	9.6(1.72)ab
With lees	3	9(1.57)b	11.1(1.60)a
With lees, enzyme	3	9.3(1.91)b	9.8(0.89)ab
Model wine	3	nd	Nd
Without lees	6	4.5(0.68)d	5(0.73)d
With lees	6	6.4(2.20)c	4.7(0.44)d
With lees, enzyme	6	5.4(0.63)cd	4.6(0.41)d
Model wine	6	nd	Nd
Without lees	12	1(0.15)e	0.8(0.07)e
With lees	12	1.1(0.16)e	0.9(0.11)e
With lees, enzyme	12	1.2(0.13)e	1(0.05)e
Model wine	12	nd	Nd

*Values are means (SD) of quadruplet analysis; different letters denote statistically significant differences at $p < 0.05$.
nd: not detected

Vasserot *et al.* (2003) proved the capability of yeast lees to consume thiols though the establishment of disulfide bridges with SH units of the cysteinyl residues of the yeast cell wall mannoprotein. The bridge formation seemed to be independent of molecular oxygen but required the presence of metallic cations. The higher GSH content for wines without lees compared to those without lees may possibly be ascribed to the sorption of GSH by the yeast lees. This trait, however, seems to be strain specific since it was only observed for Cross Evolution. After 6 months of ageing, a further decrease in GSH content was observed for both strains to concentrations ranging from 4.5 mg/L to 6.4 mg/L. No statistical differences in GSH content were observed between treatments, except for wines fermented with Cross Evolution in the case of wine aged without lees and with lees. In this specific case, the yeast lees may have had a protective effect on GSH. This is in partial agreement with findings by Lavigne *et al.* (2007) who also reported a significant decrease in GSH content for racked wines compared to wines aged on the lees when aged in barrels. This phenomenon is ascribed to the oxygen consumption capacity of yeast lees (Salmon *et al.*, 2000) limiting the oxidation of GSH.

Quite surprisingly, the GSH content (20 mg/L) of wine aged in the presence of lees in used barrels, stayed constant over a 7 month ageing period (Lavigne *et al.*, 2007). This is in stark contrast to our study

Stellenbosch University http://scholar.sun.ac.za

that clearly shows a significant decrease in GSH content over a 6 month period. Moreover, our study was performed under reductive conditions, limiting the oxidation of GSH. Ugliano *et al.* (2011), however, also found significant decreases in GSH content over a 6 month bottle storage period of Sauvignon blanc wine which corroborates with our data. Interestingly, the authors did not detect the GSH dimer, GSSG, after 6 months of ageing, and suggested that the loss in GSH was not attributed to dimerization of GSH, but rather to the formation of 2-S-glutathionyl caftaric acid (GRP) although this compound was not measured. Lavigne and Dubourdieu (2004) postulated that GSH is released upon yeast autolysis in the same manner in which pools of amino acids are released during autolysis. Our hypothesis was that an enzyme which accelerates autolysis and the subsequent release of intracellular compounds, might lead to higher extracellular GSH levels, being an cytoplasmic compound. However, no significant differences in GSH content were observed in treatments that were supplemented with Extralyse enzyme. Furthermore, no GSH was detected over the course of the experiment in the model wine that was aged in the presence of lees. The limit of detection for the UPLC method used in this experiment is 0.017 mg/L (Fracassetti *et al.*, 2011), which suggests that GSH that may have been released from the yeast during autolysis, was in negligible amounts. After 12 months of ageing, the GSH content in all treatments decreased to levels below 1.2 mg/L and no significant differences were observed between the different treatments. It is clear that the effect yeast lees has on GSH levels in wine needs to be further investigated, in combination with other factors such as different yeast strains and oxygen levels.

3.3.4 INFLUENCE OF DIFFERENT YAN LEVELS ON GSH EVOLUTION DURING FERMENTATION

To assess whether different yeast assimilable nitrogen (YAN) levels have an influence on the GSH content present in synthetic wine after alcoholic fermentation, the YAN content of a chemically defined grape juice medium was adjusted to three different levels and fermented with yeast strain QA23. Two different levels of GSH were also tested, with all fermentations completing alcoholic fermentation. Different initial GSH levels did not affect fermentation rate (results not shown). However, the yeast assimilable nitrogen content affected the fermentation rate with the lowest YAN level (100 mg/L) completing alcoholic fermentation four days after treatments with a YAN content of 250 mg/L and 350 mg/L. Treatments with an initial YAN content of 350 mg/L supplemented with the yeast nutrient, FermaidK®, finished alcoholic fermentation three days before the treatments with the same YAN content not supplemented with FermaidK® (results not shown).

Figure 3.3 presents the GSH evolution during alcoholic fermentation for the different treatments. The GSH content for all the treatments decreased considerably during alcoholic fermentation, consistent with findings for juice B of previous work. At the end of alcoholic fermentation, no significant

Stellenbosch University http://scholar.sun.ac.za

differences in GSH content were observed between treatments for the low GSH wines, suggesting that YAN had no influence on extracellular GSH levels present in the synthetic wines. This was also the case for the high GSH treatment, with no significant differences observed between the low, medium and high YAN treatments. The HHF treatment supplemented with FermaidK®, did not differ significantly from the HH treatment, suggesting that supplementation with a yeast nutrient at such a high YAN level would not lead to increased extracellular GSH levels. It thus seems that the higher YAN content in the form of free alpha amino nitrogen and NH_4Cl did not lead to higher extracellular GSH levels present after alcoholic fermentation. This is contrary to results by Park *et al.* (2000a) who reported that GSH production is directly correlated with both total nitrogen and assimilable amino acid content of grape juice fermented with *S. cerevisiae* strain Montrachet. However, this study was not conducted in the same grape juice of which the YAN levels had been adjusted. Instead, the authors used various grape musts with variable YAN contents. It should be taken into consideration that several other factors in the different musts might have influenced GSH production. It could be argued that the yeast strain used in the study by Park *et al.* (2000a) may have influenced the GSH contents observed. This is hence a factor that necessitates further investigation. Several authors are of the opinion that GSH content in grapes is closely linked to the vine nitrogen status estimated as the yeast assimilable nitrogen content (Dubourdieu & Lavigne., 2004; Choné *et al.*, 2006; Lacroux *et al.*, 2008).

It is important to note that the higher GSH content in grapes is a function of more favourable nitrogen conditions during the growth phase of the grapes and that the manipulation of the YAN content of a juice in the cellar, would not necessarily result in higher GSH levels in wine. Different results may have been obtained if the study was to be repeated with other yeast strains. Strains differ in their nitrogen requirements which may possibly influence the GSH content present after alcoholic fermentation.

Stellenbosch University http://scholar.sun.ac.za

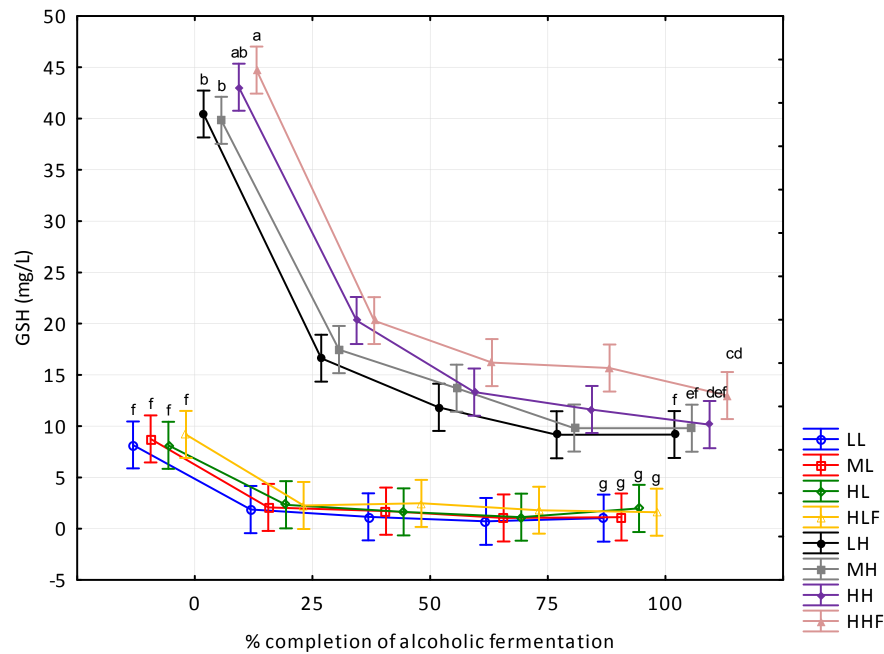


Figure 3.3 Evolution of GSH during alcoholic fermentation with different yeast assimilable nitrogen and GSH contents at the onset of fermentation.

The hypothesis that increased YAN levels would lead to enhanced GSH synthesis by the yeast with the subsequent secretion thereof into the wine medium, was thus proven wrong. It can be argued that ammonium ions suppressed the uptake of free amino acids (Roon *et al.*, 1975) such as the precursor amino acids cysteine, glutamate, glycine and serine, which might have limited the synthesis of GSH. However, ammonium phosphate, being the most readily assimilable nitrogen source to yeast, is a common constituent of nitrogen supplementation products used in cellars. Future research in this aspect should thus include assessing different YAN levels in the absence or presence of ammonia.

3.3.5 INFLUENCE OF DIFFERENT INTRACELLULAR GSH CONTENTS ON OXIDATIVE STRESS RESISTANCE

To test the oxidative stress response of yeast cells with different intracellular GSH levels, a classic approach was followed which did not involve the use of GSH depleting agents or GSH deficient mutants. Firstly, it was to be determined whether yeast cells would attain different intracellular GSH levels when grown in media supplemented with different GSH concentrations. YNB media was spiked with GSH to 0 mg/L, 20 mg/L and 120 mg/L GSH, inoculated with *S. cerevisiae* strain QA23 and grown to the mid log phase (OD_{600nm} ~ 1) where after the intracellular GSH content was determined. The results are shown in **Table 3.9**. The cells grown in media deficient of GSH, accumulated approximately 0.56 mg GSH/ g cells (as wet weight). Interestingly, although significantly different, the difference in intracellular GSH contents between cells grown in the presence of 20 mg/L and 120 mg/L did not differ greatly, being 2.06

Stellenbosch University http://scholar.sun.ac.za

mg/ g cells and 2.45 mg/ g cells, respectively. These values translate to approximately 1 % of the cell dry weight as reported in literature (Penninckx, 2000).

Table 3.9 Intracellular GSH concentration* of cells grown in media with different GSH concentrations (treatment).

Treatment	mg GSH/ g cells
0 mg/L GSH	0.56(0.05) a
20 mg/L GSH	2.06 (0.04) b
120 mg/L GSH	2.45 (0.04) c

*Values are means(SD) of triplicate analysis; different letters denote significant differences at $p<0.05$.

It has been reported that *S. cerevisiae* cells entering stationary phase acquire resistance against several environmental stresses, such as heat, osmotic and oxidative stress (Jamieson, 1992; Schenberg-Frascino & Moustacchi, 1972). For this reason, the cells were harvested at mid log phase to circumvent this resistance. To test whether different intracellular GSH contents would result in different oxidative stress responses, cultures of the strains with different intracellular GSH content were spotted in four serial 10x dilutions on plates containing different H_2O_2 concentrations. On plates containing 2 mM and 4 mM H_2O_2, no growth was observed (results not shown). Growth on plates containing 1 mM H_2O_2 are shown in **Figure 3.4**. It is evident that cells grown to the log phase in the presence of different GSH concentrations, resulting in different intracellular GSH contents, displayed no difference in cell viability. This suggests that the cells grown in the media deficient of GSH, synthesized enough GSH to acquire sufficient stress resistance to H_2O_2 under the conditions used in this experiment.

This experiment was, however, only conducted with one yeast strain. Different result may be observed when this experiment is to be repeated with more strains and different fermentation conditions, such as grape juice. To our knowledge, this is the first reported study evaluating the oxidative stress response of a commercial wine yeast strain with different intracellular GSH levels.

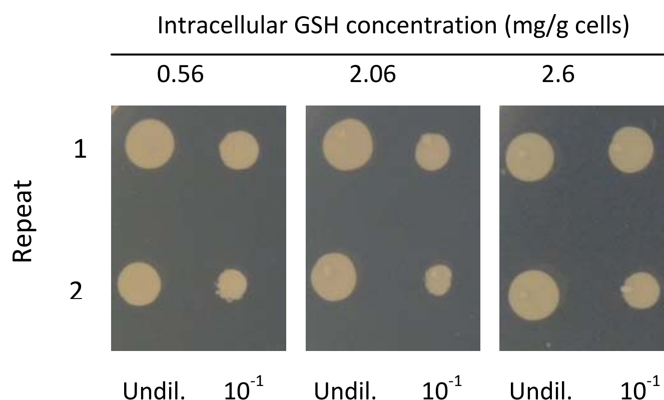


Figure 3.4 The effect of different intracellular GSH levels on oxidative stress resistance of *S. cerevisiae* strain QA23 for undiluted and 10^{-1} dilutions. Plates contain 1 mM H_2O_2. No growth was observed for the other dilutions.

Stellenbosch University http://scholar.sun.ac.za

3.4 CONCLUSIONS

This work provides a first direct comparison of several commercial yeast strains in terms of GSH utilization during alcoholic fermentation in a chemically defined grape juice medium. Differences in GSH content could be observed for synthetic wines fermented with different strains, with some strains resulting in a seven fold higher synthetic wine GSH content. However, when these strains were inoculated in Sauvignon blanc juice, they did not necessarily result in the highest wine GSH concentration.

During this study, important trends regarding GSH evolution during alcoholic fermentation was observed. It is evident that the GSH levels fluctuated during fermentation, depending on several factors, such as the yeast strain and initial GSH concentration of the juice. It appears from this experiment that the GSH concentration in some instances increases to levels on par or higher than those initially present in the juice, suggesting the *de novo* synthesis and secretion of GSH by the yeast. Differences in GSH content for wines fermented with different yeast strains could be observed, albeit small. Screening of more yeast strains in grape juice is recommended to produce more conclusive results. Screening in terms of yeast GSH metabolism on a molecular level would also be of great value. A major drawback of this study was the lack of knowledge on the source of GSH during alcoholic fermentation i.e. what was derived from the grape juice and what amount was produced by the yeast. Future work should include the use of labelled GSH (35^S) which could elucidate the fate of GSH during alcoholic fermentation. In this manner, the incorporation of GSH in 2-*S*-glutathionyl caftaric acid (GRP) and uptake by the yeast can be monitored. With this being said, another drawback of this study was the lack of a method to analyse GRP. Fracassetti (2010) reported that GRP levels stay relatively constant during alcoholic fermentation, but further investigation into the evolution of this compound, especially with regards to GSH incorporation is necessary.

With regards to the influence of ageing on GSH concentrations in wine, it is evident that GSH decreases during ageing, regardless of the yeast strain or presence of the lees. The influence of lees on the preservation of GSH in wine during ageing, seems to be negligible. Furthermore, it seems highly unlikely that GSH is released from the yeast lees upon yeast autolysis as has been previously suggested (Lavigne & Dubourdieu, 2004).

The influence of different YAN levels on GSH concentration in wine also seems to be insignificant. Manipulating the nitrogen content of juice by supplementation with, for example diammonium phosphate, would not necessarily lead to increased GSH levels. However, results may prove different when this experiment is repeated with different yeast strains.

Although the role of GSH in oxidative stress resistance has been well researched, it seems that yeast cells grown in a medium deficient of GSH are capable of producing sufficient GSH to protect the

Stellenbosch University http://scholar.sun.ac.za

cell against oxidative damage. No significant differences were observed between strains with different intracellular GSH contents. Differences may have been observed if the experiment was to be repeated with other yeast strains and fermentation conditions.

This study improved our knowledge of the behaviour of GSH during alcoholic fermentation and highlights the influence of certain vinification practices on its levels in wine. However, there is much room for further investigation into other aspects that may influence the GSH content in wine, such as the amino acid content and phenolic composition of the juice. The influence of viticultural practices, such as leaf removal and water deficit treatments, and more specifically, the locality of the vineyard, on GSH concentrations in the juice would also make for some interesting studies.

3.5 ACKNOWLEDGEMENTS

We would like to thank De Grendel and Boschendal wineries for the donation of the Sauvignon blanc juice as well as Lallemand, Laffort, Anchor, Springer Oenologie and Enartis for the donation of the yeast strains used in this study. We would also like to thank Lallemand, Winetech, THRIP and the NRF for financial support. Nina Lawrence and Drs Daniela Fracassetti and Astrid Buica are thanked for their technical assistance with the analyses of samples as well as Prof Martin Kidd for his valuable input and support with statistical analyses.

3.6 LITERATURE CITED

Adams, D. O. & Liyanage, C. ,1993. Glutathione increases in grape berries at the onset of ripening. Am. J. Enol. Vitic. 44, 333-338.

Anderson, M. E., 1998. Glutathione: an overview of biosynthesis and modulation. Chemico-Biolog. Interactions 112, 1-14.

Andújar-Ortiz, I. Pozo-Bayón, M. A., Moreno-Arribas, M. V., Martín-Álvarez, P. J. & Rodríguez-Bencomo, J. J., 2011. Reversed-Phase High-Performance Liquid Chromatography–Fluorescence detection for the analysis of glutathione and its precursor γ-glutamyl cysteine in wines and model wines supplemented with oenological inactive dry yeast preparations. Food Anal. Methods DOI 10.1007/s12161-011-9230-4.

Bely, M., Sablayrolles, J. M. & Barre., 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions, J. Ferment. Bioeng. 70, 246-252.

Bourbouloux, A., Shahi, P., Chakladar, A., Delrot, S. & Bachhawat, A. K., 2000. Hgt1p, a high affinity glutathione transporter from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 275,13259–13265.

Camera, E. & Picardo, M., 2002. Analytical methods to investigate glutathione and related compounds in biological and pathological processes: A Review. J. Chrom. B. 781, 181-206.

Carmel-Harel O. & Storz G., 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Ann. Rev. Microbiol. 54, 439-461.

Cheynier, V. F., Trousdale, E. K., Singleton, V. L., Salgues, M. J., & Wylde, R., 1986. Characterization of 2-S-glutathioylcaftaric acid and its hydrolysis in relation to grape wines. J. Agric. Food Chem. 34, 217–221.

Cheynier, V., Souquet, J. M. & Moutounet, M., 1989. Glutathione content and glutathione to hydroxycinnamic acid ratio in *Vitis vinifera* grapes and musts. Am. J. Enol. Vitic. 40, 320-324.

Choné, X., Lavigne-Cruège, V., Tominaga, T., Van Leeuwen, C., Castagnede, C., Saucier, C. & Dubourdieu, D., 2006. Effect of vine nitrogen status on grape aromatic potential: Flavor precursors (S-cysteine conjugates),

Stellenbosch University http://scholar.sun.ac.za

glutathione and phenolic content in *Vitis vinifera* L. cv. Sauvignon blanc grape juice. J. Int. Sci. Vigne Vin 40, 1-6.

Coetzee, C. 2011. Oxygen and sulphur dioxide additions to Sauvignon blanc: effect on must and wine composition. MScAgric thesis, Stellenbosch University, Private Bax X1, 7602 Matieland, South Africa.

Dhaoui, M., Auchère, F., Blaiseau, P. L., Lesuisse, E., Landoulsi, A., Camadro, J. M., Haguenauer-Tsapis, R. & Belgareh-Touzé, N., 2011. Gex1 is a yeast glutathione exchanger that interferes with pH and redox homeostasis. Mol. Biol. Cell. 22, 2054-2067.

Dubernet, M. & Ribéreau-Gayon, P., 1973. Presence et significance dans les mouts et les vins de la tyrosinase du raisin. Conn. Vigne Vin. 7, 283-302

Dubourdieu, D. & Lavigne, V., 2004. The role of glutathione on the aromatic evolution of dry white wine. Vinidea.net. 2, 1–9

Dukes, B. C., & Butzke, C. E., 1998. Rapid determination of primary amino acids in grape juice using an o phthaldialdehyde/N-acetyl-Lcysteine spectrophotometric assay. Am. J. Enol. Vitic. 49, 125-134.

Du Toit, W. J., Marais, J., Pretorius, I. S. & Du Toit, M., 2006. Oxygen in wine: A review. S. Afr. J. Enol. Vitic. 27, 76-94.

Du Toit, W. J., Lisjak, K., Stander, M. & Prevoo, D., 2007. Using LC-MSMS to assess glutathione levels in South African white grape juices and wines made with different levels of oxygen. J. Agric. Food Chem. 55, 2765-2769.

Elskens, M.T., Jaspers, C.J. & Penninckx, M.J., 1991. Glutathione as an endogenous sulfur source in the yeast *Saccharomyces cerevisiae*. J. Gen. Microbiol. 137, 637-644.

Fracassetti, D., 2010. Investigation on cysteinyl thiol compounds from yeast affecting wine properties. PdD thesis in Food Science, Technology and Biotechnology, University of Milan, Italy.

Fracassetti, D., Lawrence, N., Tredoux, A. G. J., Tirelli, A., Nieuwoudt, H. H., Du Toit, W. J., 2011. Quantification of glutathione, catechin and caffeic acid in grape juice and wine by a novel ultra-performance liquid chromatography method. Food Chem. 128, 1136–1142.

Freedman, B. J.,1980. Sulphur dioxide in foods and beverages: Its use as a preservative and its effect on asthma. Br. J. Dis. Chest 74, 128-134.

Grant, C. M., 2001. Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. Mol. Microbiol. 39, 533-541.

Henschke, P.A. & Jiranek. V., 1993. Yeast- metabolism of nitrogen compounds. Fleet, G. H. ed. Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, Switzerland, pp. 77-164. Guerrini, S., Mangani, S., Granchi, L. & Vincenzini, M., 2002. Biogenic amine production by *Oenococcus oeni.* Curr. Microbiol. 44, 374-378.

Hornsey, I., 2007. The chemistry and biology of winemaking. The Royal Society of Chemistry, Cambridge, UK.

Kumar, G. R., Ramakrishnan, V. & Linda F. Bisson, L. F., 2010. Survey of hydrogen sulfide production in wine strains of *Saccharomyces cerevisiae*. Am. J. Enol. Vitic. 61, 365-371.

Jackson, R. S. Wine Science. Principles and Applications; Academic Press: Burlington, MA, 2008; pp 316 and 697.

Jamieson, D. J., 1992. *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. J. Bacteriol. 174, 6678-6681.

Lacroux, F., Tregoat, O., Van Leeuwen, C., Pons, A., Tominaga, T., Lavigne-Cruege, V. & Dubourdieu, D., 2008. Effect of foliar nitrogen and sulphur application on aromatic expression of *Vitis vinifera* L. cv. Sauvignon blanc. J. Int. Sci. Vigne Vin 42, 125-132.

Lavigne, V., Pons, A. & Dubourdieu, D., 2007. Assay of glutathione in must and wines using capillary electrophoresis and laser-induced fluorescence detection - Changes in concentration in dry white wines during alcoholic fermentation and aging. J. Chrom. A 1139, 130-135.

Lavigne-Cruège, V. & Dubourdieu, D., 2002. Role of glutathione on development of aroma defects in dry white wines. In: Trogus, H., Gafner, J. and Sutterlin, A. (eds). 13th International Enology Symposium, International Association of Enology: Montpellier, France, pp 331-347.

Leustek, T., Martin, M., Bick, J. & Davies, J., 2000. Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Ann. Rev. Plant Physiol. Plant Mol. Biol. 51, 141-165.

Maggu, M., Winz, R., Kilmartin, P. A., Trought, M. C. T. & Nicolau, L., 2007. Effect of skin contact and pressure on the composition of Sauvignon Blanc must. J. Agric. Food Chem. 55, 10281-10288.

Marchand, S. & de Revel, G., 2010. A HPLC fluorescence-based method for glutathione derivatives quantification in must and wine. Anal. Chim. Acta 660, 158-163.

Miyake, T., Hazu, T., Yoshida, S., Kanayama, M., Tomochika, K., Shinoda, S. & Ono, B., 1998. Glutathione transport systems of the budding yeast *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. 62, 1858-1864.

Stellenbosch University http://scholar.sun.ac.za

Miyake T., Kanayama, M., Sammoto, H. & Ono, B., 2002. A novel cis-acting cysteine responsive regulatory element of the gene for the high affinity glutathione transporter of *Saccharomyces cerevisiae.* Mol. Genet. Genomics 266, 1004-1011.

Nieuwoudt, H. H., Prior, B. A., Pretorius, I. S., Manley, M. & Bauer, F. F., 2004. Principle component analysis applied to fourier transform infrared spectroscopy for the design of calibration sets for glycerol prediction models in wine and for the detection and classification of outlier samples. J. Agric. Food Chem. 52, 3728-3735.

Noctor, G. & Foyer, C. H., 1998. Ascorbate and glutathione: keeping active oxygen under control. Ann. Rev. Plant Physiol. Plant Mol. Biol. 49, 249–279.

Nykanen, L., 1986. Formation and occurrence of flavor compounds in wine and distilled alcoholic beverages. Am. J Enol. Vitic. 37, 84–96.

Okuda T. & Yokotsuka, K., 1999. Levels of glutathione and activities of related enzymes during ripening of Koshu and Cabernet Sauvignon grapes and during winemaking. Am. J. Enol. Vitic. 50, 264-270.

Papadopoulou, D. & Roussis, I. G., 2001. Inhibition of the decline of linalool and α-terpineol in muscat wines by glutathione and N-acetyl-cysteine. It. J. Food Sci. 13, 413-419.

Papadopoulou, D. & Roussis, I. G., 2008. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a model wine medium by glutathione and N-acetyl-cysteine. It. J. Food Sci. Technol. 43, 1053-1057.

Park, S. K., Boulton, R. B. & Noble, A. C., 2000a. Formation of hydrogen sulfide and glutathione during fermentation of white grape musts. Am. J. Enol. Vitic. 51, 91-97.

Park, S. K. , Boulton, R. B. & Noble, A. C., 2000b. Automated HPLC analysis of glutathione and thiol-containing compounds in grape juice and wine using pre-column derivatization with fluorescence detection. Food Chem. 68, 475-480.

Patel, P., Herbst-Johnstone, M., Lee, S. A., Gardner, R.C., Weaver, R., Nicolau, L. & Kilmartin, P. A., 2010. Influence of juice pressing conditions on polyphenols, antioxidants and varietal aroma of Sauvignon blanc microferments. J. Agric. Food Chem. 58, 7280-7288.

Penna, N. C., Daudt, C. E., Brendel, M. & Henriques, J. A. P., 2001. Evolución de los niveles de glutathiona y ésteres hidroxicinámicos durante la vinificación de Sauvignon Blanc, Chenin Blanc, Niágara y Tannat. Alimentaria 326, 147–151.

Penninckx, M. J., 2000. A short review on the role of glutathione in the response of yeasts to nutritional, environmental, and oxidative stresses. Enz. Microbial Techn. 26, 737–742.

Penninckx M. J., 2002. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. FEMS Yeast Res. 2, 295–305.

Perrone, G. G, Grant, C. M. & Dawes, I. W., 2005. Genetic and environmental factors influencing glutathione homeostasis in *Saccharomyces cerevisiae*. Mol. Biol. Cell. 16, 218–230.

Roon, R. R., Larimore, F., Jane, S. & Leyy, J. S., 1975. Inhibition of amino acid transport by ammonium ion in *Saccharomyces cerevisiae.* J Bacteriol. 124, 325-331.

Roussis, I. G., Papadopoulou, D. & Sakarellos-Daitsiotis, M., 2009. Protective effect of thiols on wine aroma volatiles. Open Food Sci. J. 3, 98-102.

Salmon, J.M., Fornairon-Bonnefond, C., Mazauric, J.P. & Moutounet, M., 2000. Oxygen consumption by wine lees: impact on lees integrity during wine ageing. Food Chem. 71,519-528.

Sauvage, L., Frank, D., Stearne, J. & Millikan, M. B., 2002. Trace metal studies of selected white wines: an alternative approach. Anal. Chim. Acta 458, 223-230.

Schenberg-Frascino, A. & Moustacchi, E., 1972. Lethal and mutagenic effects of elevated temperature on haploid yeast. Variations in sensitivity during the cell cycle. Mol. Gen. Genet. 115, 243-257.

Singleton, V. L., Salgues, J., Zaya, J. & Trousdale, E., 1985. Caftaric acid disappearance and conversion to products of enzymatic oxidation in grape must and wine. Am. J. Enol. Vitic. 36, 50-56.

Sonni F., Clark, A. C., Prenzler, P. D., Riponi, C. & Scollary, G. R., 2011a. Antioxidant action of glutathione and the ascorbic acid/glutathione pair in a model white wine. J. Agric. Food Chem. 59, 3940-3949.

Sonni, F., Moore, E. G., Clark, A. C., Chinnici, F., Riponi, C. & Scollary, G. R., 2011b. Impact of glutathione on the formation of methylmethine- and carboxymethine-bridged (+)-catechin dimers in a model wine system. J. Agric. Food Chem. 59, 7410-7418.

Srikanth, C. V., Vats, P., Bourbouloux, A., Delrot, S. & Bachhawat, A. K., 2005. Multiple cis-regulatory elements and the yeast sulphur regulatory network are required for the regulation of the yeast glutathione transporter, Hgt1p. Curr. Genet. 47, 345–358.

Tirelli, A., Fracassetti, D., & De Noni, I., 2010. Determination of reduced cysteine in oenological cell wall fractions of *Saccharomyces cerevisiae*. J Agric. Food Chem. 58, 4565–4570.

Stellenbosch University http://scholar.sun.ac.za

Ugliano, M., Kwiatkowski, M. J., Vidal, S., Capone, D., Siebert, T., Dieval, J. B., Aagaard, O. & Waters, E. J., 2011. Evolution of 3-mercatohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. J. Agric. Food Chem. 59, 2564–2572.

Vasserot, Y., Steinmetz, V. & Jeandet, P., 2003. Study of thiol consumption by yeast lees. Antonie Van Leeuwenhoek International J. Gen. Mol. Microbiol. 83, 201-207.

Wen, S., Zhang, T. & Tan, T., 2004. Utilization of amino acids to enhance glutathione production in *Saccharomyces cerevisiae.* Enz. Microbial Techn. 35, 501–507.

Wang, Z. Tan, S. & Song, J., 2007. Effect of amino acids addition and feedback control strategies on the high-cell-density cultivation of *Saccharomyces cerevisiae* for glutathione production. Process Biochem. 42, 108–111.

Stellenbosch University http://scholar.sun.ac.za

Chapter 4

Research results

Novel UPLC-MS/MS method to assess the effect of glutathione-enriched inactive dry yeast preparation or yeast strains on glutathione levels in juice, wine and yeast cells during alcoholic fermentation

Stellenbosch University http://scholar.sun.ac.za

4. RESEARCH RESULTS

NOVEL UPLC-MS/MS METHOD TO ASSESS THE EFFECT OF GLUTATHIONE-ENRICHED INACTIVE DRY YEAST PREPARATION OR YEAST STRAIN ON GLUTATHIONE LEVELS IN JUICE, WINE AND YEAST CELLS DURING ALCOHOLIC FERMENTATION

ABSTRACT

A simple, robust and fast ultra-high performance liquid chromatography-mass spectrometry method has been developed for the simultaneous quantification of reduced (GSH) and oxidized glutathione (GSSG) in grape juice, wine and model wine solution. In addition, the method also allows the determination of intracellular GSH and GSSG contents. Sample preparation is minimal and does not require derivatization. The method presents very good performances in terms of sensitivity and selectivity. The limit of detection is 0.002 µg/L and 0.001 µg/L for GSH and GSSG, respectively. This method was applied to assess the amount of GSH and GSSG released by commercial glutathione-enriched inactivated dry yeast preparations (GSH-IDYs) into model solution. The GSH and GSSG levels in grape juice fermentations supplemented with GSH-IDY were also assessed in relation to different addition times during fermentation. GSH-IDY addition could lead to elevated wine GSH levels, provided the supplementation is done early during alcoholic fermentation. In an additional study, the must originating from a Sauvignon blanc block which received different leaf removal treatments, was fermented with two different yeast strains and monitored for GSH and GSSG evolution during fermentation. The intracellular GSH contents were also evaluated for the first time during winemaking. Extracellular GSH levels fluctuated depending on the yeast strain and canopy treatment applied. Intracellular GSH levels increased as fermentation progressed whereas GSSG levels remained relatively stable.

4.1 INTRODUCTION

The anti-oxidant activity of reduced glutathione (GSH) in must and wine has been well established. During enzymatic oxidation of white must, hydroxycinnamates such as caftaric acid and coutaric acid, are oxidized to the corresponding *ortho*-quinones by grape polyphenoloxidase (Singleton *et al.*, 1985; Cilliers & Singleton, 1990). *Ortho*-quinones can polymerize and undergo condensation reactions with other compounds to form brown pigments (Li *et al.*, 2008). GSH plays an integral role during oxidation of white musts where it reacts with the *ortho*-quinones to generate 2-S-glutathionyl caftaric acid, also

Stellenbosch University http://scholar.sun.ac.za

known as the Grape Reaction Product (GRP). This product is resistant to further oxidation by grape polyphenoloxidase (Cilliers & Singleton, 1990). In this manner, GSH traps the *ortho*-quinones in a colourless form and the formation of brown polymers is limited to a certain extent (Singleton *et al*., 1985). GSH can thus impede the browning of white must. It has also been shown that its addition to must or wine prior to bottling resulted in better colour stability during ageing (Vaimakis & Roussis, 1996; Dubourdieu & Lavigne, 2004.

During ageing, GSH helps to preserve characteristic aroma compounds in wine, including volatile thiols, esters and terpenes (Lavigne-Cruège & Dubourdieu, 2002; Dubourdieu & Lavigne, 2004; Ugliano *et al.*, 2011; Papadopoulou & Roussis, 2001, 2008; Roussis *et al.*, 2009), while at the same time preventing and the formation of atypical aromas associated with wine deterioration such as sotolon and 2-aminoacetophenone (Dubourdieu & Lavigne, 2004).

Reduced glutathione (GSH), the dominant form in grape berries (Okuda & Yokotsuka, 1999) has been found to either increase (Park *et al.*, 2000a,b; Fracassetti, 2010; Andujar-Ortiz *et al.*, 2011) or decrease during alcoholic fermentation (Okuda & Yokotsuka, 1999; du Toit *et al*., 2007; Coetzee, 2011). The evolution of GSSG, formed upon oxidation of GSH, during alcoholic fermentation has, to our knowledge, only been reported by Okuda & Yokotsuka (1999). They found that GSSG levels initially increased and then decreased during fermentation of Koshu juice. Du Toit *et al*. (2007) reported GSSG levels in white grape juice with different oxygen additions to range from 0.46 mg/L to 2.93 mg/L. Oxidative handling of the juice led to significantly higher GSSG levels. The quantification of GSSG may prove interesting as it could possibly be used as an indicator for oxidation.

Elevated wine GSH levels may indirectly contribute to wine quality. However, the addition of food grade GSH to must or wine is not allowed by current wine regulation (Ugliano *et al*., 2011). There are, however, other permitted additives available on the market which claim to increase wine GSH levels and have positive effects on wine quality, especially in terms of aroma and colour preservation (Pozo-Bayón *et al*., 2009a). These products, referred to as GSH-enriched inactive dry yeast preparations (GSH-IDYs) are incorporated during alcoholic fermentation and claim to increase wine GSH content either by the liberation of GSH into the wine, or by allowing the yeast to assimilate GSH precursors for GSH synthesis during alcoholic fermentation. GSH, which presents more than 95% of the non-protein thiols in *Saccharomyces cerevisiae* (Elskens *et al*., 1991), may account for 0.5 to 1% of the cell dry weight, depending on the growth conditions (Penninckx, 2000). GSH-IDYs are manufactured from the thermal inactivation of S. *cerevisiae* cultivated under specific conditions that stimulate the intracellular accumulation of GSH.

To date, little work has been done on GSH-IDYs and only one study has evaluated the GSH levels released from GSH-IDYs. When added at 0.3 g/L to model wine, Andujar-Ortiz *et al*. (2011) reported the

Stellenbosch University http://scholar.sun.ac.za

GSH levels released from four different GSH-IDYs ranged between 1 and 2 mg/L whereas the total GSH levels ranged between 1.82 mg/L and 2.72 mg/L. Furthermore, the effects of certain wine parameters, such as ethanol and sulphur dioxide (SO_2), on the release of GSH by GSH-IDYs have not been elucidated. Similarly, the optimal addition time of this product during alcoholic fermentation, has not been determined in detail.

In view of the increasing interest in GSH, various analytical methods have been developed for the determination of GSH, GSSG and total GSH in grapes, must and wine. Methods reported in literature include enzymatic methods (Adams & Liyanage, 1991; Cassol & Adams, 1995), high performance liquid chromatography (HPLC) with diode array detection (DAD) (Cheynier *et al*., 1989), HPLC with with fluorescence detection (Park *et al*., 2000b; Marchand & de Revel, 2010; Janes *et al*., 2010) and HPLC with tandem mass spectrometry (du Toit *et al*., 2007). Recently an ultra-high performance liquid chromatography (UPLC) method coupled with a photo array detector (PAD) has also been developed (Fracassetti *et al*., 2011). Lavigne *et al*. (2007) used capillary electrophoresis coupled with laser-induced fluorescence detection and Bramanti *et al.* (2008) atomic absorption spectrometry. Many of the above mentioned methodologies are time-consuming and require extensive sample preparation. The need thus arises for a fast, accurate and robust method requiring minimal sample preparation for the simultaneous determination of GSH and GSSG in grape juice and wine.

The aim of this study was hence to develop a reliable, fast and selective UPLC-MS/MS method for the simultaneous determination of GSH and GSSG in grape juice, must and wine. The method also allows for the simultaneous determination of GSH and GSSG in yeast cells as well as GSH-IDYs. This method was applied to evaluate extracellular GSH and GSSG levels during alcoholic fermentation of a must supplemented with GSH-IDY. In addition, the effects of yeast strain and vine canopy treatments on both extra- and intracellular GSH and GSSG levels in a Sauvignon blanc must during alcoholic fermentation were assessed.

4.2 MATERIALS AND METHODS

4.2.1 UPLC-MS/MS method

UPLC-MS/MS analyses were performed on a Waters Acquity UPLC (Milford, MA) connected to a Waters Xevo triple-quadrupole mass spectrometer using electrospray ionization in the positive mode. Separation was achieved on a Waters Acquity BEH Phenyl column (100 x 2.1 mm, 1.7 µm), using a 0.4% trifluoroacetic acid (Solvent A) to acetonitrile (Solvent B) gradient (**Table 4.1**). The injection volume was 3 µL.

Stellenbosch University http://scholar.sun.ac.za

Table 4.1 Solvents concentration in the eluting gradient; A: trifluoroacetic acid 0.4% (v/v), B: acetonitrile.

Time (min)	Flow (mL/min)	% A	% B
0	0.3	100	0
0.1	0.3	100	0
3.5	0.5	98	2
3.8	0.5	0	100
4	0.5	0	100
4.01	0.3	100	0
5.5	0.3	100	0

The MS settings were optimized for best sensitivity, a Cone voltage of 18V was used for reduced GSH and 20V for oxidized GSSG. Data was acquired in multiple reaction monitoring mode (MRM). A MRM transition of 308.1>179.1 at a collision energy of 17V was used for GSH. A MRM transition of 613.1>355.1 at a collision energy of 20V was used for GSSG. Recoveries were tested in various matrices including white and red grape juice and wine, chemically defined grape juice media, model wine solution and phosphate buffer saline. It was found that a dilution of five times in water was optimal to give the best recoveries and peak shapes.

4.2.2 DETERMINATION OF GSH RELEASED FROM VARIOUS GSH-IDYs

The GSH, GSSG and total GSH levels (GSH + 2 X GSSG as molar equivalents) released into model solution by five different commercial GSH-IDYs were evaluated using a model solution. The GSH-IDYs were supplied by different manufacturers (Lallemand, Laffort, Springer Oenologie and Enartis). The model solution consisted of 5 g/L tartaric acid adjusted to pH 3.3 using 5 M NaOH (Merck Chemicals). N_2 gas (Afrox, South Africa) was bubbled through the model solutions to attain an O_2 concentration < 1 mg/L (NomaSense oxygen meter with dipping probe, Nomacorc). 1 g GSH-IDY was transferred quantitatively into a 100 mL volumetric flask, filled to the mark with model solution and stirred for 10 minutes where after sampling for GSH analyses was done. This experiment was performed in triplicate.

4.2.3 INFLUENCE OF TIME, SO_2 AND ETHANOL ON GSH RELEASE BY GSH-IDYs INTO MODEL SOLUTION

In order to ascertain the effects of sulphur dioxide (SO_2) and ethanol (EtOH) on the release of GSH in model solution (described in section 4.2.2), SO_2 (50 mg/L, $K_2S_2O_5$, EVERINTEC, Italy) and EtOH (12 % v/v, Merck, Darmstadt, Germany) additions were made to the model solution according to **Table 4.2.** 1 g GSH-IDY was transferred quantitatively into a 100 mL volumetric flask, filled to the mark with model solution and stirred for 10 minutes where after the first GSH sample was drawn. The model wines were kept at 20 °C after which additional samples were drawn after 1 hour and 24 hours of contact time. The

Stellenbosch University http://scholar.sun.ac.za

GSH content was determined as mg/L GSH or GSSG released from 0.3 g/L GSH-IDY. This experiment was performed in triplicate for two GSH-IDYs.

Table 4.2 Codes and description of the different model wines prepared.

Code	EtOH (% v/v)	SO_2 (mg/L)
-EtOH/-SO_2	0	0
+EtOH/-SO_2	12	0
-EtOH/+SO_2	0	50
+EtOH/+SO_2	12	50

*Added as tartaric acid

4.2.4 INFLUENCE OF GSH-IDY ADDED AT DIFFERENT FERMENTATION STAGES ON GSH LEVELS IN WINE

4.2.4.1 Small scale wine production

The grapes were grown and processed according to standard viticultural and oenological practices. Settled Sauvignon blanc juice was obtained from KWV cellar, Paarl in 20 litre stainless steel canisters. Upon crushing, a dosage of 50 mg/L SO_2 was added to the juice. The air in the canisters had previously been displaced by CO_2 gas (Afrox SA). Additional CO_2 gas was blown on the headspace to exclude any oxygen contact and the canisters were transported to the Stellenbosch University experimental cellar. Upon arrival at the experimental cellar, standard wine analytical parameters (°Brix, pH, titratable acid as tartaric acid equivalents) were measured using a GrapeScan FT 120 instrument (Foss Electric, Denmark) (Nieuwoudt *et al.*, 2004). Ammonia concentration was quantified using the Glutamate Dehydrogenase Enzymatic Bioanalysis UV-method (Roche, Mannheim, Germany). Free α-amino acid nitrogen was determined by the o-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay described by Dukes & Butzke (1998). YAN is expressed as the sum of the α-amino acid nitrogen (FAN) plus ammoniacal nitrogen. Free and bound SO_2 of the juices were measured using the aspiration method. The values are presented in **Table 4.3**.

Table 4.3 Composition of the Sauvignon blanc must.

Free SO_2 (mg/L)	Total SO_2 (mg/L)	YAN[a] (mg/L)	°Brix	pH	TA[b] (g/L[c])
12.5	44	380	21.8	3.38	6.68

[a] YAN: yeast assimilable nitrogen (ammonia + α-amino acid nitrogen)
[b] Titratable acidity
[c] Tartaric acid equivalents

Stellenbosch University http://scholar.sun.ac.za

The juice was then displaced with CO_2 gas into 2 litre glass bottles to a volume of 1.8 litres per bottle. The glass bottles had been previously filled with water which was then displaced with CO_2 gas (Afrox SA) to achieve inert atmosphere. The free SO_2 concentration of each treatment was adjusted to 30 mg/L by means of 2.5% SO_2 addition ($K_2S_2O_5$ solution, EVERINTEC, Italy) and the SO_2 levels were confirmed by a Metrohm titration unit (Metrohm, Ltd., Switzerland). The juice was then inoculated with *S. cerevisiae* yeast QA23 (Lallemand) at 0.3 g/L rehydrated in GoFerm Protect® (Lallemand) according to the supplier's recommendation. GSH-IDY-4 addition at 0.3 g/L was made to the different treatments as listed in **Table 4.4**. Each bottle was then sealed with a rubber bung equipped with an airlock to release CO_2 during fermentation and then weighed. Fermentation was initiated at room temperature after which the bottles were transferred to a temperature controlled room set at 15°C. FermaidK® yeast nutrient (Lallemand) at 0.25 g/L was added to the fermenting must after 5 °Brix had fermented out. The bottles were weighed each day to follow the progress of fermentation. When mass loss stopped for three consecutive days, GrapeScan FT 120 (Nieuwoudt *et al.*, 2004) results confirmed that all wines were fermented dry to a residual sugar content < 5 g/L. This experiment, all controls and treatments, was performed in triplicate. Samples were taken three times during the course of the experiment; juice samples drawn before inoculation, must samples in the middle of alcoholic fermentation and wine samples after completion of alcoholic fermentation.

Table 4.4 Time of GSH-IDY-4 additions to Sauvignon blanc must during alcoholic fermentation.

Code	Description
Control	No addition made
Juice	Addition made to settled juice directly before inoculation with yeast
1/3	Addition made 1/3 through fermentation (at 14.5 °Brix)
2/3	Addition made 2/3 through fermentation (at 7.3 °Brix)

4.2.4.2 Large scale wine production

Larger scale fermentations were conducted at two different commercial cellars to asses the influence of GSH-IDY additions to musts on GSH levels in wine. The same Sauvignon blanc juice used in the small-scale ferments as described in 4.2.4.1. was used to produce wine on a larger scale at KWV, a commercial cellar in Paarl, South Africa. In the other large scale experiment, conducted at Hartenberg cellar, Stellenbosch, South Africa, Riesling juice was used. 1 600 litre fermentation tanks were used at KWV whereas 10 000 litre tanks were used at Hartenberg cellar. The tanks (control and GSH-IDY treatment) were filled alternately with the settled juice to ensure homogeneity. The Sauvignon blanc and Riesling

Stellenbosch University http://scholar.sun.ac.za

juices were inoculated with 0.3 g/L *S. cerevisiae* yeast QA23 and D254 (Lallemand), respectively, rehydrated in GoFerm Protect® (Lallemand) according to the supplier's recommendations. GSH-IDY-4 addition at 0.3 g/L was made to the GSH-IDY treatment a third into alcoholic fermentation (corresponding to "1/3" as listed in **Table 4.4**). FermaidK® yeast nutrient (Lallemand) at 0.25 g/L was added to the fermenting must after 5 °Brix had fermented out. Due to capacity constraints of the commercial wineries, the large scale fermentations were not replicated. Three samples were taken during the course of the experiment; juice samples drawn before inoculation, must samples in the middle of alcoholic fermentation and wine samples after completion of alcoholic fermentation.

4.2.5 INFLUENCE OF YEAST STRAIN AND CANOPY TREATMENTS IN VINEYARD ON EXTRA- AND INTRACELLULAR GSH LEVELS DURING ALCOHOLIC FERMENTATION

4.2.5.1 Vineyard experimental design

This trial was conducted in 2011 on a commercial Sauvignon blanc (*Vitis vinifera* L.) block located in the Overberg region (Western coastal area, South Africa). The vines (clone 316 grafted on rootstock 101.14 Ruggeri) were planted in 2004. The row direction was NW-SE (2.8m x 1.5m). The training system was the vertical shoot positioned (VSP) trellis system (2 levels of movable wires) and the pruning system the split cordon. Two canopy treatments were applied to the Sauvignon blanc: 100 % exposed bunches morning side and 100 % shaded bunches. A total leaf and lateral removal in the bunch zone (40 cm height from the cordon) was applied from 17 December 2010 at (E-L 29). The shaded treatment was a permanent thick canopy at the bunch zone during the berry growth and ripening. The experimental plot was laid out in a randomized block design. The experiment was designed over 5 rows with 4 replicates of 5 vines per treatment and per row. Only fully exposed and fully shaded bunches were collected from the respective treatments and used to produce wine from.

4.2.5.2 Fermentation conditions

Both treatments were hand-harvested on 1 March 2011 at ±23 °Brix. The yields for the two treatments were 203.6 kg and 350.9 kg for the exposed and shaded treatments, respectively. Grapes of the two treatments were de-stemmed and crushed separately. Thirty mg/L SO_2 was added to the grape mass by means of 2.5% SO_2 addition ($K_2S_2O_5$, EVERINTEC, Italy). The grapes were pressed to 0.5 bars and the juice collected in containers previously lined with dry ice (Sidewinder, South Africa). Pectolytic enzyme (Rapidase® Vino Super, DSM Oenology) was added at 0.03 mL/L to the juice, the headspace of the container filled with CO_2 gas (Afrox, South Africa), sealed and left to settle overnight at 4 °C. The clear juice was racked into a container under CO_2 gas, mixed homogenously and divided into 20 litre stainless steel canisters (which had been previously filled with CO_2 gas) to a volume of approximately 15 litres and

Stellenbosch University http://scholar.sun.ac.za

closed with an airlock. The °Brix and free SO_2 levels were measured at this stage. The sugar content for the exposed and shaded treatments was 23.5 and 22.7 °Brix, respectively, with the free SO_2 level being 25 mg/L for both treatments. The juice was then inoculated with *S. cerevisiae* VIN7 (Anchor) and Cross Evolution (Lallemand) at 0.3 g/L according to the suppliers' recommendations and fermentations were performed at 15 °C. Diammonium phosphate at 0.3 g/ L was added to the fermenting must after 5 °Brix had fermented out. GrapeScan FT 120 analyses of the wines confirmed the completion of alcoholic fermentation, with residual sugar concentration of all the treatments being lower than 2 g/L. The wine was racked off the lees with CO_2 gas, an addition of 40 mg/L SO_2 was made to the wine after which it was cold stabilized at -4 °C for 1 week. All the wines were bottled under CO_2 gas in green 750 mL bottles and sealed with screw caps. Juice and wine samples for the determination of GSH and GSSG were drawn five times during alcoholic fermentation corresponding to 0%, 25%, 50%, 75% and 100% completion of fermentation in terms of sugar loss. Yeast samples for the determination of intracellular GSH and GSSG were drawn four times during alcoholic fermentation corresponding to 25%, 50%, 75% and 100% completion of fermentation in terms of sugar loss.

4.2.6 SAMPLING PROCEDURE, SAMPLE PREPARATION AND GSH ANALYSIS

4.2.6.1 Model solution, juice and wine sampling

Samples destined for GSH analyses were drawn at the stages described in sections 4.2.4.1, 4.2.4.2 and 4.2.5.2. This was done by transferring the required volume under CO_2 gas into plastic sampling bottles which had been previously filled with CO_2 gas. Samples in section 4.2.3 and 4.2.4 were drawn with a syringe through the rubber septum under CO_2 backflow. Prior to sampling, 1000 mg/L SO_2 ($K_2S_2O_5$, EVERINTEC, Italy) and 500 mg/L ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) additions were made to the sampling bottles in order to prevent oxidation. Additional CO_2 gas was blown into the sampling bottle's headspace and then immediately frozen at -20 °C until sample analyses. Sampling in the commercial cellars was done in exactly the same manner as described above with the exception that 500 mL samples were directly collected from the tanks using the sampling taps.

4.2.6.2 Sample preparation for extracellular GSH analysis

Samples were thawed on the day of analyses and 2 mL centrifuged (Centrifuge 5415 D, Eppendorf, Hamburg, Germany) at 12 800 rpm for 5 minutes at 20°C. The supernatant was diluted five times with HPLC grade water (Millipore Filter Corp., Bedford, MA, USA) supplemented with 1000 mg/L SO_2 (EVERINTEC, Italy) and a freshly prepared 500 mg/L ascorbic acid solution (Sigma-Aldrich, St. Louis, MO, USA) to protect the sample against oxidation. The samples drawn to determine the GSH and GSSG

Stellenbosch University http://scholar.sun.ac.za

content released from the GSH-IDYs were centrifuged immediately (Centrifuge 5415 D, Eppendorf, Hamburg, Germany) at 12 800 rpm for 5 minutes at 20 °C. The supernatant was also diluted five times with HPLC grade water containing SO_2 and ascorbic acid as described above.

4.2.6.3 Sample preparation for intracellular GSH analysis

Must and wine samples for the determination of intracellular GSH content were drawn in the following manner. The musts were mixed homogenously by sparging the canisters with N_2 gas (Afrox, SA). A 2 mL aliquot was drawn and centrifuged (Hermle Z233 M-2, Germany) at 5 000 rpm for 5 minutes. The supernatant was discarded and the pellet washed with 1 mL of a 1 M EDTA and 20 mM Tris (pH 8.5) solution. The washed cells were resuspended in PBS (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl and 2.0 mM KCl, pH 7.4) containing 10% metaphosphoric acid (Sigma-Aldrich, St. Louis, MO, USA) and disrupted with glass beads (500 µm diameter) for 20 minutes at 8 °C. Cell debris was removed by centrifugation (12 000 rpm, 10 minutes, 12°C). Glutathione concentration was determined in the supernatant and expressed as mg GSH (or GSSG) per g cells (as wet weight). The supernatant was not diluted but injected directly.

4.2.7 STATISTICAL ANALYSES

All analyses were done using Statistica V. 10 software (Statsoft Inc., Tulsa, OK). One-way ANOVA's , three-way ANOVA's and mixed model repeated measures ANOVA's were used for statistical analyses. Significant differences were judged on a 5% significance level ($p<0.05$).

4.3 RESULTS AND DISCUSSION

4.3.1 UPLC-MS/MS METHOD

Compared to conventional HPLC, UPLC offers improved sensitivity, speed and resolution for analytical determinations, particularly when coupled with mass spectrometers capable of high-speed acquisitions (Churchwell *et al*., 2005). UPLC-MS/MS chromatograms for GSH and GSSG standards are shown in **Figure 4.1.** The elution times were 2.1 and 1.65 min for GSSG and GSH, respectively.

Stellenbosch University http://scholar.sun.ac.za

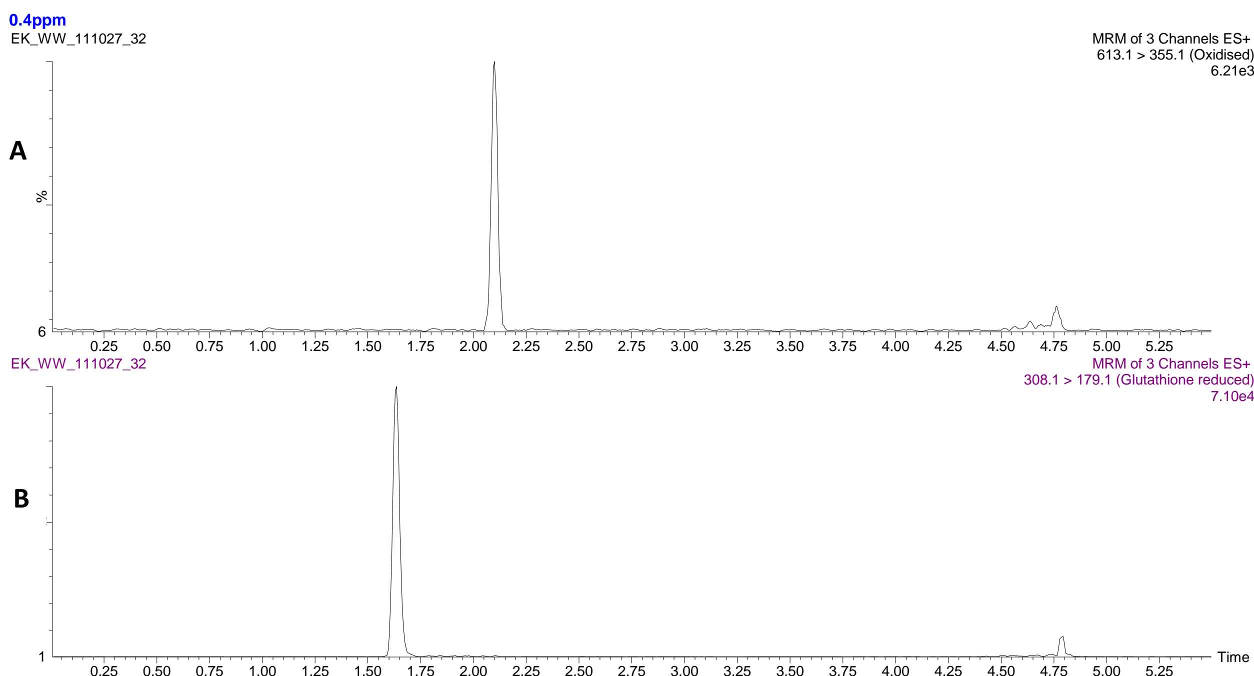


Figure 4.1 UPLC-MS/MS chromatograms of (A) GSSG (2.10 min) and (B) GSH (1.65 min) standards.

Recoveries for GSH and GSSG were tested in various matrices including white and red grape juice and wine, chemically defined grape juice media and phosphate buffer saline and were proven to be better than 80%.

The limit of detection (LOD) for GSH was 0.002 mg/L whereas the limit of quantification (LOQ) was 0.01 mg/L. The LOD and LOQ of this method for GSSG were 0.001 mg/L and 0.005 mg/L, respectively. LOD was taken at a signal to noise of 3 and LOQ at a signal to noise of 10. Du Toit *et al*. (2007) reported a LOD for GSSG of 0.2 mg/L in grape juice and wine whereas Marchand & de Revel (2010) reported a LOD of 2.45 mg/L in white wine and 3.06 mg/L in red wine.

The method yielded linear responses up to 20 mg/L and 40 mg/L for GSSG and GSH, respectively. Taken into consideration the five fold dilution, these are improved concentration ranges compared to those previously reported (Park *et al*., 2000b; Marchand & de Revel, 2010; Fracassetti *et al*., 2011). The rapid sample preparation which involves a quick centrifugation step followed by dilution allowed GSH and GSSG to be quantified in less than 8 minutes. This analysis time can even be shortened if the sample is microfiltered (0.22 µm, PVDF, Millipore) in stead of centrifuged for 5 minutes.

This method represents significant advantages over other methods for the determination of GSH and GSSG in grape juice and wine. Firstly, the method allows for the direct and simultaneous quantification of GSH and GSSG without the need of derivatization or a reduction step to quantify GSSG. No sample preparation is required apart from a rapid centrifugation step and dilution. Furthermore, to our knowledge, this method currently has the shortest analysis time for the quantification of GSH and

Stellenbosch University http://scholar.sun.ac.za

GSSG in juice and wine samples. In view of the increasing environmental pressure, the reduction in solvent generation with this technique is thus another advantage.

4.3.2 DETERMINATION OF GSH RELEASED FROM VARIOUS GSH-IDYs

The GSH and GSSG contents in this section are reported as a 0.3 g/L GSH-IDY addition in order to compare the data with that and the Sauvignon blanc trial in section 4.2.4 and the study by Andujar-Ortiz *et al.* (2011) where the same dosage was used. Significant differences ($p<0.05$) in the amount of GSH released by the different GSH-IDYs, can be seen in **Figure 4.2.** The GSH released by the various preparations ranged between 1.45 mg/L and 2.53 mg/L, which corroborates with data by Andujar-Ortiz *et al.* (2011) whom reported four GSH-IDY preparations to release between 1 mg/L and 2 mg/L GSH into synthetic wine solutions. These authors proposed that differences in the amounts released might be attributed to differences in the manufacturing processes among the preparations, especially in terms of the nutrients provided during the growth of the yeast culture. Several cultivation strategies to optimize the fermentative production of GSH by *S. cerevisiae* have been reported in literature (Li *et al*., 2004), with cysteine being established as a key amino acid addition for GSH production (Alfafara *et al*., 1992; Wen *et al*., 2004; Wang *et al*., 2007; Li *et al*., 2004). The possibility that strain differences could also have been accountable for the differences observed in the amount of GSH released, should not be neglected. Furthermore, thermal damage during the drying process may also reduce the GSH content present in GSH-IDYs (Tirelli *et al*. 2010; Andujar-Ortiz *et al.,* 2011) which may also account for the large variation reported for the GSSG contents (0.04 mg/L to 0.88 mg/L GSSG).

Stellenbosch University http://scholar.sun.ac.za

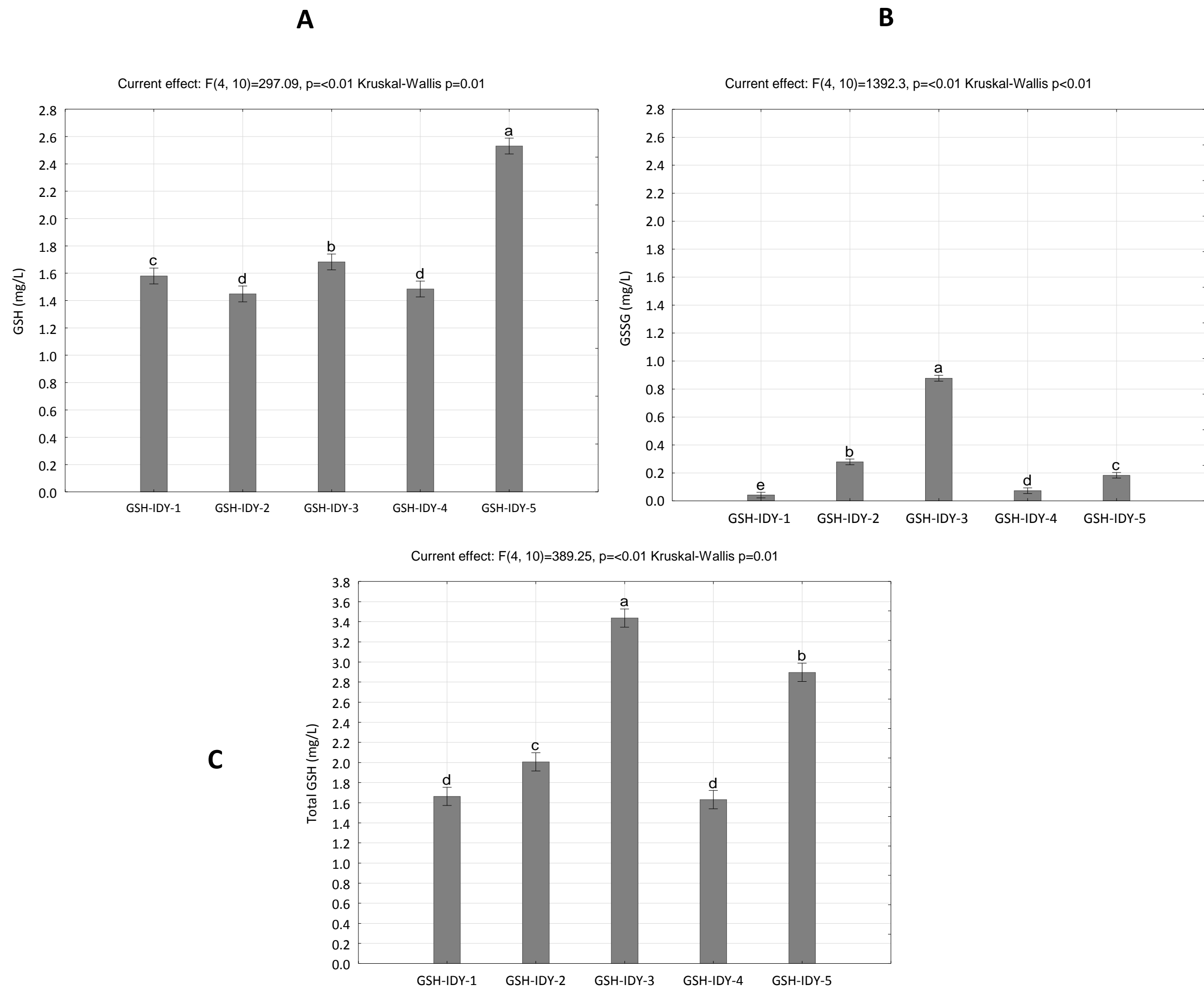


Figure 4.2 (A) Reduced (GSH), (B) oxidized (GSSG) and (C) total glutathione content released by various GSH-IDYs. Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% significance level.

The total GSH contents released, ranged from 1.63 mg/L to 3.44 mg/L which is also similar to results by Andujar-Ortiz *et al.* (2011) reporting total GSH levels in the range of 1.82 mg/L to 2.72 mg/L. GSH-IDY-3 displayed the highest total GSH content, however, the high GSSG content of this product attributed to the high total GSH observed. This data illustrates the variation that exists among GSH-IDYs in terms of GSH content. Moreover, it shows the importance to differentiate between GSH and total GSH contents since it is the reduced form that is the active antioxidant in wine.

No literature could be found on the industrial preparation of GSH-IDYs, and it is not known whether exogenous GSH enrichment is allowed during the manufacturing process, although it has been previously proposed by Tirelli *et al*. (2010). From this data it can be deducted that the production process of GSH-IDY-5 is optimized to such an extent that it results in a considerably higher GSH content.

Stellenbosch University http://scholar.sun.ac.za

That enrichment with exogenous GSH occurred during the manufacture of this product can only be speculated. Nevertheless, this product could likely be more efficient in reducing the oxidation phenomena in wines when compared to the other GSH-IDYs in this study. However, further investigation to establish the effectiveness of the GSH released by these preparations in inhibiting wine oxidation, is necessary.

4.3.3 INFLUENCE OF TIME, SO_2 AND ETHANOL ON GSH RELEASE BY GSH-IDYs INTO MODEL WINE SOLUTION

Figure 4.3 presents the levels of GSH released into a model solution for the respective treatments (with or without SO_2/EtOH) 10 minutes after the addition of the GSH-IDY. The conditions in the model solution in terms of O_2 concentration, pH, tartaric acid, ethanol and SO_2 content were manipulated to simulate grape juice or wine (depending on the ethanol content present). This was done in order to establish the effect of ethanol and SO_2 on GSH release under grape-juice or wine-like conditions. SO_2, being an antioxidant, may impede the oxidation of GSH, whereas ethanol might influence the solubility or extraction of GSH into the model solution. It is evident that, irrespective of the treatment, GSH-IDY-5 released significantly more GSH into the model solution compared to GSH-IDY-4. It seems as if though SO_2 addition to the model solution resulted in slightly higher released GSH values, although the differences were not in all cases significant. In the absence of ethanol, GSH-IDY-5 released significantly more GSH in the -EtOH/+SO_2 treatment compared to the -EtOH/-SO_2 treatment. Equal amounts of GSH were released by GSH-IDY-5 in the -EtOH/-SO_2, +EtOH/-SO_2 and +EtOH/+SO_2 treatments. Exactly the opposite was observed in the case of GSH-IDY-4 where SO_2 addition did not result in significantly higher GSH values in the treatments lacking ethanol. Instead, it was in the ethanol containing treatments where significantly more ($p<0.05$) GSH was released in the +EtOH/+SO_2 treatment, compared to the +EtOH/-SO_2 treatment. However, the GSH content of the +EtOH/+SO_2 treatment did not differ significantly from the -EtOH/-SO_2 and -EtOH/+SO_2 treatments.

Theoretically speaking, SO_2, being an antioxidant, may indirectly protect GSH against oxidation, by reacting with oxidants such as H_2O_2, capable of oxidizing GSH, but whether this happened under our conditions is unclear. However, this protective effect was only observed in the absence of ethanol for GSH-IDY-5 and in the presence of ethanol for GSH-IDY-4 . It is thus evident that the two products behave differently in response to ethanol and SO_2 additions. However, since no significant difference in released GSH could be observed between the -EtOH/+SO_2 and +EtOH/+SO_2 treatments for GSH-IDY-4, the effect of ethanol on GSH release in the short term seems insignificant. The GSSG levels were all below 0.2 mg/L, consistent with data for GSH-IDY-4 and GSH-IDY-5 in **Figure 4.2,** with no significant differences observed between the different treatments (results not shown). The significant lower GSH

Stellenbosch University http://scholar.sun.ac.za

contents for GSH-IDY-5 in the -EtOH/-SO_2 treatment and GSH-IDY-4 in the +EtOH/-SO_2 treatment were thus not associated with higher GSSG contents, suggesting that oxidation was not accountable for the lower GSH values obtained. Consequently, it is postulated that the extraction of GSH from the GSH-IDYs was affected in these two treatments, leading to lower GSH values compared to the other treatments.

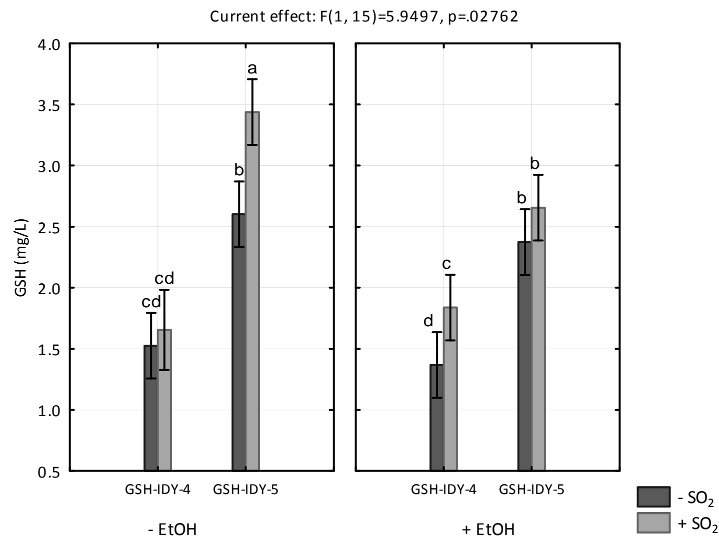


Figure 4.3 GSH content released into model solution 10 minutes after the addition of the GSH-IDYs. Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% significance level.

Interestingly, when a time factor was introduced into the experiment by analysing the GSH content released by GSH-IDYs after one hour and 24 hours of contact time, the effect of SO_2 had no significant influence on GSH levels (results not shown). **Figure 4.4** thus only shows the effect of ethanol and GSH-IDY. With a longer contact time, the GSH-IDYs might have released soluble compounds such as cysteine with reducing properties, which might have overshadowed the protective effect of SO_2 on GSH. Tirelli *et al*. (2010) reported some IDY preparations to contain sufficient amounts of reduced protein cysteine (RPC) to serve as effective antioxidant. However, in another study by Pozo-Bayón *et al*. (2009b) which investigated the soluble compounds released into a model solution by IDY preparations, no mention was made of cysteine and uncertainty remains whether it was not detected or simply not measured. Nevertheless, over a longer contact time, ethanol seems to have had a greater effect on GSH release by the GSH-IDYs, especially in the case of GSH-IDY-5. The GSH contents for GSH-IDY-4 stayed constant in the 1 hour of the experiment with no significant differences observed between the treatment lacking ethanol and the treatment containing ethanol. Interestingly, a significant difference ($p<0.05$) in released GSH content could be observed after 24 hours of contact time with the ethanol containing treatment displaying a higher GSH content compared to the treatment lacking ethanol.

Stellenbosch University http://scholar.sun.ac.za

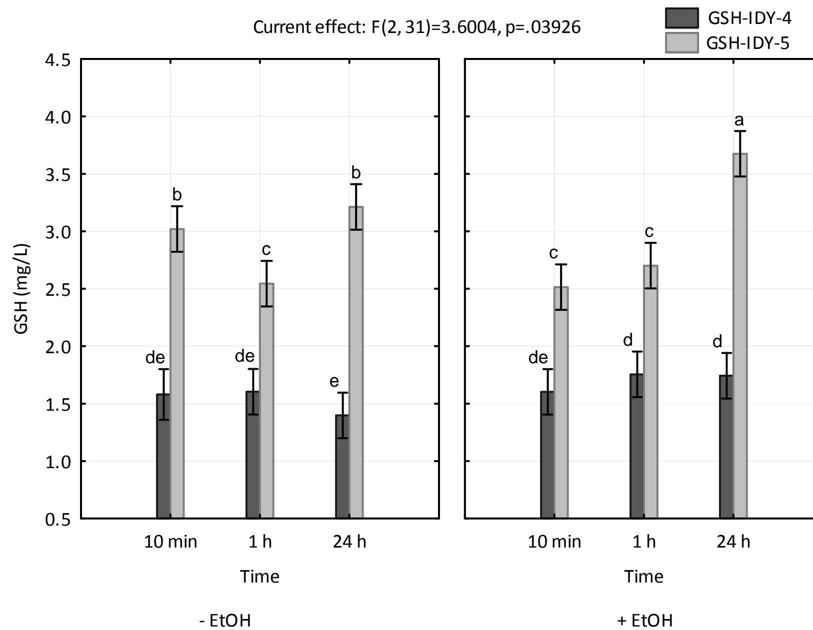


Figure 4.4 GSH content released into model solution 10 minutes, 1 hour and 24 hours after the addition of the GSH-IDYs. Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% significance level.

In the case of GSH-IDY-5, fluctuating GSH contents were observed over the course of the experiment and the contents differed significantly ($p<0.05$) between the treatment containing ethanol and the treatment lacking ethanol. After 10 minutes of GSH-IDY addition, significantly lower amounts of GSH were released into the treatment with ethanol compared to the treatment without ethanol. However, after one hour of contact time, the GSH content of the treatment lacking ethanol had significantly decreased whereas that of the treatment containing ethanol, remained constant. After 24 hours of contact time, the GSH values increased for both treatments, but the GSH content of the ethanol containing treatment was significantly ($p<0.05$) higher than the treatment without ethanol, similar to results obtained for GSH-IDY-4. Moreover, the GSH content for the treatment without ethanol only increased to the level initially present after 10 minutes of its addition whereas that of the ethanol containing treatment, increased to levels significantly higher than initially present. It thus seems as if ethanol may rather have a stimulatory effect on GSH release when GSH-IDYs are subjected to longer contact times.

The GSSG contents of the respective treatments again were all below 0.2 mg/L and did not increase or decrease during the course of the experiment (results not shown). The reduction in the GSH content for the treatment lacking ethanol, could thus not be attributed to the oxidation of GSH to GSSG. The possibility that the GSH was temporarily bound by another substance in the GSH-IDY and later released, should not be excluded. This is partially supported by work done by Andujar-Ortiz *et al.* (2011) who

Stellenbosch University http://scholar.sun.ac.za

reported that the amount of GSH released into a model wine solution remained quite stable over a nine day period whereas a slight reduction was observed for the total GSH content. Nevertheless, this study provides the first preliminary results on the effects of SO_2 and ethanol on GSH release by GSH-IDYs.

4.3.4 INFLUENCE OF GSH-IDY ADDITIONS AT DIFFERENT FERMENTATIONS STAGES ON GSH LEVELS IN WINE

4.3.4.1 GSH levels for small scale fermentation

All treatments achieved dryness (residual sugar content < 5 g/L). Supplementation with GSH-IDY preparation had no effect on fermentation rate (results not shown), despite possibly contributing to yeast nutrients in the form of amino acids and peptides. **Figure 4.5** presents the GSH concentrations for the different treatments during alcoholic fermentation. It is evident that the GSH content increased during fermentation, regardless of the treatment applied. Although the interaction between the time of GSH-IDY supplementation and time during fermentation is not supported by a p-value <0.05, important trends regarding the influence of the treatments on the GSH content can still be seen which will be supported by literature. From this data it seems as if the "juice" and "1/3" treatment resulted in higher GSH contents after fermentation compared to the control and "2/3" treatment. The final GSH concentrations for the "juice" and "1/3" treatments were 59.9 mg/L and 58.5 mg/L, respectively, whereas those of the control and "2/3" treatment were 51.6 mg/L and 51.8 mg/L. The difference between the GSH content of the control and "juice" or "1/3" treatment was thus approximately 7-8 mg/L. This was quite intriguing, taken into consideration the amount of GSH released from GSH-IDY-4 into the model solution as determined in sections 4.3.2 and 4.3.3 (**Figure 4.2 and 4.3**), which was approximately 1.6 mg/L.

Andujar-Ortiz *et al.* (2011) analyzed the nitrogen contents of a control and GSH-IDY supplemented wine which revealed considerably higher peptide and amino acid contents for the GSH-IDY wine. Similarly, Pozo-Bayón *et al*. (2009b) showed that numerous soluble nitrogenous compounds were released by inactive dry yeast preparation when tested in model wine solutions, some having a stimulating effect on GSH synthesis by the yeast (Wen *et al*., 2004; Andujar-Ortiz *et al.*, 2011). This led to the hypothesis that the nutrients provided by the GSH-IDY preparation (free amino acids, peptides ect) supplemented at the onset or after a third of alcoholic fermentation had been completed led to increased GSH synthesis and subsequent release by the yeast compared to the control and "2/3" treatment. The fact that the GSH content of the "2/3" treatment did not differ from the control may have been due to the fact that the supplementation was made too late during alcoholic fermentation for the yeast to benefit from the increased nutrients to synthesize and release GSH. Indeed, ethanol

Stellenbosch University http://scholar.sun.ac.za

inhibits the hydrogen ion-coupled import of amino acids (Bisson, 1996) and it is likely that the yeast cells were unable to assimilate the amino acids provided by the GSH-IDY to produce and secrete GSH at such a late stage during alcoholic fermentation. GSH-IDY-4 released approximatley 1.6 mg/L GSH in model solution in section 4.3.2 and 4.3.3. However, the difference in GSH concentration between the control and "2/3" treatment was not significantly different.

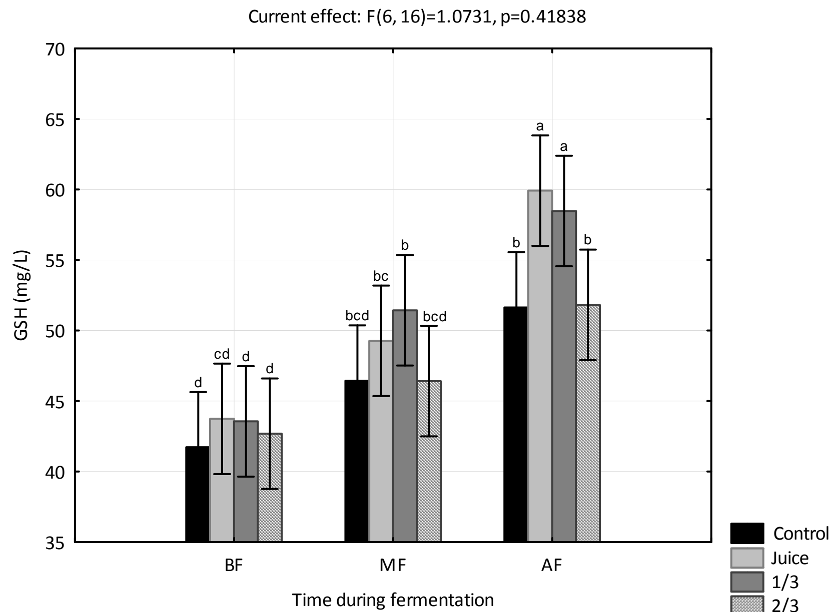


Figure 4.5 Reduced glutathione (GSH) evolution during alcoholic fermentation for Sauvignon blanc juice supplemented with GSH-IDY-4 at different stages during fermentation. Vertical bars denote 95% confidence interval for the means.

Another explanation for the increased GSH levels observed for the "juice" and "1/3" treatment is the preferential uptake of the nutrients provided by the GSH-IDY preparation over GSH early during fermentation, which resulted in higher GSH levels at the end of alcoholic fermentation. The lack of nutrient enrichment during the early stages of fermentation in the control and "2/3" treatment may have caused the uptake of GSH by the yeast with the consequent decrease in GSH levels in the must and/or wine.

Nevertheless, this study shows that it is not necessarily the GSH content released from in the specific GSH-IDY that leads to increased GSH levels in wine. It seems that it is rather a function of addition time permitting the uptake of certain nutrients by the yeast which leads to increased GSH levels in wine. Whether this is as a result of increased GSH synthesis and secretion by the yeast or preferential uptake of the nutrients over GSH, still remains unclear and further investigation is thus necessary. The current recommended supplementation time of the specific GSH-IDY is a third into alcoholic fermentation. We have shown that its supplementation to juice directly after yeast inoculation

Stellenbosch University http://scholar.sun.ac.za

resulted in equal wine GSH content compared to the "1/3" treatment. This could simplify the winemaking process in the sense that the winemaker does not have to monitor fermentations especially for the addition of the GSH-IDY at the correct stage. The yeast inoculation and addition of GSH-IDY can thus be made consecutively with the GSH-IDY that we investigated.

4.3.4.2 GSSG levels for small scale fermentation

Figure 4.6 presents the GSSG values during alcoholic fermentation for the different treatments. Again, the interaction between the stage of GSH-IDY supplementation and sampling stage during fermentation is not supported by a p-value < 0.05 and consequently, only trends can be discussed. The GSSG concentration was initially very low (<0.7 mg/L), which is in agreement with other studies. Du Toit *et al*. (2007) reported GSSG levels in reductively treated Sauvignon blanc juice to range from 0.46 mg/L to 1.43 mg/L whereas Okuda & Yokotsuka (1999) measured GSSG levels below 1 mg/L in a Koshu grape juice at the onset of fermentation.

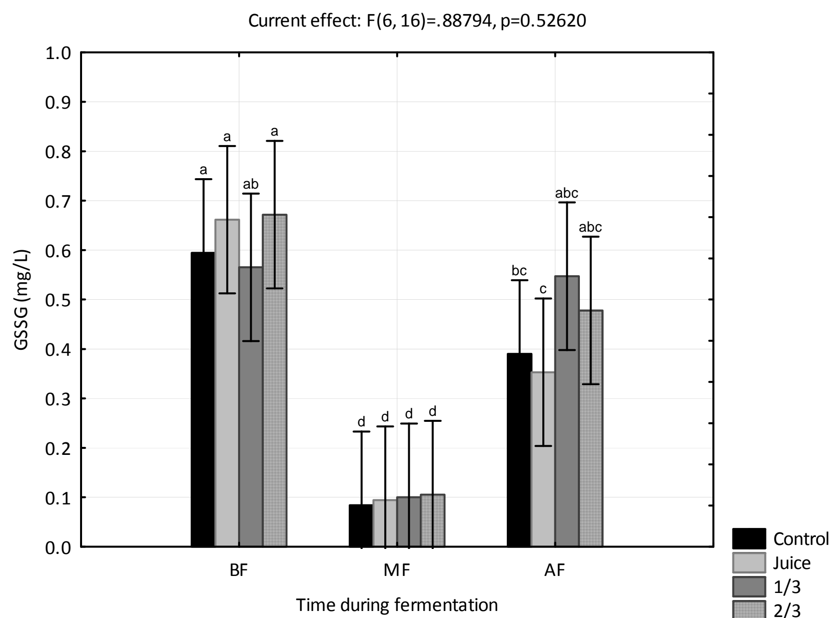


Figure 4.6 Oxidized glutathione (GSSG) evolution during alcoholic fermentation for Sauvignon blanc juice supplemented with GSH-IDY-4 at different stages during fermentation. Vertical bars denote 95% confidence interval for the means.

At the middle of fermentation, the GSSG levels for all the treatments had decreased to approximately 0.1 mg/L. At the end of fermentation it had increased again to reach levels ranging from 0.35 mg/L to 0.56 mg/L. Early work done by Cassol & Adams (1995) suggested that glutathione is present in white wine as the disulfide, GSSG. However, it seems as if though GSSG levels remain very low during alcoholic fermentation. This is supported by Lisjak (2007) who reported that GSSG levels in Sauvignon

Stellenbosch University http://scholar.sun.ac.za

blanc and Colombar wines were below the limit of detection of 0.2 mg/L for the LC-MS/MS method used. Although GRP was not measured during this study, it has been shown by other authors that GRP levels did not vary drastically during alcoholic fermentation (Fracassetti, 2010). Low oxygen levels and inhibition of polyphenol oxidase with SO_2 inhibits the formation of GRP to a large extent (Singleton *et al.*, 1985; du Toit *et al.*, 2006). Several measures were also taken to prevent the formation of GRP and the subsequent loss in GSH. The juice was treated very reductively and the SO_2 levels were also adjusted to 30 mg/L free and 60 mg/L total SO_2. Dubernet & Ribéreau-Gayon (1973) reported that the addition of 25 to 75 mg/L SO_2 to clarified juices led to inhibitions of 75% to 97% in polyphenol oxidase activity. Andujar-Ortiz *et al.* (2011) observed no significant differences in GSH content after alcoholic fermentation between a control and GSH-IDY supplemented wine. However, the total GSH for the GSH-IDY supplemented wine was double that of the control wine (16 mg/L compared to 8 mg/L). Although the GSSG content of the wines were not measured, the authors explained this large difference in GSH by a rapid oxidation of GSH to GSSG. This is in contrast to our findings which show that GSH-IDY supplementation to juice or fermenting must does not lead to severely elevated GSSG levels compared to the control treatment. Alcoholic fermentation is normally a reductive environment, with little oxygen coming into contact with the wine, which could explain the low GSSG levels observed in our study.

4.3.4.3 GSH levels for large scale fermentations

Due to capacity constraints of the commercial wineries, the large scale fermentations were not replicated (**Figure 4.7**). These data should therefore only be viewed as preliminary results. The addition of GSH-IDY-4 to the respective juices was done according to manufacturers' recommendations i.e. after a third of alcoholic fermentation has been completed. Juices supplemented with GSH-IDY-4 resulted in higher wine GSH contents compared to the control wine. This is contrary to data by Andujar-Ortiz *et al*. (2011) who found no statistical difference in GSH between a control and GSH-IDY supplemented commercially manufactured Rosé wine.

Stellenbosch University http://scholar.sun.ac.za

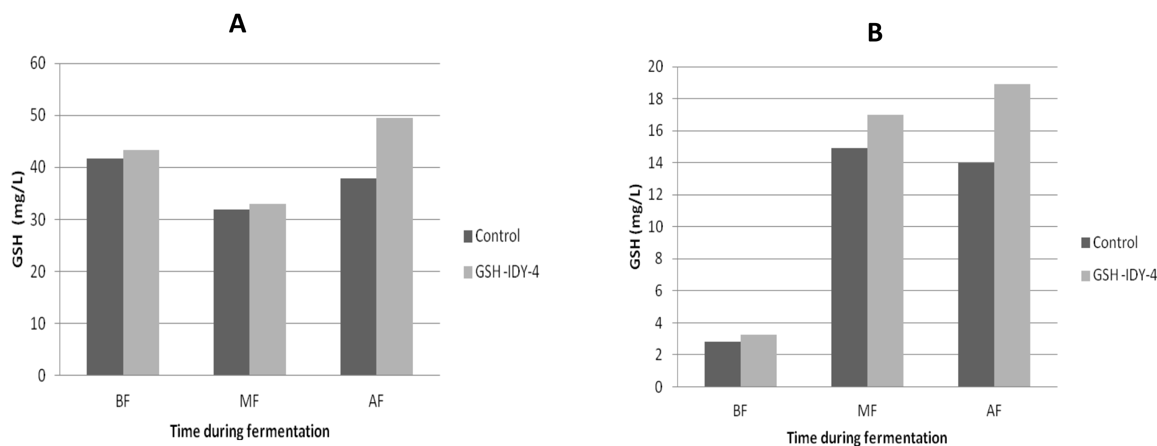


Figure 4.7 GSH contents of commercial scale ferments for (A) Sauvignon blanc and (B): Riesling supplemented with GSH-IDY-4 at 30 g/hL a third into alcoholic fermentation.

The GSH content of the Sauvignon blanc must was initially 42 mg/L, similar to the GSH contents of the small scale fermentations. However, contrary to the increased levels observed at the middle of fermentation in the small scale fermentations compared to the juice GSH levels, the GSH content of the larger scale ferments were approximately 10 mg/L lower. The final GSH content for the control wine was 37 mg/L whereas that of the wine to which GSH-IDY was added had a GSH concentration of 49 mg/L. The GSH content at the end of alcoholic fermentation for the small and large scale ferments of the same juice differed by almost 10 mg/L. Various factors in the commercial scale ferments could have possibly influenced the GSH concentration, for instance higher oxygen exposure, fermentation temperature differences and higher pressure values in larger tanks.

Nevertheless, it is evident that supplementation with GSH-IDY resulted in higher GSH contents after alcoholic fermentation. The same observation in terms of increased GSH levels was made in the case of the Riesling must supplemented with GSH-IDY. Interestingly, the low initial GSH content of the Riesling must of approximately 3 mg/L increased considerably during alcoholic fermentation to reach a concentration of 14 mg/L and 19 mg/L for the control and supplemented wine, respectively. It is thus clear from this data that supplementation of must with GSH-IDY early during alcoholic fermentation leads to increased GSH levels in the resulting wines.

The proposed stimulating effect of amino acids on GSH synthesis by the yeast under winemaking conditions with the subsequent increase in wine GSH levels should be further examined. Although several authors have used amino acid addition strategies to enhance GSH production in *S. cerevisiae* (Wen *et al*., 2004; Wang *et al*., 2007), the GSH produced had always been in the intracellular form. Even

Stellenbosch University http://scholar.sun.ac.za

though Gex1, a GSH/proton antiport transporting GSH to the extracellular medium, has recently been identified in *S. cerevisiae* (Dhaoui *et al.*, 2011), little or no information exists as to how and under which conditions GSH is secreted by yeast. The exact mechanism by which GSH-IDY preparations leads to increased GSH levels in wine thus needs to be elucidated.

4.3.5 INFLUENCE OF YEAST STRAIN AND CANOPY TREATMENTS IN VINEYARD ON EXTRA- AND INTRACELLULAR GSH LEVELS DURING ALCOHOLIC FERMENTATION

Figure 4.8 shows the duration of fermentation for the different treatments. The fermentation rate of the different treatments differed slightly. Cross Evolution fermented slightly faster than VIN7, while the duration of fermentation was also longer for the exposed treatments, which can be attributed to the higher reduced sugar content of the must originating from the exposed treatments.

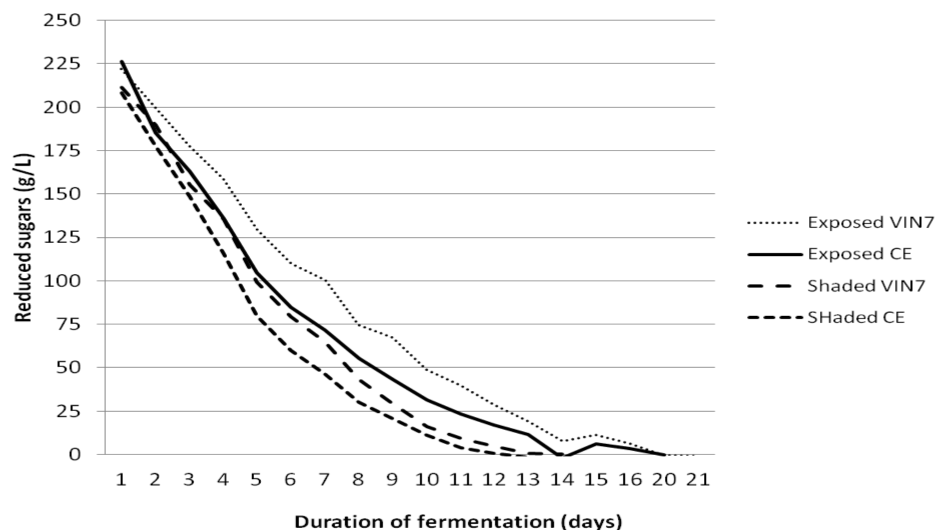


Figure 4.8 Consumption of reduced sugars during alcoholic fermentation for exposed and shaded treatments fermented with strains Cross Evolution and VIN7.

4.3.5.1 Extracellular GSH levels

The evolution of GSH during alcoholic fermentation for the different treatments is shown in **Figure 4.9**. The average GSH content of the juice for the exposed and shaded treatments was 75 mg/L and 70 mg/L, respectively. Such high levels have been reported before in grape juice that had been crushed and treated reductively (Cheynier *et al*., 1989; du Toit *et al*., 2007).

Stellenbosch University http://scholar.sun.ac.za

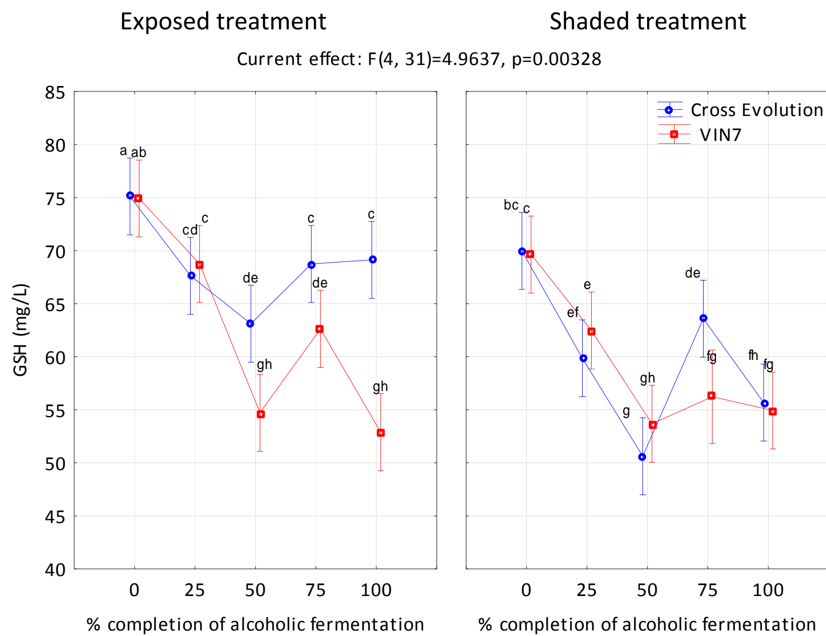


Figure 4.9 GSH evolution during alcoholic fermentation for different yeast strains and different canopy treatments. Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% significance level.

The average °Brix for the juice of the exposed and shaded treatment was 22.4 and 21, respectively. The leaf removal treatment (exposed treatment) thus resulted in berries with a higher soluble solid content, consistent with findings by Bledsoe *et al*. (1988). It thus seems as if the higher soluble solids content of the juice from the exposed treatment was correlated with a higher GSH content. Adams & Liyanage (1993) and Okuda & Yokotsuka (1999) have shown that an increase in soluble solids in grape berries is accompanied by an increase in GSH content. However, this correlation only persisted until the berries reached 16 °Brix, where after the GSH content stabilized. Our results suggest that higher sun exposure in the vineyard might lead to increased GSH accumulation in the berries, but this postulation necessitates further investigation. Nevertheless, this is the first time GSH juice content in relation to a leaf removal treatment in the vineyard, is reported.

The extracellular GSH content differed significantly ($p<0.05$) during alcoholic fermentation for the different yeast strains and canopy treatments applied. During the first half of alcoholic fermentation, a decrease in GSH content was observed for all the treatments. However, the GSH content of the juice from of the exposed treatment, fermented with Cross Evolution, did not decrease to the same extent as the other treatments. Extracellular GSH content then increased, with differences again observed between treatments. In the last quarter of fermentation, a decrease in GSH content was again observed in the exposed VIN7 and shaded Cross Evolution treatments. Interestingly, the GSH content of the juice

Stellenbosch University http://scholar.sun.ac.za

fermented with Cross Evolution in the exposed treatment stayed constant, resulting in the highest GSH content after alcoholic fermentation. No significant differences in GSH content could be observed between the other treatments. The GSH evolution thus differed according to the yeast strain used and juice composition. A possible explanation for this is the different behaviour of the same yeast in the different conditions of the two juices (higher sugar content etc), possibly influencing the GSH metabolism on intracellular level which was then also expressed in the extracellular medium.

4.3.5.2 Extracellular GSSG levels

The evolution of extracellular GSSG during alcoholic fermentation for the different treatments is shown in **Figure 4.10**. The extracellular GSSG levels were initially very low (<0.2 mg/L) and stayed relatively constant up to 75% completion level of alcoholic fermentation. The last quarter of fermentation was associated with an increase in GSSG content with significant differences ($p<0.05$) observed between the strains and canopy treatments applied. For both the exposed and shaded treatments, Cross Evolution resulted in wines with a significantly lower extracellular GSSG levels at the end of alcoholic fermentation compared to VIN7. The exposed treatment fermented with VIN7 fermented the slowest, which could have led to more oxygen coming into contact with the wine at the latter stages of this treatment, leading to higher GSSG levels. However, the GSSG levels were, in general, still relatively low compared to GSH levels at the end of fermentation.

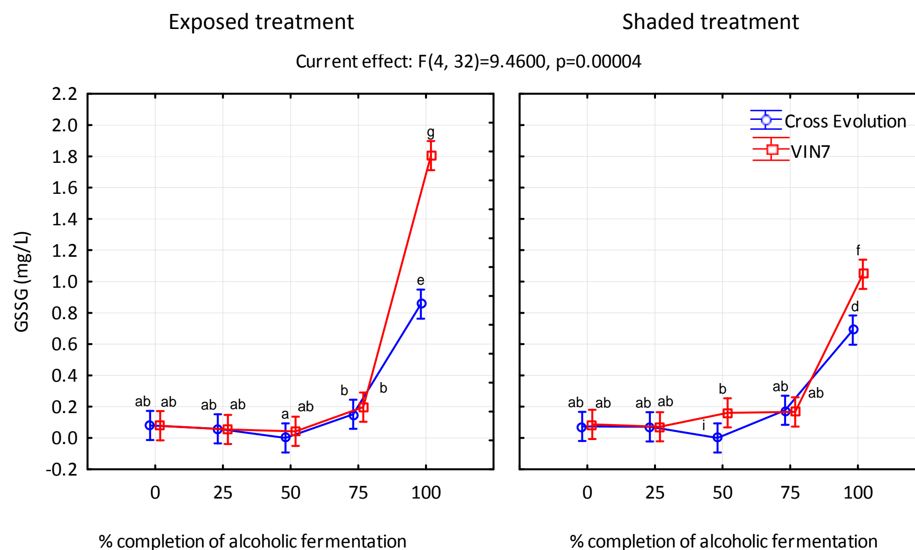


Figure 4.10 Extracellular GSSG evolution during alcoholic fermentation for different yeast strains and different canopy treatments. Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% significance level.

Stellenbosch University http://scholar.sun.ac.za

It is thus clear that maximum extracellular GSSG levels (0.69 - 1.8 mg/L) were reached at the end of fermentation, (corresponding to 13-21 days of fermentation) which is contrary to Okuda & Yokotsuka (1999) who reported maximum extracellular GSSG levels three days into fermentation for a Koshu ferment. Moreover, these authors reported a decrease in extracellular GSSG content towards the end of alcoholic fermentation. It should, however, be mentioned that these authors made use of a different yeast strain (Uvaferm CM) which might have had an influence on the extracellular GSSG levels observed during alcoholic fermentation. Fermentation conditions are also highly variable from one cultivar to another, which may further explain the differences observed in GSSG evolution in the two studies.

4.3.5.3 Total, reduced and oxidized intracellular glutathione levels

Although it was earlier suggested that various factors might have influenced yeast GSH metabolism which resulted in the different extracellular GSH evolutions observed for the different yeast strains and canopy treatments, no significant differences in intracellular GSH were observed between canopy treatments or different yeast strains. The evolution of total intracellular GSH for the different strains and canopy treatments is thus combined in **Figure 4.11**. Intracellular GSH homeostasis is maintained through a complex system which involves GSH uptake, usage, synthesis and secretion (Dhaoui *et al.*, 2011) and it is important to note that extracellular GSH concentration trends would not necessarily be reflected on intracellular level. Significant differences in total GSH content could, however, be observed in relation to the time during alcoholic fermentation ($p<0.05$). The total intracellular GSH content increased over the course of alcoholic fermentation, with a slight decrease observed at the end of fermentation. The total intracellular GSH content at the end of alcoholic fermentation ranged from 0.93 to 1.18 mg GSH/ gram cells as wet weight which translates to approximately 0.5% of the yeast cell dry weight as reported in literature (Penninckx, 2000). With regards to the intracellular GSH content, again, no significant differences could be observed in intracellular GSH concentrations between the different yeast strains and canopy treatments over the course of alcoholic fermentation. However, significant differences ($p>0.05$) were observed over the course of alcoholic fermentation. The intracellular GSH content increased as alcoholic fermentation progressed and stabilized at the 75 % completion level of alcoholic fermentation.

Stellenbosch University http://scholar.sun.ac.za

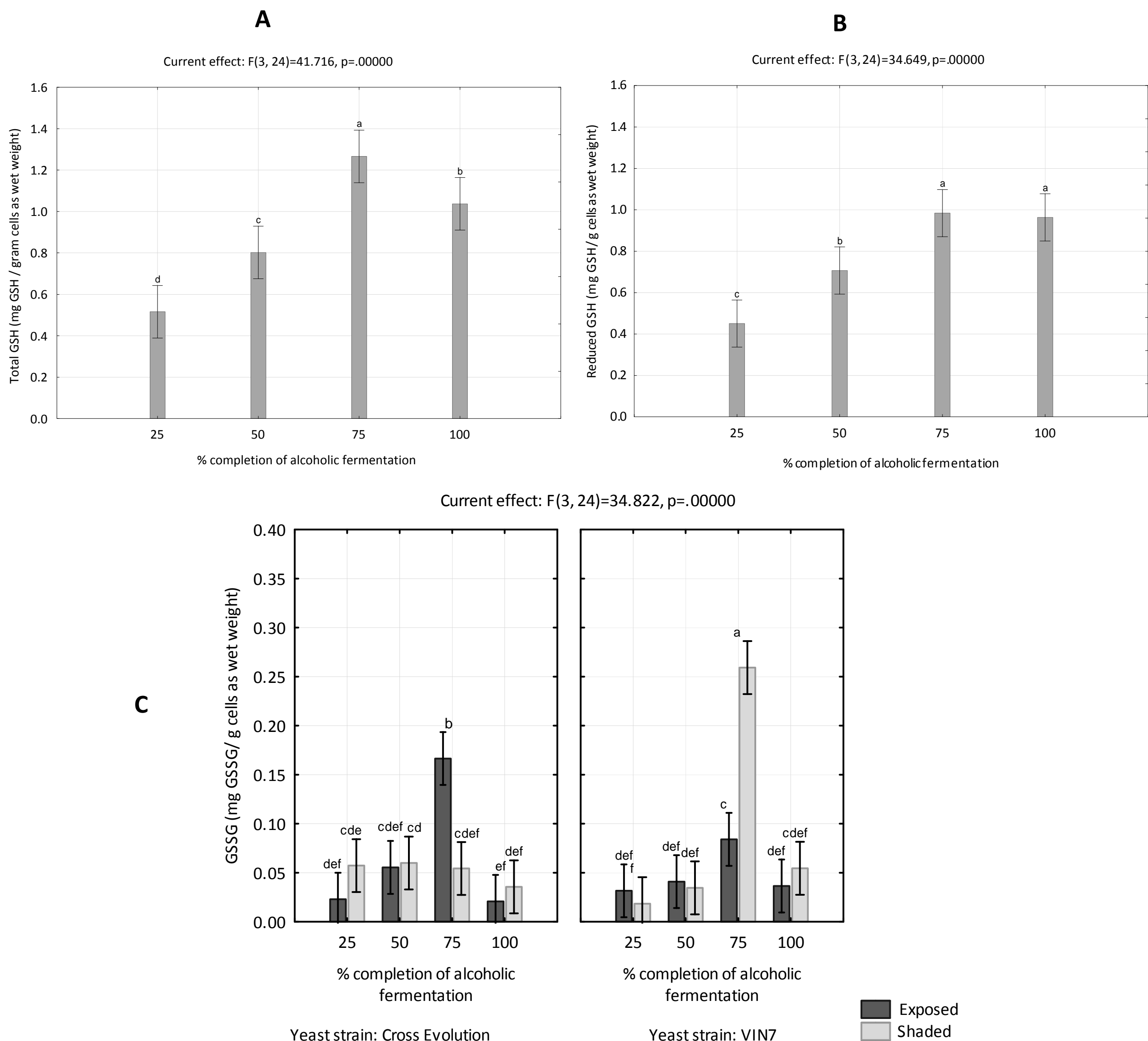


Figure 4.11 Evolution of (A) total (all treatments combined), (B) reduced (all treatments combined) and (C) oxidized intracellular glutathione during alcoholic fermentation. Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% significance level.

The total intracellular GSH content consisted mostly of reduced GSH whereas GSSG only accounted for a small amount of the total GSH. This is consistent with other studies reporting that the intracellular redox status of glutathione is predominantly in a reduced form (redox ratio of GSH:GSSG ~11 ± 16:1) (Hwang *et al*., 1992; Muller, 1996; Grant *et al.*, 1996). In general, the intracellular GSSG levels were very low throughout alcoholic fermentation and little differences could be observed between the yeast strains and the canopy treatments. However, at the 75% completion level of alcoholic fermentation, the GSSG contents differed significantly ($p<0.05$) for two ferments. The must of the exposed treatment

Stellenbosch University http://scholar.sun.ac.za

fermented with strain Cross Evolution displayed a significantly higher intracellular GSSG content compared to the must of the shaded treatment. On the contrary, significantly higher GSSG content for the must fermented with strain VIN7, was observed for the shaded treatment. Random stress factors on intracellular level could have been responsible for the increased intracellular GSSG levels in the two specific ferments. However, taken into consideration the very low concentrations range of GSSG, these significant differences did not have a major impact on the overall intracellular GSH evolution observed. To our knowledge, this is the first report of intracellular GSH and GSSG levels of commercial wine yeast strains in fermenting must.

4.4 CONCLUSIONS

This work presents a novel method for the simultaneous determination of GSH and GSSG in juice, wine, model wine and yeast fractions. The short analysis time and excellent sensitivity of the method allow the routine analysis of a large number of samples which could add significant value to GSH related research in wine.

The commercial GSH-IDYs tested, differed significantly in the amount of GSH and GSSG levels released into a model solution, which highlights the variability between the products in terms of their antioxidant potential. Depending on the type of GSH-IDY and contact time with the product, SO_2 and ethanol may affect the GSH levels observed in model solution. Further research is required to determine the effects of other parameters, such as pH and O_2 concentration on GSH release by GSH-IDYs.

GSH-IDY supplementation could result in elevated wine GSH levels, provided the supplementation is made within the first third of alcoholic fermentation. Moreover, the difference in GSH content between the control and GSH-IDY supplemented wine was five fold higher than the GSH content released into a model solution. Further investigation into GSH-IDYs, especially with regards to their influence on yeast metabolism, is needed to elucidate the exact mechanism by which GSH-IDY s leads to increased GSH levels in wine. Future research will also benefit from a comprehensive sensorial evaluation of the wine to establish the influence GSH-IDY supplementation has on the sensory profile of wines. More GSH-IDY products should also be employed in alcoholic fermentations to validate the early addition strategy and to assess the GSH levels present after fermentation.

Canopy treatments may affect the GSH levels present in juice, since the juice from the exposed bunches had a significantly higher GSH content compared to that of the shaded bunches. It was shown that extracellular GSSG levels, in general, remained low (<2 mg/L) during alcoholic fermentation, indicating that the decrease in wine GSH levels is not solely attributable to the oxidation of GSH to

Stellenbosch University http://scholar.sun.ac.za

GSSG. Intracellular GSH levels increased as alcoholic fermentation progressed whereas intracellular GSSG levels remained relatively constant throughout alcoholic fermentation. A future prospect of this work is to assess the intracellular GSH and GSSG levels in a fermentation supplemented with GSH-IDY to determine whether these products have an influence on intracellular level.

4.5 ACKNOWLEDGEMENTS

Hartenberg and KWV cellars are thanked for the donation of juice and their willingness to partake in the large scale experiments. Carien Coetzee and Jaco Franken are thanked for technical inputs as well as Prof Martin Kidd for statistical analyses of data. This research was financed by Lallemand, Winetech, THRIP and the NRF.

4.6 LITERATURE CITED

Adams, D. O. & Liyanage, C., 1991. Modification of an enzymatic glutathione assay for determination of total glutathione in grapevine tissues. Am J. Enol. Vitic. 42, 137-140.

Adams, D. O. & Liyanage, C., 1993. Glutathione increases in grape berries at the onset of ripening. Am. J. Enol. Vitic. 44, 333-338.

Alfafara, C. G., Kanda, A., Shioi, T., Shimizu, H., Shioya, S. & Suga, K., 1992. Effect of amino acids on glutathione production by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 36, 538-40.

Andújar-Ortiz, I., Pozo-Bayón, M. A., Moreno-Arribas, M. V., Martín-Álvarez, P. J. & Rodríguez-Bencomo, J. J., 2011. Reversed-Phase High-Performance Liquid Chromatography-Fluorescence detection for the analysis of glutathione and its precursor γ-glutamyl cysteine in wines and model wines supplemented with oenological inactive dry yeast preparations. Food Anal. Methods DOI 10.1007/s12161-011-9230-4.

Bisson, L. F., 1996. Yeast and biochemistry of ethanol formation. In: Boulton, R.B., Singleton, V.L., Bisson, L.F. and R.E. Kunkee, (eds). Principles and Practices of Winemaking. Chapman & Hall, New York. p. 140.

Bledsoe, A. M., Kliewer, W. M. & Marois, J. J., 1988. Effects of timing and severity of leaf removal on yield and fruit composition of Sauvignon blanc grapevines. Am. J. Enol. Vitic. 39, 49-54.

Bramanti, E., Cavallaro, R., Onor, M., Zamboni, R., & D'Ulivo, A., 2008. Determination of thiolic compounds as mercury complexes by cold vapour atomic absorption spectrometry and its application to wine. Talanta 74, 936–943.

Camera, E., Rinaldi, M., Briganti, S., Picardo, M. & Fanali, S., 2001. Simultaneous determination of reduced and oxidized glutathione in peripheral blood mononuclear cells by liquid chromatography-electrospray mass spectrometry, J. Chromatogr. B 757, 69–78.

Cassol, T. & Adams, D. O., 1995. Detection of glutathione in white wines using an enzymatic analytical method. Am. J. Enol. Vitic. 46, 410.

Coetzee, C. 2011. Oxygen and sulphur dioxide additions to Sauvignon blanc: effect on must and wine composition. MScAgric thesis, Stellenbosch University, Private Bax X1, 7602 Matieland, South Africa.

Cheynier, V., Souquet, J. M. & Moutounet, M., 1989. Glutathione content and glutathione to hydroxycinnamic acid ratio in *Vitis vinifera* grapes and musts. Am. J. Enol. Vitic. 40, 320-324.

Churchwell, M. I., Twaddle, N. A., Meeker, L. R. & Doerge, D. R., 2005. Improving LC–MS sensitivity through increases in chromatographic performance: Comparisons of UPLC–ES/MS/MS to HPLC–ES/MS/MS. J. Chromatogr. B, 825, 134–143.

Cilliers, J. J. L. & Singleton, V. L., 1990. Caffeic acid autoxidation and the effects of thiols. J. Agric. Food Chem. 38, 1789-1796.

Dhaoui, M., Auchère, F., Blaiseau, P. L., Lesuisse, E., Landoulsi, A., Camadro, J. M., Haguenauer-Tsapis, R. & Belgareh-Touzé, N., 2011. Gex1 is a yeast glutathione exchanger that interferes with pH and redox homeostasis. Mol. Biol. Cell. 22, 2054-2067.

Dubourdieu, D. & Lavigne, V., 2004. The role of glutathione on the aromatic evolution of dry white wine. Vinidea.net 2, 1-9.

Stellenbosch University http://scholar.sun.ac.za

Du Toit, W. J., Lisjak, K., Stander, M. & Prevoo, D., 2007. Using LC-MSMS to assess glutathione levels in South African white grape juices and wines made with different levels of oxygen. J. Agric. Food Chem. 55, 2765-2769.

Elskens, M.T., Jaspers, C.J. & Penninckx, M.J., 1991. Glutathione as an endogenous sulfur source in the yeast *Saccharomyces cerevisiae*. J. Gen. Microbiol. 137, 637-644.

Fracassetti, D., 2010. Investigation on cysteinyl thiol compounds from yeast affecting wine properties. PhD (Food Science) dissertation, Technology and Biotechnology, University of Milan, Italy.

Fracassetti, D., Lawrence, N., Tredoux, A. G. J., Tirelli, A., Nieuwoudt, H. H. & du Toit, W. J., 2011. Quantification of glutathione, catechin and caffeic acid in grape juice and wine by a novel ultra-performance liquid chromatography method. Food Chem. 128, 1136–1142.

Grant, C.M., Collinson, L.P., Roe, J. H., & Dawes, I. W. 1996. Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation. Mol. Microbiol. 21, 171-179.

Hwang, C., Sinskey, A. J. & Lodish, H. F. 1992. Oxidized redox state of glutathione in the endoplasmic reticulum. Science 257, 1496–502.

Janes, L., Lisjak, K. & Vanzo, A., 2010. Determination of glutathione content in grape juice and wine by high-performance liquid chromatography with fluorescence detection. Anal. Chim. Acta 674, 239-242.

Lavigne-Cruège, V. & Dubourdieu, D., 2002. Role of glutathione on development of aroma defects in dry white wines. In: Trogus, H., Gafner, J. and Sutterlin, A. (eds). 13th International Enology Symposium, International Association of Enology: Montpellier, France, pp 331-347.

Lavigne, V., Pons, A. & Dubourdieu, D., 2007. Assay of glutathione in must and wines using capillary electrophoresis and laser-induced fluorescence detection - Changes in concentration in dry white wines during alcoholic fermentation and aging. J. Chromatogr. A 1139, 130-135.

Li, Y., Wei, G. & Chen, J., 2004. Glutathione: a review on biotechnological production. Appl. Microbiol. Biotechnol. 66, 233–242.

Li, H., Guo, A. & Wang, H., 2008. Mechanisms of oxidative browning of wine-A Review. Food Chem. 108, 1-13.

Lisjak, K., 2007. The role of oxygen in new vinification technologies of white and red wines. PhD (Food Science and Technology) dissertation, Biotechnical Faculty, University of Ljubljana, Slovenia.

Marchand, S. & de Revel, G., 2010. A HPLC fluorescence-based method for glutathione derivatives quantification in must and wine. Anal. Chim. Acta 660, 158-163.

Muller, E. G. D., 1996. A glutathione reductase mutant of yeast accumulates high levels of oxidized glutathione and requires thioredoxin for growth. Mol. Biol. Cell. 7, 1805- 1813.

Nieuwoudt, H. H., Prior, B. A., Pretorius, I. S., Manley, M. & Bauer, F. F., 2004. Principle component analysis applied to fourier transform infrared spectroscopy for the design of calibration sets for glycerol prediction models in wine and for the detection and classification of outlier samples. J. Agric. Food Chem. 52, 3728-3735.

Okuda T. & Yokotsuka, K., 1999. Levels of glutathione and activities of related enzymes during ripening of Koshu and Cabernet Sauvignon grapes and during winemaking. Am. J. Enol. Vitic. 50, 264-270.

Papadopoulou, D. & Roussis, I. G., 2001. Inhibition of the decline of linalool and α-terpineol in muscat wines by glutathione and N-acetyl-cysteine. Int. J. Food Sci. 13, 413-419.

Papadopoulou, D. & Roussis, I. G., 2008. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a model wine medium by glutathione and N-acetyl-cysteine. Int. J. Food Sci. Technol. 43, 1053-1057.

Park, S. K., Boulton, R. B. & Noble, A. C., 2000a. Formation of hydrogen sulfide and glutathione during fermentation of white grape musts. Am. J. Enol. Vitic. 51, 91-97.

Park, S. K. , Boulton, R. B. & Noble, A. C., 2000b. Automated HPLC analysis of glutathione and thiol-containing compounds in grape juice and wine using pre-column derivatization with fluorescence detection. Food Chem. 68, 475-480.

Penninckx, M. J., 2000. A short review on the role of glutathione in the response of yeasts to nutritional, environmental, and oxidative stresses. Enz. Microbial Techn. 26, 737–742.

Penninckx M. J., 2002. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. FEMS Yeast Res. 2, 295–305.

Pozo- Bayón, M. A., Andújar-Ortiz, I. & Moreno-Arribas, M. V., 2009a. Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. Food Res. Int. 42, 754-761.

Pozo-Bayón, M. A., Andujar-Ortiz I., Alcaide-Hidalgo, J. M., Martin-Alvarez P. J. & Moreno-Arribas, M. V., 2009b. Characterization of commercial inactive dry yeast preparations for enological use based on their ability to release soluble compounds and their behavior toward aroma compounds in model wines. J. Agric. Food Chem. 57, 10784-10792.

Stellenbosch University http://scholar.sun.ac.za

Roussis, I. G., Papadopoulou, D. & Sakarellos-Daitsiotis, M., 2009. Protective effect of thiols on wine aroma volatiles. Open Food Sci. J. 3, 98-102.

Singleton, V. L., M. Salgues, J. Zaya, & Trousdale, E., 1985. Caftaric acid disappearance and conversion to products of enzymic oxidation in grape must and wine. Am. J. Enol. Vitic. 36, 50-6.

Sonni F., Clark, A. C., Prenzler, P. D., Riponi, C. & Scollary, G. R., 2011a. Antioxidant action of glutathione and the ascorbic acid/glutathione pair in a model white wine. J. Agric. Food Chem. 59, 3940-3949.

Sonni, F., Moore, E. G., Clark, A. C., Chinnici, F., Riponi, C. & Scollary, G. R., 2011b. Impact of glutathione on the formation of methylmethine- and carboxymethine-bridged (+)-catechin dimers in a model wine system. J. Agric. Food Chem. 59, 7410-7418.

Tirelli, A., Fracassetti, D., & De Noni, I., 2010. Determination of reduced cysteine in oenological cell wall fractions of *Saccharomyces cerevisiae*. J. Agric. Food Chem. 58, 4565–4570.

Ugliano, M., Kwiatkowski, M. J., Vidal, S., Capone, D., Siebert, T., Dieval, J. B., Aagaard, O. & Waters, E. J., 2011. Evolution of 3-mercatohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. J. Agric. Food Chem. 59, 2564–2572.

Vaimakis, V. & Roussis, I. G., 1996. Must oxygenation together with glutathione addition in the oxidation of white wine. Food Chem. 57, 419-422.

Wang, Z., Tan, T. & Song, J., 2007. Effect of amino acids addition and feedback control strategies on the high-cell -density cultivation of *Saccharomyces cerevisiae* for glutathione production. Process Biochem. 42, 108–111.

Wen, S., Zhang, T. & Tan, T., 2004. Utilization of amino acids to enhance glutathione production in *Saccharomyces cerevisiae*. Enzyme Microb. Tech. 35, 501-507.

Stellenbosch University http://scholar.sun.ac.za

Chapter 5

General discussion and conclusions

Stellenbosch University http://scholar.sun.ac.za

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 CONCLUDING REMARKS AND FUTURE WORK

Over the last few decades the competitive nature of the global wine market has increased, with quality wines entering the market from all over the world. The consumer's understanding of product value has increased resulting in a discriminating demand for wine quality (Bisson *et al*., 2002). This translates into wineries being challenged to produce wines of consistent and, if possible, higher quality. Similarly, consumer demands for healthful wines resulted in pressure to reduce the use of chemical preservatives, such as sulphur dioxide (SO_2) in wine. An improved product, both in terms of quality and consumer acceptance, would hence confer a competitive advantage to the winemaker (Bisson *et al*., 2002). The significance of glutathione (GSH) in limiting juice and wine oxidation, along with its protective effect on various positive aroma compounds and inhibiting effect on the formation of ageing off-flavours, confer unique quality enhancing and quality preserving possibilities in wine. Furthermore, higher antioxidant levels in wine, such as GSH, could permit the use of reduced SO_2 dosages without compromising the sensory quality of the wine (Roussis *et al*., 2007).

The overriding aim of this research was to gain a better understanding of the fate of GSH during alcoholic fermentation and to ascertain the influence of certain oenological factors on its levels in wine. Factors studied included yeast strain, extended lees contact, different YAN levels and supplementation with a glutathione-enriched inactive dry yeast preparation (GSH-IDY).

During this study it was shown that synthetic wines fermented with different wine yeast strains differed significantly in terms of GSH content, with certain strains resulting in a seven fold higher wine GSH content. However, when selected strains were inoculated in grape juice, the trends with regards to final GSH content were not always confirmed. The reasons for the variation in final GSH levels in different fermentation media remains unexplained, but are likely linked to grape juice metabolic complexity. Indeed, it is well established that many grape and yeast metabolites are either taken up or released by yeast during fermentation depending on complex, non-linear interactions between the grape juice initial metabolic composition, the genetic background of the yeast strain and the many environmental parameters that affect yeast growth such as temperature, pH and osmotic pressure. The grape juice fermentations, nevertheless, delivered valuable information regarding the evolution of GSH during alcoholic fermentation. It was shown that the GSH content fluctuated during alcoholic fermentation, with the time stage and degree of fluctuation depending on the yeast strain and initial GSH content of the juice. Not only does this observation suggest that strains differ in their GSH metabolism and that GSH is assimilated at certain stages and released again at others during alcoholic fermentation, but also that the metabolism of the yeast is likely to be influenced by the extracellular GSH content.

Stellenbosch University http://scholar.sun.ac.za

Compared to GSH levels before fermentation, lower, on par and higher levels were observed after fermentation, highlighting the complexity and variability of the behaviour of GSH during fermentation. Small, but significant differences in GSH content for wines fermented with different yeast strains were observed. It should, however, be taken into consideration that the GSH levels after alcoholic fermentation were not strictly attributable to a specific yeast strain alone. Other factors in the juice and fermenting must could also have influenced the GSH levels observed after alcoholic fermentation and further investigation is necessary to elucidate these factors. Nevertheless, the observation of wines exhibiting higher GSH levels after alcoholic fermentation, proposes the *de novo* synthesis of GSH by the yeast, followed by secretion into the extracellular medium. Taken into consideration the lack of literature on the extracellular accumulation of GSH by *Saccharomyces cerevisiae* (Wei *et al*., 2003; Li *et al*., 2004) and the general view of authors that GSH produced by yeast is always in the intracellular form (Wei *et al*., 2003), this observation may have interesting repercussions for future research. Future work should thus be aimed at studying the factors that may favour GSH secretion by wine yeast strains under winemaking conditions.

Regarding the ageing of Sauvignon blanc wine, this work showed that GSH decreased during ageing, consistent with findings by Ugliano *et al*. (2011). It was also shown that the presence of yeast lees during wine ageing did not have a profound protective effect on GSH levels as has been suggested by Lavigne *et al*. (2007). Furthermore, this study indicated that no GSH was released upon yeast autolysis during the ageing period, which implies that the GSH content cannot be manipulated by the *sur lie* practice.

The influence of different YAN levels in a chemically defined grape juice medium on GSH levels after alcoholic fermentation seems to be insignificant. It thus seems that nitrogen supplementation to grape juice would not lead to higher wine GSH levels. However, it would be advisable to repeat this experiment in grape juice with more yeast strains before final conclusions are drawn.

Preliminary results obtained also showed that yeast cells attained significantly different intracellular GSH contents when grown in media with different GSH concentrations. However, cells grown in media deficient of GSH produced sufficient amounts of intracellular GSH to confer oxidative stress resistance, which implies that extracellular GSH levels would not necessarily impact on the oxidative stress ability of yeast cells. This study should, however, be repeated with more yeast strains, since the intrinsic oxidative stress resistance of wine yeast strains differs (Carrasco *et al*., 2001).

During this study it was shown that the glutathione contents [both GSH and oxidized glutathione (GSSG)] released from different GSH-IDYs into model solution, were highly variable, which indicate that the inherent oxidative buffer capacities of the various GSH-IDYs are likely to differ. The addition of GSH-IDY during alcoholic fermentation could lead to increased GSH levels in wine, provided the supplementation is done early in the process. This observation was also confirmed in two commercial scale wines. Further investigation is necessary to determine whether the product leads to increased

Stellenbosch University http://scholar.sun.ac.za

GSH synthesis and secretion by the yeast or whether soluble compounds from the GSH-IDY are preferentially assimilated above GSH naturally present in the juice. Sensory analysis of such wines would also provide valuable information regarding the organoleptic impact GSH-IDY supplementation has on wines.

This study also showed that canopy treatments in vineyard could influence the GSH content of juice, with the leaf removal treatment leading to elevated levels in juice. However, this effect did not always present in the resulting wines. Valuable insights into extracellular GSSG levels during alcoholic fermentation were gained. The extracellular GSSG levels remained very low during fermentation but increased levels were observed at the end of alcoholic fermentation. The levels also seemed to be influenced by the particular yeast strain. However, the GSSG content could not account for the reduction observed in GSH content. Incorporation of GSH into the Grape Reaction Product, yeast assimilation and conjugation with both intra- and extracellular compounds, could all contribute to lower GSH levels. This study also provides the first report on intracellular GSH and GSSG contents during alcoholic fermentation in wine. It was shown that intracellular GSH levels increased as alcoholic fermentation progressed whereas GSSG contents stayed relatively constant during fermentation.

During this study, a novel UPLC-MS/MS method for the simultaneous detection and quantification of both GSH and GSSG was developed. The method allows for rapid and accurate analysis of GSH and GSSG in juice, wine, model wine solution and intracellular fractions without the need for GSH derivatization. The method showed excellent sensitivity and the minimal sample preparation, together with the shortened analysis time, renders this method suitable for routine analysis of a large number of samples which could add significant value to GSH research in wine. Another advantage is of the shortened analysis duration and reduced solvent consumption, leading to a reduction in analysis cost.

In conclusion, this study contributes to the foundation required to understand the evolution of GSH during alcoholic fermentation and the oenological factors that impact its levels in wine. In addition, several novel research topics that merit further investigation were generated in this study.

5.2 LITERATURE CITED

Bisson, L. F., Waterhouse, A. L., Ebeler, S. E., Walker, M. A., Lapsey, J. T., 2002. The present and the future of the international wine industry. Nature 418, 696- 699.

Carrasco, P., Querol, A. & del Olmo, M., 2001. Analysis of the stress resistance of commercial wine yeast strains. Arch. Microbiol. 175, 450–457.

Li, Y., Wei, G. & Chen, J., 2004. Glutathione: a review on biotechnological production. Appl. Microbiol. Biotechnol. 66, 233–242.

Ugliano, M., Kwiatkowski, M. J., Vidal, S., Capone, D., Siebert, T., Dieval, J. B., Aagaard, O. & Waters, E. J., 2011. Evolution of 3-mercatohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. J. Agric. Food Chem. 59, 2564–2572.

Wei, G., Li, Y., Du, G. & Chen, J., 2003. Effect of surfactants on extracellular accumulation of glutathione by *Saccharomyces cerevisiae*. Process Biochem. 38, 1133–1138.