Glycerol and wine

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

Key research areas in modern enology are related to the production of wine of consistent quality, as well as to the improvement of existing wine quality through the enhancement of the sensory properties of wine. The formation of glycerol during alcoholic fermentation is highly relevant to both these issues. Since the early years of the 20th century, glycerol has been positively associated with the quality of wine and it is thought to impart important mouth-feel sensations such as "viscosity", "smoothness" and "body". In general, it is considered that glycerol concentrations higher than those normally found in wine, can contribute towards the improvement of wine quality. It has also been suggested that increased concentrations of glycerol can enhance the aroma of wine. On the basis of these perceptions, several strategies have been developed to favour the production of glycerol during the fermentation process and over a period of years, a large volume of data has been collected that relates to various aspects regarding glycerol production during alcoholic fermentation.

To date, however, several aspects regarding the relationship between glycerol and wine quality remain unclear. The reasons for this situation can mainly be ascribed to the lack of reliable analytical data to serve as a basis for investigating the relationship between glycerol and wine quality, as well as the preponderance of empirical and anecdotal evidence. Despite numerous opinions regarding optimal glycerol concentrations in wine, glycerol is indeed seldom assayed on a routine basis and targets with respect to specific wine grape cultivars and glycerol concentrations have largely remained unspecified. To date, very little information regarding glycerol concentrations in South African wines has been published. The analytical techniques that are most frequently used for the quantification of glycerol in grape juice, fermenting must and wine are not easily automated and this aspect placed severe limitations on the generation of large volumes of analytical data on glycerol concentrations in these matrices.

This project was undertaken with the aim to holistically address some of the unresolved issues relating to the relationship between glycerol and wine quality. This also implied the development and optimisation of analytical techniques suitable for the rapid and accurate determination of glycerol in fermentation media, as well as in finished wine. In the first stage of this project a quantitative database was established that contained the analytical data on the glycerol concentrations of a statistically significant number of wines of adjudged quality, as well as additional information for each wine regarding the geographic origin, vintage, routine chemical analyses and the yeast strain(s) used for the production of the wine. The relevance of glycerol in wine for the modern South African winemaker was evaluated through the establishment of a quantitative database that contained the opinions of an expert panel of 15 South African winemakers, enologists and wine chemists on topics relating to glycerol in wine.

In the second stage of the project the data captured in the databases were used to investigate aspects regarding the relationship between glycerol and wine

quality. From the data captured in the qualitative database, it was clear that the topic regarding glycerol in wine was important to the South African winemakers and it was also evident that there was a need for the development and optimisation of methods suitable for the routine analysis of the glycerol concentrations in grape juice, fermenting must and wine. The opinions of the panel members also highlighted the issue that the mouth-feel property of wine was considered to be an area where the quality of some wines could be further improved.

The quantitative database contained the information on the glycerol concentrations of 450 commercial South African table wines of adjudged quality. The premium cultivars Chardonnay, Chenin blanc, Sauvignon blanc, Cabernet Sauvignon, Merlot, Shiraz and Pinotage were used for the purpose of investigating the relationship between glycerol concentration and wine quality. The wines represented a wide variety of wine styles, including dry white, off-dry white, dry red and late harvest wines. The average glycerol concentration was significantly associated with the wine style. In white wines the average glycerol concentration was much lower than in the red wines (6.82 g/L versus 10.49 g/L, respectively). No significant relationship between the final glycerol concentration and the geographic origin, vintage and the yeast strain used for the fermentation was found. Wine quality could not be significantly associated with glycerol concentration in the red wines. In the white wines, the relationship between glycerol concentration and wine quality was significant, but due to the very small differences in the average glycerol concentrations of the wines of different quality ratings, the statistical significance is probably of little practical value.

The effect of glycerol on the volatility of a selection of esters and higher alcohols was also investigated. Solid-phase microextraction, followed by gas chromatography, was used to analyse the composition of the headspace at equilibrium between the liquid phase and the gas phase of a model wine, and a dry white wine that contained a basal concentration of 5.4 g/L glycerol. Results showed that incremental increases in the glycerol concentrations over a range of 1 –10 g/L in the model wine and in a Chenin blanc wine, were not accompanied by a proportional increase or decrease in the abundance of the aroma components in the headspace. The volatile components tested were isoamyl acetate, ethyl butyrate, ethyl valerate, ethyl lactate, ethyl hexanoate, hexyl acetate, isoamyl alcohol and isobutanol. For all the volatile components tested, the difference between the headspace composition of samples containing the lowest glycerol concentration, and those containing the highest glycerol concentration, was not significant. However, sufficient experimental evidence was obtained to indicate that increasing glycerol concentrations had an effect on the volatility of aroma components, and that the effect is of a complex and non-linear nature.

In the third phase of the work Fourier transform infrared spectroscopy (FT-IR) was used to establish and optimise methods for the accurate and rapid quantification of glycerol in wine. For this purpose calibrations were developed for the quantification of glycerol in dry wine and late harvest wines. The accuracy of prediction was evaluated by means of the standard error of prediction that was 0.38 g/L for the dry wines and 0.65 g/L for the sweet wines. Large variations are introduced in the FT-IR spectra of wine by factors such as process technology, cultivar and geographic origin, and this variation can have an effect on the accuracy of the analytical data generated when employing FT-IR spectroscopy. Using glycerol prediction in wine as a model system, principal component analysis of the FT-IR spectra was done in order to establish quality control measures for the detection of poorly predicted, or outlier samples. A classification model, based on principal component analysis, was established that enabled the interpretation and classification of the outlier samples in the data set in 100% of the cases tested. This work forms the basis for expanding the quality control measures for the detection of wines of which the FT-IR spectra are highly unnatural, as well as for establishing quality control measures to ensure that accurate analytical data are generated when FT-IR is used.

FT-IR spectroscopy was also used to develop a rapid screen for the evaluation of the fermentation profiles of wine yeasts. For this purpose, a selection of wine yeasts, which included commercial wine yeasts frequently used in winemaking in South Africa, as well as a selection of hybrid *Saccharomyces cerevisiae* yeasts, that were obtained through a selective breeding strategy aimed at increasing glycerol concentrations were used. Calibrations necessary for the accurate quantification of glycerol, volatile acidity, ethanol, reducing sugar and glucose, in Chenin blanc must and a synthetic must were developed and optimised. This work forms the basis upon which the scope of the analysis, both in terms of the number of components that can be measured, as well as the medium in which the yeasts are being evaluated, can be enlarged. This would be valuable for future applications in both the research as well as the industrial environment. The method that was developed serves to illustrate how this application can play a supportive role in yeast development programmes, through the speeding up of the initial stages of yeast strain evaluation.

OPSOMMING

Navorsing in moderne wynkunde is sterk gefokus op die produksie van wyn waarvan die kwaliteit van 'n volhoubare goeie peil is, sowel as die verdere verbetering van bestaande wynkwaliteit, deur 'n verhoging van die sensoriese eienskappe van wyn. Die vorming van gliserol tydens alkoholiese fermentasie het betrekking op beide hierdie aspekte. 'n Opvatting wat wyd gehuldig word en wat reeds sedert die vroeë jare van die 20e eeu geld, impliseer dat gliserol 'n positiewe bydrae lewer tot wynkwaliteit. Oor die algemeen word dit beskou dat gliserolvlakke hoër as wat normaalweg in wyn aangetref word, kan bydra om die kwaliteit van die wyn nog verder te vehoog. Daar is ook spekulasie dat verhoogde gliserolvlakke in wyn die intensiteit van die aroma van wyn kan verhoog. Hierdie opvattings het tot gevolg gehad dat veskeie strategieë ontwikkel is om die gliserol vlakke wat tydens die fermentasieproses gevorm word, te verhoog.

Baie min inligting oor die gliserolinhoud van Suid-Afrikaanse wyn is tot onlangs gepubliseer. Ten spyte van die opvattings oor gliserol en wynkwaliteit wat reeds oor 'n lang tydperk gehuldig word, bly sekere aspekte van die verwantskap tussen gliserol en wynkwaliteit nog steeds onduidelik. Redes vir hierdie situasie kan hoofsaaklik toegeskryf word aan die totale afwesigheid van betroubare en substansiële eksperimentele data wat as basis kan dien vir die evaluering van die algemene opvattings aangaande die verwantskap tussen gliserol en wynkwaliteit. Die inligting wat wel beskikbaar is, is verder ook oorwegend van 'n empiriese aard. Huidig word gliserol selde op 'n roetine basis in die analitiese laboratorium bepaal, hoofsaaklik omdat die bestaande metodes tydrowend is en nie maklik ge-outomatiseer kan word nie.

In hierdie studie is 'n kwantitatiewe databasis opgestel waarin die inligting ten opsigte van die gliserolvlakke van 450 kommersiële Suid-Afrikaanse tafelwyne waarvan die kwaliteit beoordeel is, vervat is. Die kultivars Chardonnay, Chenin blanc, Sauvignon blanc, Cabernet Sauvignon, Merlot, Shiraz en Pinotage is gebruik vir hierdie doel en die seleksie van wyne was verteenwoordigend van 'n wye verskeidenheid wynstyle, insluitende droë wit-, halfdroë wit-, droë rooi- en laatoeswyne. Die gemiddelde gliserolvlakke in die witwyne was heelwat laer as die gemidelde gliserolvlakke in die droë rooiwyne (6.82 g/L teenoor 10.49 g/L, onderskeidelik). Geen beduidende verwantskap kon aangetoon word tussen die gliserolkonsentrasie in die wyn en die geografiese oorsprong, oesjaar, en die gisras wat gebruik is in die produksie van die wyn nie. Die kwaliteit van rooiwyn kon nie beduidend met die gliserolkonsentrasie geassosieer word nie. In die geval van wit wyn was die verwantskap statisties beduidend, maar die verskille was klein en moontlik nie van veel praktiese waarde nie.

Die aktualiteit van gliserol vir die moderne Suid-Afrikaanse wynmaker is geëvalueer op grond van die opinies van 'n paneel van 15 Suid-Afrikaanse kundiges,

rakende aspekte wat verband hou met die algemene opvattings oor gliserol en wyn. Die paneel het bestaan uit wynmakers, wynkundiges en chemici. Die opinie van die panel is deur middel van 'n vraelys bekom en is vervat in 'n kwalitatiewe databasis. Die resultate van hierdie menigspeiling het getoon dat gliserol wel vir die Suid-Afrikaanse wynmakers belangrik is en dit het ook die behoefte uitgewys vir die ontwikkeling en optimisering van metodes wat geskik is vir die roetine analyses van gliserol in wyn.

Die invloed van gliserol op die vlugtigheid van 'n seleksie van hoëralkohole en esters is ondersoek in 'n model wyn sowel as 'n wit wyn, waarvan die basiese gliserolkonsentrasie 5.4 g/L was. Soliede-fase mikroekstraksie van die gasfase van wyn is opgevolg met 'n gaschromatografiese analise. Resultate het getoon dat 'n stapsgewyse toename in die gliserol konsentrasie, oor 'n konsentrasie reeks van 0 – 10 g/L in die model wyn, en 0 – 15 g/L in die wit wyn, nie gepaard gegaan het met 'n reglynige toename of afname in die konsentrasie van die aromakomponente nie. Vir al die komponente wat ondersoek is, was die samestelling van die gasfase in monsters wat die laagste gliserol konsentrasie gehad het, teenoor dié wat die hoogste gliserol konsentrasie gehad het, nie beduidend nie. Nietemin het die resultate getoon dat gliserol wel 'n effek het op die vlugtigheid van die aroma komponente wat in hierdie ondersoek gebruik is, maar dat die aard van die effek kompleks en nie-linié is.

Fourier-transformasie-infrarooispektroskopie (FT-IR) is gebruik om die metodes vir die analise van gliserol in wyn sodanig te optimiser, dat vinnige en akkurate bepalings op 'n roetine basis in wyn gedoen kan word. Kalibrasies is ontwikkel vir die kwantifisering van gliserol in droëwyn en laatoeswyn. Die standaard voorspellingsfout van die kalibrasies wat ontwikkel is, was 0.38 g/L in droëwyn en 0.65 g/L in die geval van laatoeswyn. Inherente variasie in die FT-IR-spektra word deur eksterne faktore soos die proses tegnologie wat gebruik is om die wyn te berei, die kultivar en geografiese oorsprong van die wyn veroorsaak en hierdie variasie kan 'n effek hê op die voorspelingsakkuraatheid van FT-IR spektroskopie. Om die effek van hierdie variasie op die akkuraarheid van die analitiese data te evalueer, is die voorspelling van gliserol as modelsisteem gebruik. Hoofkomponentanalise van die FT-IR spektra is gedoen om dié wyne met onnatuurlike FT-IR-spektra te identifiseer, ten einde beheer oor die akkuraatheid van die analitiese data uit te oefen. 'n Verdere model wat ook gebaseer is op hoofkomponentanalise van die FT-IR-wynspektra is ontwikkel vir die interpretasie en klassifikasie van wynmonsters met onnatuurlike FT-IR-spektra. Met die model wat ontwikkel is kon onnatuurlike wynmonsters met 'n akkuraatheid van 100% geïdentifiseer word. Hierdie werk vorm die basis waarop kwaliteitsbeheer verder uitgebrei kan word sodat wyne met onnatuurlike spektra geïdentifiseer kan word, sowel as om te verseker dat akkurate data gegenereer word as FT-IR as analitiese metode gebruik word vir die kwantifisering van gliserol in wyn.

FT-IR spektroskopie is ook gebruik om 'n vinnige skandeerproses te ontwikkel waarmee die fermentasieprofiele van wyngiste geëvalueer kan word. Die seleksie giste wat vir hierdie doel gebruik is, sluit kommersiële Suid-Afrikaanse wyngiste in, sowel as hibriede *Saccharomyces cerevisia*-giste wat normaalweg hoër vlakke van gliserol tydens die fermentasieproses produseer. Kalibrasies is ontwikkel vir die akkurate kwantifisering van gliserol, vlugtige suur, alkohol, reduserende suiker en glukose, in Chenin blanc-mos sowel as in 'n sintetiese medium. Hierdie werk vorm die basis waarop verdere uitbreidings gedoen kan word, sodat meer metaboliete gemeet kan word en 'n groter verskeidenheid van fermentasiemedia gebruik kan word. Hierdie ontwikkeling is waardevol vir toekomstige toepassings in die navorsings- sowel as die industriële omgewing. Die metode wat ontwikkel is illustreer ook hoe hierdie toepassing 'n ondersteunende rol kan speel in wyngis-ontwikkelingsprogramme deur die aanvanklike evalueringsproses van die giste te versnel.

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PREFACE

This dissertation is presented as a compilation of seven chapters and two appendices. Each chapter is introduced separately and is written according to the style of the journal to which the chapter was submitted for publication.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

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1.1 AN INTRODUCTION TO GLYCEROL AND WINE

Glycerol (CH_2OH-CHOH-CH_2OH) is quantitatively a major component of wine, and after ethanol and water it is the most abundant wine constituent. In beverages such as wine and beer, glycerol is thought to impart important sensory properties to the product and these properties are usually described in terms of mouth-feel sensations such as "smoothness", "viscosity" and "body" (Scanes *et al.*, 1998). Glycerol can also contribute to the sweetness of wine (Hinreiner *et al.*, 1955). Glycerol is not considered to contribute directly to the aroma of wine, but it has been suggested that increased glycerol concentrations enhance the flavour of beverages such as wine, saké and shochu (Omori *et al.*, 1997). This could suggest that glycerol has an indirect effect on aroma and flavour components in these beverages. The presence of glycerol in wine can mainly be ascribed to its formation by the wine yeast during the fermentation process (Gancedo *et al.*, 1968; Scanes *et al.*, 1998). Glycerol concentrations in grape juice are usually below 1 g/L, but in exceptional cases where the grapes are affected by fungal growth, especially *Botrytis cinerea,* significant amounts of glycerol are formed in the berries (Mühlberger and Grohmann, 1962; Zoecklein *et al.*, 2000). Glycerol concentrations in dry table wines typically range from 5 - 14 g/L, whereas concentrations in excess of 15 g/L are frequently encountered in botrytised wines (Rankine and Bridson, 1971; Ough *et al.,* 1972; Nieuwoudt *et al.*, 2002).

Interest in the formation of glycerol by yeasts can be traced back to 1858, when Pasteur reported on its formation as a by-product of alcoholic fermentation (Prescott and Dunn, 1959). Since that time, its formation has been a widespread point of interest amongst biochemists and enologists alike. The perception that a correlation between glycerol concentration and the quality of wine exists was already firmly established in the early years of the 20^{th} century (Hickinbotham and Ryan, 1948; Amerine, 1954). During the first half of the 20^{th} century, glycerol formation during alcoholic fermentation was studied from three major angles, which were: (i) investigations into enological factors that affected the concentrations of glycerol formed by wine yeasts, with the aim to manipulate (mostly increase) the final glycerol concentrations formed in wine; (ii) the development of analytical techniques to quantify glycerol in fermentation media and in wine; and (iii) large scale investigations into the glycerol concentrations of finished wines from various countries.

During the second half of the 20^{th} century (and including the present), glycerol production by *Saccharomyces cerevisiae* has been the focus of intense research and major advances have been made towards understanding the physiological, biochemical and genetic aspects of yeast glycerol metabolism (for recent reviews see Bakker *et al.*, 2001; Hohmann, 2002). Based on the perception that glycerol concentrations higher than those normally formed during alcoholic fermentation were required to improve the quality of wine, several strategies were developed to manipulate the fermentation conditions in favour of glycerol formation, or alternatively, to manipulate the metabolic pathways of the wine yeast to produce higher concentrations of glycerol (Scanes *et al.*, 1998). Researchers have also chosen glycerol formation as a vehicle for disposing of surplus carbon, which would otherwise be destined for the formation of ethanol, in attempts to decrease the final ethanol concentrations in wines where this would be advantageous (Michnick *et al.*, 1997; de Barros Lopes *et al.*, 2000).

With respect to progress in analytical techniques for the quantification of glycerol in fermentation media and wine, several chromatographic and spectroscopic techniques were developed during the second half of the previous century (Drawert and Kupfer, 1963; Klein and Leubolt, 1993). In the last decade or two the conventional chemical analyses of wine (including the quantification of glycerol) has been revolutionised through the use of computerised technologies (Cordella *et al.*, 2003; Esti *et al.*, 2003). However, with respect to our understanding of the nature of the relationship between glycerol and wine quality, very little progress has been made during the last four to five decades and only a limited number of studies relating to this aspect have been reported.

A critical review of the available literature on glycerol and wine quality showed that much anecdotal evidence exists from enologists suggesting a strong positive correlation between glycerol concentration and wine quality (Rankine and Bridson, 1971; Eustace and Thornton, 1987; Omori *et al.*, 1995). The sample sets of wines used in these studies to investigate the possible relationships were frequently small and selective, and in many cases, only experimental wines were used. In some instances, clear anomalies exist between the perceptions of winemakers and enologists and the actual data that have been obtained through experimental work (Noble and Bursick, 1984). Despite numerous popular opinions on the "optimal" glycerol concentrations in wine, glycerol has indeed until very recently, seldom been assayed on a routine basis in finished wine, and hence, due to the lack of substantial analytical data, discussions of this nature not only remain speculative, but specific "targets" for improvement, in terms of wine cultivars or styles and specific glycerol concentrations, remain undefined. Although there is growing evidence that interactions occur between volatile aroma components and other non-volatile components in wine (Voilley *et al.*, 1991; Lubbers *et al.*, 1994), very little work has been undertaken to investigate the interactions between glycerol and the aroma components in wine (Lubbers *et al.*, 2001).

Against this background, the need to holistically investigate the relationship between glycerol and wine quality is clear. This also implied the generation of a substantial volume of analytical data on the glycerol concentrations in wine and in fermentation media, and therefore the establishment and optimisation of analytical methods suitable for rapid and accurate glycerol determinations in these matrices were also necessary.

1.2 PROJECT AIMS AND RESEARCH STRATEGIES

At the outset of this study very little published information regarding the glycerol concentrations in South African wines was available. The importance and relevance of the topic regarding glycerol and wine quality for the modern South African winemaker were also not known. The first phase of this project was therefore to establish databases containing the required information. The next phase of the work focused on investigating the relationship between glycerol and wine quality (using the information contained in the established databases) and this phase also included a project on the interactions between glycerol and aroma components in wine. The third phase of the work involved the establishment and optimisation of analytical techniques suitable for the routine analysis of glycerol in wine and fermentation media. The particular aims of the three different phases are listed below.

1.2.1 THE ESTABLISHMENT OF DATABASES

1.2.1.1 Quantitative database

The aim was to establish a quantitative database containing the analytical data of the glycerol concentration of a significantly large number of South African wines of which the quality was officially rated by the South African National Wine Show Association. For each wine, additional information pertaining to the cultivar, style, geographic origin, vintage, yeast strain(s) used for the production of the wine and the quality rating of the wine was also captured. Data on the routine chemical analyses of the wines (upon certification of the wines) were also included.

1.2.1.2 Qualitative database

The aim of this project was to collect in a database, the opinions of an expert panel of South African individuals on aspects regarding the importance and relevance of glycerol in wine. This included opinions on some of the commonly expressed perceptions regarding glycerol and wine quality. The panel members included leading South African winemakers, experts in the field of wine chemistry, individuals involved with the training of future winemakers, and individuals actively involved in the international marketing of South African export wines.

1.2.2 INVESTIGATIONS INTO THE RELATIONSHIP BETWEEN GLYCEROL AND WINE QUALITY

1.2.2.1 An assessment of the relationship between glycerol concentration and quality in South African table wines

The aims of this work were to investigate the relationships between glycerol concentrations and the wine style, geographic origin, vintage, wine cultivar and yeast strain(s) used for the production of the wine. The possibility of using the glycerol concentration as a predictor for wine quality was investigated using discriminant analysis. For this purpose the information contained in the quantitative database was used.

1.2.2.2 An evaluation of the importance and relevance of glycerol in wine for the modern South African winemaker

The aims of this work were (i) to establish the importance attributed by modern South African winemakers to glycerol and its relationship to wine quality; (ii) to identify which wine styles were perceived to benefit from increased glycerol concentrations; (iii) to assess the opinions with respect to optimal glycerol concentrations in wine; and (iv) to assess the need for the routine analysis of glycerol in finished wine. The information obtained through the establishment of the qualitative database was also used to critically evaluate some of the commonly held perceptions regarding glycerol and wine quality, by evaluating the perceptions against the quantitative data.

1.2.2.3 An investigation of the effect of glycerol on the volatility of aroma components in wine

The aim of this project was to investigate the effects of glycerol concentration on the volatility of aroma components in wine. For this purpose the glycerol concentration of a model wine and of a white wine was increased in a stepwise manner through the addition, to the respective matrices, of glycerol from an external source. The effect of the increase in the glycerol concentration on the volatility of aroma components was investigated by using headspace solid-phase microextraction followed by gas chromatography. The analytical data obtained through the headspace analysis were correlated to the sensory evaluation of the wine.

1.2.3 THE ESTABLISHMENT AND OPTIMISATION OF ANALYTICAL TECHNIQUES SUITABLE FOR THE ROUTINE ANALYSIS OF GLYCEROL IN WINE AND IN FERMENTATION MEDIA

The specific aims of this part of the work were to establish and optimise the application of Fourier transform infrared spectroscopy (FT-IR), using the WineScan FT 120 instrument (Foss Electric, Denmark), for the routine quantification of glycerol in finished wine. This work involved (i) the establishment of calibrations suitable for the accurate quantification of glycerol in wines of different styles; and (ii) the establishment of quality control measures suitable for the detection, interpretation and classification of samples that were poorly predicted by the established calibrations. A further aim of the application of FT-IR was to develop a rapid screen for evaluating the fermentation profiles of wine yeasts in fermenting must and in a synthetic must. In this particular application, the focus was on the quantification of glycerol, volatile acidity, ethanol, glucose and residual sugar in the fermentation media.

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CHAPTER 2

LITERATURE REVIEW

Glycerol and its relevance to wine

LITERATURE REVIEW

2.1 INTRODUCTION

Glycerol (CH_2OH-CHOH-CH_2OH) belongs to a family of simple alcohols, commonly referred to as the sugar alcohols, or polyols. Since the report on its formation by Pasteur in 1858 (Prescott and Dunn, 1959), glycerol production by yeast during alcoholic fermentation has generated considerable interest amongst scientists, winemakers, wine writers and biotechnologists alike. Glycerol is an economically important alcohol due to its wide application in the food, beverage, pharmaceutical and chemical industries (Scanes *et al.*, 1998). The formation of glycerol during the production processes of beverages such as wine, beer, saké and sochu, has received considerable attention based on the perceptions amongst winemakers and enologists that glycerol contributes to the quality of these products. The presence of glycerol in these beverages can mainly be ascribed to the metabolic activity of microorganisms associated with the fermentation process. Of these microorganisms, the yeast *Saccharomyces cerevisiae* has been the focus of intense research and scientific data relating to glycerol formation during alcoholic fermentation have been generated from a variety of scientific disciplines.

This review presents a brief overview of the major advances that have been made towards an understanding of the fundamental aspects regarding glycerol production by the wine yeast, as well as the relevance of glycerol production by microorganisms associated with grape and wine spoilage, for the purpose of quality control (section 2.2). The progress towards the manipulation of the glycerol levels formed by wine yeasts during the fermentation process (section 2.3) and the relationship between glycerol and wine quality are discussed (section 2.4).

In modern winemaking, the control of the quality of the end product is a prominent issue. In comparatively recent times, major technological advances have been made in the field of analytical chemistry and related instrumentation, which provide valuable tools with which to address several aspects regarding quality control during the winemaking process. These advances include continuous monitoring of the progress of the fermentation process through *on line* measurements of critical parameters such as glycerol; instrumentation designed for high-speed, multi-component routine analyses in grape juice, fermenting must and finished wine (section 2.5); and software applications designed for quality control and authenticity testing of grape-derived products, including verification of the source of glycerol in wine (section 2.6). The last section of this review (section 2.7) presents a general discussion on future perspectives regarding wine quality and highlights some novel strategies that are aimed at finding solutions for some of the challenges that face the modern wine industry.

2.2 GLYCEROL METABOLISM BY MICROORGANISMS ASSOCIATED WITH THE WINEMAKING PROCESS

The origin of glycerol in wine can almost exclusively be ascribed to the metabolism of microorganisms that are associated with the various stages of the winemaking process. These stages start with the raw material (the grapes) in the vineyard, and proceed through the harvesting and transportation of grapes, the preparation of must, the fermentation stage and finally to the maturation and storage of wine (Du Toit and Pretorius, 2000). The microorganisms therefore are those that are present on the vine and grape bunches, those associated with the harvester and transport equipment, the cellar equipment, and the yeast strain(s) introduced by the winemaker into the grape juice for the purpose of the fermentation (in the case of an inoculated fermentation).

2.2.1 GLYCEROL DERIVED FROM GRAPES

Glycerol is one of several polyols, which include 2,3-butanediol and erythritol (C_4 polyols), arabitol (C_5 polyol), sorbitol, mannitol and *meso*-inositol (C_6 polyols), commonly found in grapes (Ribéreau-Gayon *et al.*, 2000b). Of these, glycerol is quantitatively predominant, and levels most frequently measured in healthy grapes range from *ca*. 1 mg/L to 0.5 g/L. In the special case where the grapes are affected by rot, significant increases in the glycerol levels in the grape juice, together with several other complex chemical changes, were observed (Ravji *et al.*, 1988; Donèche, 1993; Sponholz, 1993; Zoecklein *et al.*, 2000). These aspects are discussed in more detail in section 2.2.4.1.

Recently it has been shown that the exposure of intact, healthy grapes to excessive high temperatures during transit from the vineyard to the cellar, resulted in complex chemical changes in their metabolic profiles (Dubernet *et al.*, 2001), including significant increases in the glycerol, arabitol, *meso*-inositol, mannitol and 2,3-butanediol levels, as well as significant decreases in the malic acid and lactic acid levels (Table 1).

These results were obtained during the harvest season of 2000 in Argentina, in one instance where the transport of the grapes, which lasted *ca.* 3 - 4 hours, was done during a daytime maximum temperature in excess of 40°C. Careful monitoring of the metabolic changes in the grapes over shorter time intervals showed that ethanol was also produced internally in the berries, but rapidly re-consumed. It was speculated that the changes were due to a rapid anaerobic metabolism in the grapes, although the physiological basis of this phenomenon is not completely understood at present (Dubernet *et al.*, 2001). These changes in the chemical composition of the grape juice have major negative implications for the quality of the juice, and ultimately the quality of the wine. Recently, a technological innovation has been designed to process and apply data of this nature for the purposes of monitoring grape quality and this aspect is discussed in section 2.2.4.1.

Table 1. Changes in the metabolic profiles of intact healthy grapes upon transit from the vineyard to the cellar during a day-time temperature of ± 40^0C (adapted from Dubernet *et al.*, 2001).

Parameter	Vineyard	Cellar
malic acid (g/L)	2.06	0.35
lactic acid (g/L)	0.5	0.30
total acidity (g/L)	4.17	3.92
pH	3.39	3.56
ethanol (% v/v)	0	0
glycerol (g/L)	1.4	4.1
mannitol (mg/L)	43	68
2,3-butanediol (mg/L)	761	1427
arabitol (mg/L)	442	660
meso-inositol (mg/L)	617	2349
3-methyl-1-butanol (mg/L)	128	301

2.2.2 GLYCEROL PRODUCTION BY THE WINE YEAST

Glycerol is produced as a by-product of yeast glycolysis (Figure 1) and the metabolic pathway that has been proposed for this process, starts with glyceraldehyde-3-phosphate (GA-3-P) which is an intermediate of the glycolytic pathway (Pronk *et al.*, 1996; Bakker *et al.*, 2001). The interconversion of GA-3-P to dihydroxyacetonephosphate (DHAP) is mediated by a triose phosphate isomerase enzyme, which has a greater affinity for DHAP than for GA-3-P, with the result that the equilibrium of this reaction favours the formation of GA-3-P. Glycerol is produced in two steps from DHAP and in the first step which is catalysed by NAD-dependent glycerol-3-phosphate dehydrogenase (Gpd) enzymes, DHAP is converted to glycerol-3-phosphate. Subsequently, the glycerol-3-phosphatase (Gpp) enzymes catalyse the conversion of glycerol-3-phosphate to glycerol (Gancedo *et al.*, 1968; Albertyn *et al.*, 1994; Norbeck *et al.*, 1996). The yeast has two differentially expressed isoforms for each of these enzymes, encoded by the genes *GPD1* and *GPD2*, and *GPP1* and *GPP2*, respectively. *S. cerevisiae* also possesses genes that may encode the enzymes dihydroxyacetonekinase and glycerol dehydrogenase, which could, respectively, catalyse the interconversion of DHAP to dihydroxyacetone and the latter to glycerol. It has been suggested that these enzymes could form a pathway for glycerol degradation in *S. cerevisiae,* but several aspects of this proposed pathway must still be clarified (Hohmann, 2002).

Glycerol metabolism is important for phospholipid biosynthesis and both DHAP and glycerol-3-phosphate are required as precursors for this process (Scanes *et al.*, 1998). The glycerol metabolic pathway is also important for the osmotic stress response (discussed in section 2.2.2.1) and the maintenance of the redox balance in *S. cerevisiae* (discussed in section 2.2.2.2).

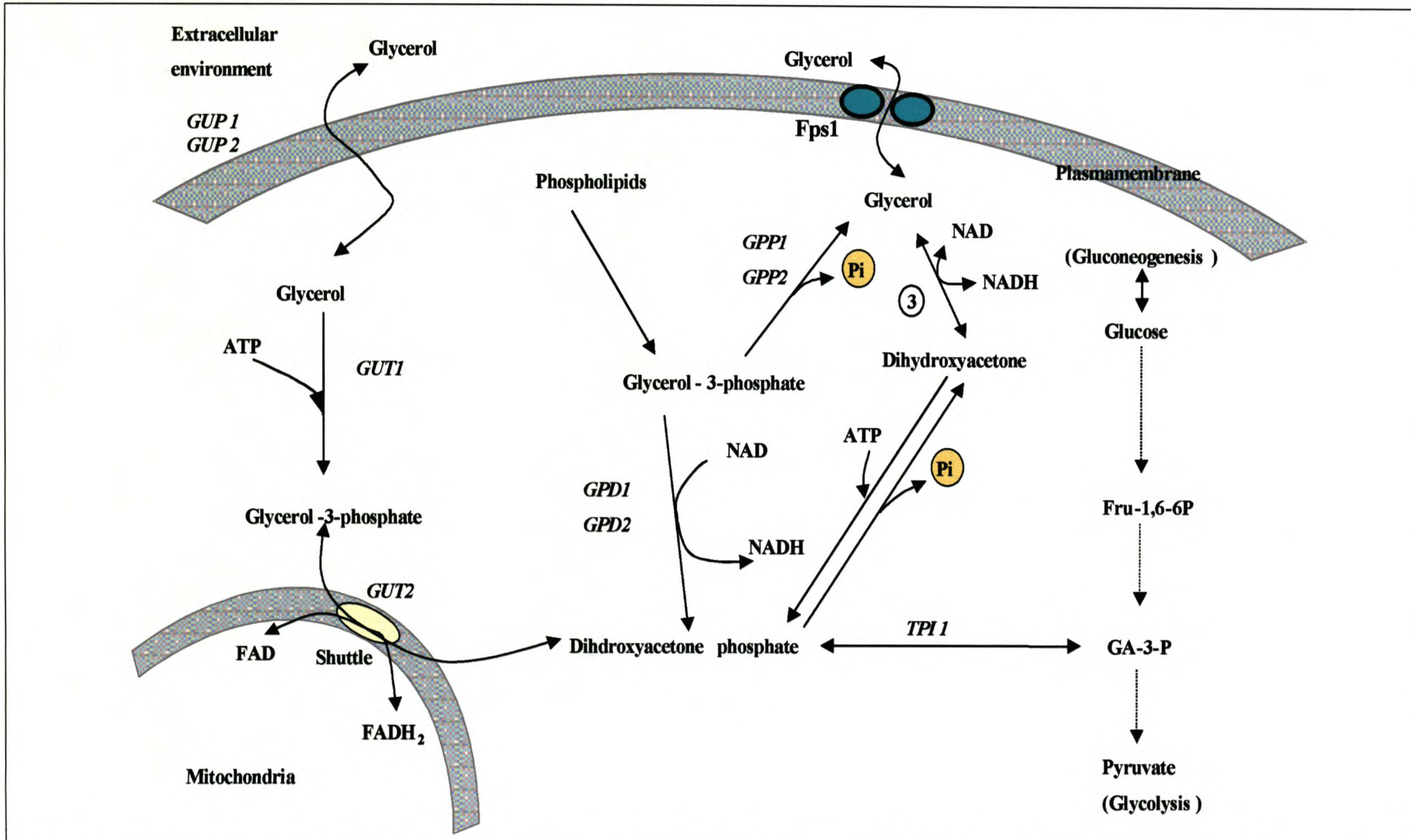


Figure 1. Proposed metabolic pathway for glycerol production by *Saccharomyces cerevisiae* (adapted from Prior and Hohmann, 1997).

The expression of *GPD1* (Albertyn *et al.*, 1994; Rep *et al.*, 1999) and *GPP2* (Norbeck *et al.*, 1996; Påhlman *et al.*, 2001) was found to be stimulated under various stress conditions, notably under osmotic stress. The expression of *GPD2* (Ansell *et al.*, 1997; Costenoble *et al.*, 2000) and *GPP1* (Costenoble *et al.*, 2000; Påhlman *et al.*, 2001) was stimulated under anaerobic conditions, pointing to a prominent role that these genes play in redox balancing.

Glycerol can also be utilised by *S. cerevisiae* during aerobic growth, as a source of carbon and energy in the absence of glucose, and it is thought that glycerol can be taken up from the external environment by the transmembrane proteins Gup1p and Gup2p which are encoded by the *GUP1* and *GUP2* genes, respectively (Holst *et al.*, 2000), (Figure 1). Upon uptake, glycerol is phosphorylated in the cytoplasm of the yeast to form glycerol-3-phosphate. This step is catalysed by glycerol kinase (Gut1p), that is encoded by the *GUT1* gene. Glycerol-3-phosphate is subsequently oxidised to DHAP in a reaction catalysed by the FAD-dependent glycerol-3-phosphate dehydrogenase enzyme (Gut2p), that is located on the inner mitochondrial membrane (Sprague and Cronan, 1977; Larsson *et al.*, 1998), (Figure 1). Gut2p is encoded by the *GUT2* gene, and forms an integral part of the glycerol 3-phosphate shuttle which plays an important role in redox balancing during the aerobic growth of *S. cerevisiae* (Larsson *et al.*, 1998).

2.2.2.1 Glycerol production in response to osmotic stress

Upon their introduction into grape juice, yeast cells are exposed to a medium that contains high concentrations of osmotically active compounds, notably glucose and fructose, which could cause a rapid leakage of water from the cell to the surrounding medium (Scanes *et al.*, 1998; Bauer and Pretorius, 2000). To prevent this, *S. cerevisiae* produces and accumulates glycerol in an attempt to equilibrate the internal osmotic pressure with that of the external medium. In this context glycerol is referred to as a compatible solute, since it is compatible with enzyme and membrane functions. The expression of the genes involved in the synthesis of glycerol in response to osmotic stress, respectively *GPD1* and *GPD2,* is partially under control of the HOG (**H**igh **O**smolarity **G**lycerol) pathway (Albertyn *et al.*, 1994; Norbeck *et al.*, 1996). The release of the cellular glycerol content to the external environment is a controlled process and is mediated via a specific export channel protein, Fps1 (Luyten *et al.*, 1995; Tamás *et al.*, 1999). All aspects of sensing and transduction of the osmotic stress response are currently the focus of intense research (Bauer and Pretorius, 2000; Hohmann, 2002).

2.2.2.2 Glycerol production in response to redox balancing

During growth on sugars, the preference of *S. cerevisiae* for NADH in dissimilatory reductions (such as the reduction of acetaldehyde to ethanol) is very strong (Pronk *et al.*, 1996; Bakker *et al.*, 2001). NADH/NAD^+ is considered as a "conserved moiety" and only catalytic quantities of these pyridine nucleotides are present in the yeast.

The yeast maintains a balance between the amounts of NADH and NAD^+ and the reduction of NAD^+ must be matched by the oxidation of NADH and *vice versa*. Under strictly anaerobic conditions, the dissimilation of sugar to pyruvate results in the reduction of NAD^+ in the conversion of glyceraldehyde-3-phosphate (GA-3-P) to glyceraldehyde-1,3-biphosphate (G-1,3-bP), (Figure 2). Pyruvate is decarboxylated to form acetaldehyde, which subsequently acts as an electron acceptor in the reoxidation of NADH.

The formation of biomass by the yeast during the early stages of the fermentation process, results in a surplus of NADH (Pronk *et al*., 1996; Bakker *et al*., 2001). Reoxidation of NADH is achieved through the formation of secondary by-products such as glycerol and 2,3-butanediol. Of these by-products, glycerol is predominant, and *ca.* 3.3% w/w of the sugar utilised under normal fermentative conditions is converted to glycerol (Oura, 1977). The redox balance therefore dictates that an increased specific rate of glycerol production (such as in strains with a stimulated glycerol production pathway), will be balanced by an increased production of oxidised metabolites, such as acetaldehyde and/or acetic acid, pyruvate, succinic acid, acetoin, diacetyl and 2,3-butanediol (Figure 2). These components have the potential to influence the sensory and flavour properties of wine. Studies aimed at the manipulation of glycerol production pathway of wine yeasts are discussed in more detail in section 2.3.

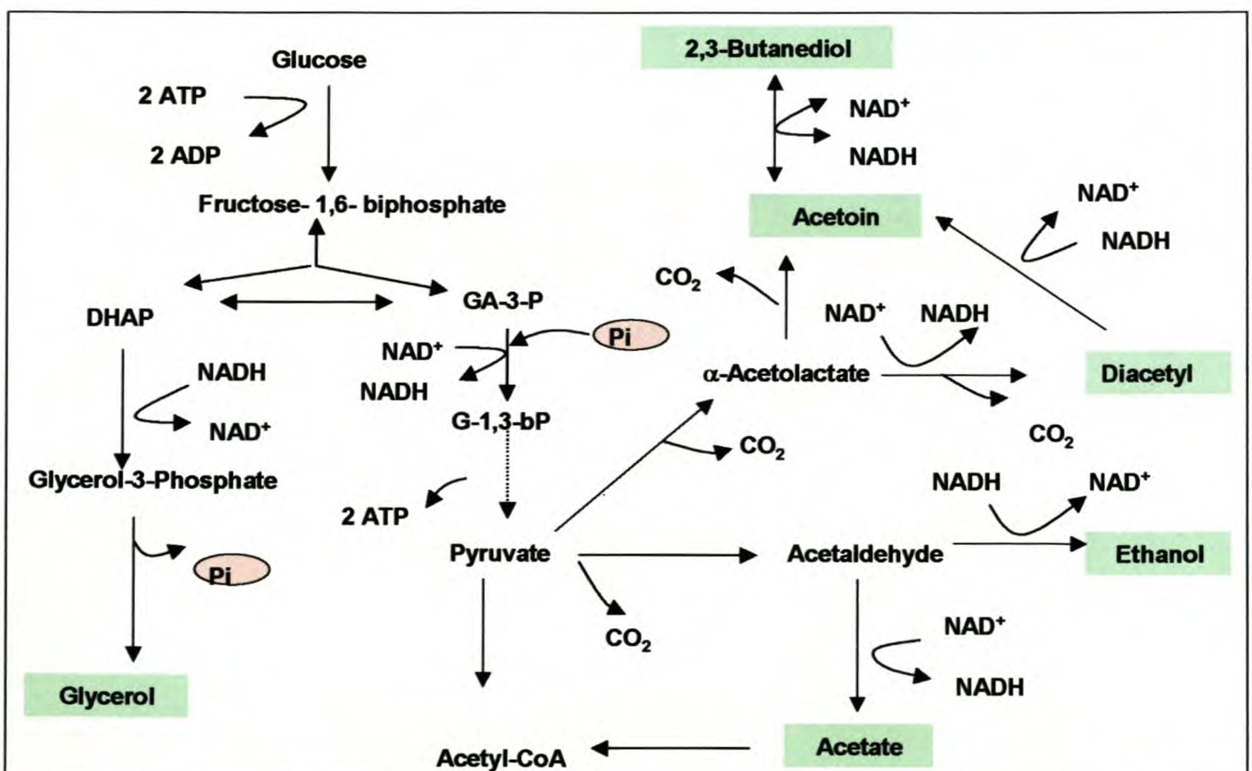


Figure 2. The formation of glycerol and other by-products in relationship to redox balancing in *Saccharomyces cerevisiae* (adapted from Pronk *et al*., 1996).

2.2.3 GLYCEROL METABOLISM BY NON- *SACCHAROMYCES CEREVISIAE* WINE YEASTS

Interest in the use of non-*Saccharomyces* yeast strains to improve the sensory qualities of wine is widespread and active research in this field has been undertaken in the last two decades (Romano *et al.*, 1992; Gil *et al.*, 1996; Ciani and Ferraro, 1998; Heard, 1999; Ferraro *et al.*, 2000; Jolly *et al.*, 2003a,b). The strains that have generated particular interest include *Candida stellata*, *C. colliculosa*, *C. pulcherrima* and *Kloeckera apiculata*. The glycerol metabolism of *C. stellata* is interesting in comparison to that of *S. cerevisiae*, since high levels of glycerol and low levels of acetic acid are formed by the former yeast during fermentative metabolism (Ciani and Ferraro, 1998). In a batch fermentation where immobilised *C. stellata* cells were used in combination with *S. cerevisiae,* the average glycerol levels formed (14.5 g/L), were 100% above those formed by *S. cerevisiae* alone, while the average levels of acetic acid and acetoin were lower (Table 2). In one study where *C. pulcherrima* was used together with *S. cerevisiae* for the production of Chenin blanc wine, the overall quality of the wine produced by the combined fermentation, was found to be higher over a consecutive production period of three years, than that produced by *S. cerevisiae* alone (Jolly *et al.*, 2003b).

Table 2. Metabolic profile (average values of duplicate fermentations) of batch fermentations in grape juice, using *Saccharomyces cerevisiae*, *Candida stellata* and combinations of these yeast strains (adapted from Ciani and Ferraro, 1998.)

Fermentation conditions	RS[a] (g/L)	Ethanol (% v/v)	Glycerol (g/L)	Acetic acid (g/L)	Succinic acid (g/L)	Acet-aldehyde (mg/L)	Ethyl-acetate (mg/L)	Acetoin (mg/L)
Saccharomyces cerevisiae control	10.6	15.4	6.4	0.45	0.45	38.4	73.8	9.2
Candida stellata immobilised cells	81.7	6.6	14.5	0.20.	1.83	130.8	22.3	60.3
Combined fermentation	0.6	14.5	12.9	0.27	0.29	24.4	84.8	16.3

[a]Residual sugar.

2.2.4 GLYCEROL METABOLISM BY MICROORGANISMS ASSOCIATED WITH WINE SPOILAGE

Glycerol is metabolised (produced and utilised) by microorganisms associated with wine spoilage, and its determination at various stages of the winemaking process provides information that is useful for the purposes of the detection and control of spoilage (Drysdale and Fleet, 1988; Donèche, 1993; Ribéreau-Gayon *et al.*, 2000a; Zoecklein *et al.*, 2000). In the next sections, the glycerol metabolism of microorganisms that are prominent during grape rot (section 2.2.4.1), and in the spoilage of wine during the fermentation process and storage of wine (section

2.2.4.2) is discussed. The relevance of glycerol as an indicator of grape- and wine spoilage is highlighted in section 2.2.4.3.

2.2.4.1 Spoilage due to grape rot

There are three stages during which the uncontrolled growth of microorganisms can alter the chemical composition of wine to such an extent that it detracts from the quality of the final product. The first stage involves the grapes that can become affected by diseases commonly referred to as "grape rot". Microorganisms that are typically associated with grape rot include moulds, acetic acid bacteria, lactic acid bacteria and yeasts. The etiology of grape rot is complex, both in terms of the factors that influence its development and in terms of the microbial population dynamics (Ravji *et al.*, 1988; Donèche, 1993; Sponholz, 1993; Zoecklein *et al.*, 2000). Complex changes in the chemical composition of grape berries that have been affected by rot have also been reported, and in several instances these changes included significant increases in the glycerol levels in affected grapes (Mühlberger and Grohmann 1962; Donèche, 1993; Zoecklein *et al.*, 2000).

Mühlberger and Grohmann (1962) reported on the glycerol levels of 11 different types of must, including samples of grapes that had been affected by rot. For the majority of the samples tested, the glycerol levels ranged from 1 - 4 g/L. In a few exceptional cases, the glycerol levels were between 5 - 10 g/L, and in one instance as high as 19.8 g/L. The latter sample was obtained from grape berries that had been affected by noble rot, and in that instance the prominent organism present in the must was *Botrytis cinerea.* The effect of sour rot on White Riesling grapes also resulted in an increase in glycerol concentration, from 1 mg/L in healthy grapes to 2.4 g/L in affected grapes (Zoecklein *et al.*, 2000). Other fungi that have been associated with glycerol production in berries affected by rot include *Rhizopus nigricans* (Rhizopus rot), *Aspergillus niger* (black-mould rot), *Penicllium italicum* (blue-mould rot), *Alternaria* and *Cladosporium* (Ravji *et al.*, 1988). Glycerol is also produced by some non-*Saccharomyces* yeasts that are frequently isolated from infected grapes, and these include microorganisms such as *Hanseniaspora uvarum* (*Kloecera apiculata*), *Metschnikowia pulcherrima*, *Hansenula anomala* and species of *Brettanomyces* (Sponholz, 1993).

Glycerol can also be utilised as a source of energy and carbon by other microorganisms that are frequently associated with grape rot, and these include acetic acid bacteria such as *Gluconobacter oxydans*, *Acetobacter pateurianus* and *A. aceti*, as well as lactic acid bacteria such as *Lactobacillus* and *Leuconostoc* (Drysdale and Fleet, 1988; Sponholz, 1993; Du Toit and Pretorius, 2000).

2.2.4.2 Spoilage during the fermentation process and upon storage of wine

One of the more serious defects caused by the spoilage of wine relate to the formation of an unpleasant bitter taste (Sponholz, 1993; Du Toit and Pretorius, 2000). This defect has been ascribed to the metabolism of glycerol by strains of

Lactobacillus, *Leuconostoc*, *Oenococcus* and *Pediococcus*. Strains that possess a dehydratase enzyme, convert glycerol into 3-hydroxypropionaldehyde, which in turn, can be converted to acrolein under certain conditions which include the exposure of wines to excessive heat during storage. The metabolic pathway that has been proposed for this reaction is shown in Figure 3. The association between acrolein and the phenolic groups of the anthocyanins is thought to be responsible for the unpleasant bitter taste, although to date several aspects of this interaction remain unclear.

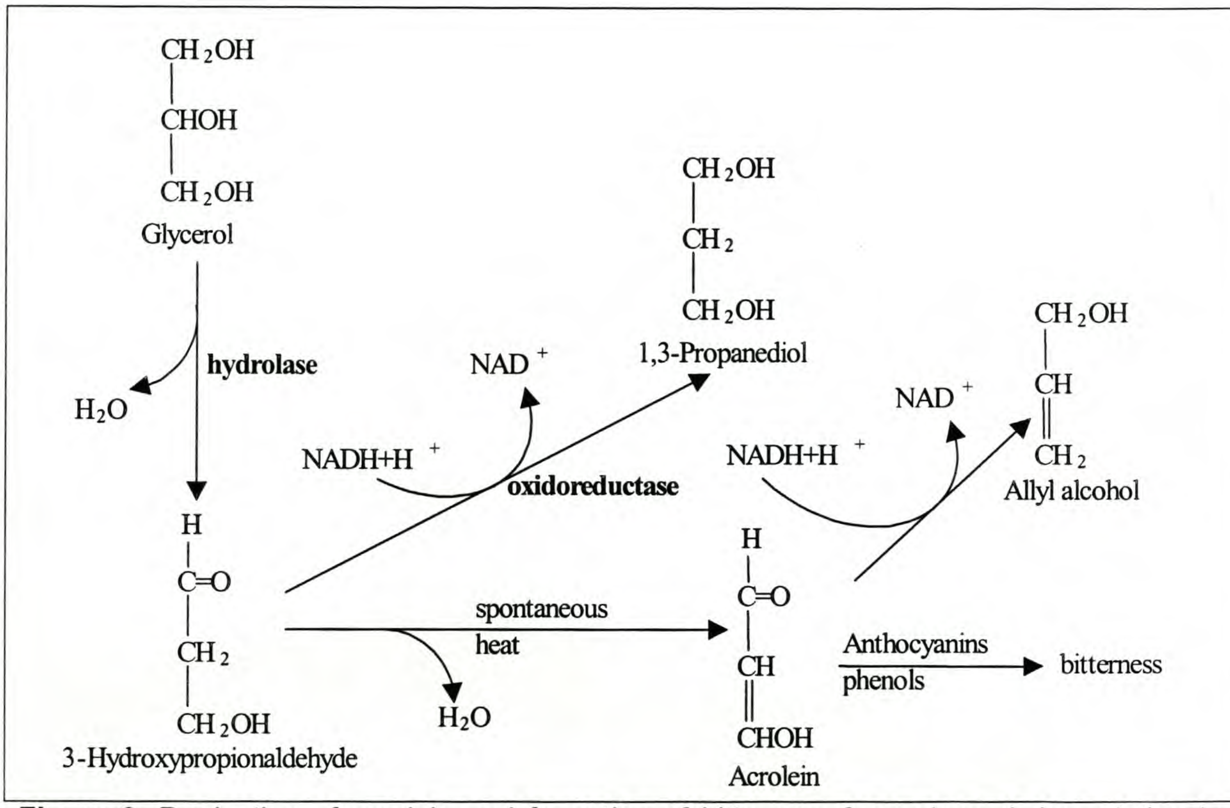


Figure 3. Production of acrolein and formation of bitterness from glycerol degradation by *L. brevis* (adapted from Sponholz, 1993).

2.2.4.3 Glycerol as an indicator of grape and wine spoilage

Throughout the history of winemaking, wine spoilage has always been an issue of high prominence. Spoilage may affect the appearance, aroma, flavour and other sensory properties of wine, and therefore has important economic implications (Sponholz, 1993). In the modern wine industry, microbial spoilage of wine is a focus area of research, and major advances have been made towards the identification of the species that cause the spoilage, as well as towards the development and use of bio-preservatives for the control of spoilage (recently reviewed by Du Toit and Pretorius, 2000).

The early detection of wine spoilage is of critical importance, although this is a challenging problem. In the case of grapes affected by rot for instance, it is not always possible to visually detect the infection, and the detection of off-odours and off-flavours by human smell and taste in the very early stages of wine spoilage, is equally challenging. The chemical changes that are observed in spoiled grape juice or wine reflect the sum total of the metabolic activity of all the microorganisms associated with the particular type of rot, but these changes are frequently also characteristic of the metabolic activity of the predominant organisms. It is therefore possible to establish relationships between the chemical changes and the type of spoilage (Sponholz, 1993; Boulton *et al.*, 1996). In this respect, glycerol is a useful metabolite to monitor, since the changes in the glycerol levels during some types of rot, e.g. rot due to *Botrytis cinerea*, are relatively big and hence significant. Furthermore, the analytical techniques available for the quantification of glycerol in grape juice and wine have been optimised during the last few years and at present, glycerol can be determined accurately at high-speed and in a continuous fashion in fermentation media and in wine (for more detail refer to section 2.5).

Grape- and/or wine spoilage can not be monitored and explained in terms of one or a few metabolites only, and relationships between spoilage and the metabolites formed under these conditions, are seldom of a linear nature (Dubernet *et al.*, 2001). Recently, innovations aimed at monitoring the quality of grape juice and wine have been introduced to the wine industry, and these include software applications that are based on the mathematical treatment of the metabolic profiles associated with different types of spoilage. One such an application that has been developed to monitor grape quality, involves the so-called *Sanitary Index*, which is an additional feature of the WineScan FT 120 instrument (Application Note 183, 2001, Foss Electric, Denmark). This instrument utilises Fourier transform infrared spectroscopy and is discussed in detail in section 2.5.3. Based on the analytical data generated by the instrument for a specific sample of grape juice, a numerical index value is calculated that provides an objective evaluation of the sanitary state and overall quality of the grapes. The index value is calculated using a mathematical equation that was developed through the application of artificial neural networks to the metabolic profiles associated with different types of grape spoilage. Future developments pertaining to the *Sanitary Index* are discussed in section 2.7.

2.3 MANIPULATION OF GLYCEROL LEVELS

2.3.1 THE MANIPULATION OF GLYCEROL LEVELS BY MEANS OF ENVIRONMENTAL FACTORS AND PROCESS TECHNOLOGY

In small-scale laboratory fermentations of must, the amount of glycerol formed by *S. cerevisiae* varies considerably, and concentrations ranging from 4 g/L – 11 g/L have been reported (Rankine and Bridson, 1971; Ough *et al.*, 1972; Prior *et al.*, 2000; Ribéreau-Gayon *et al.*, 2000a). This variation has been ascribed to the influence that the environmental parameters prevailing during the fermentation process, as well as the yeast strain that dominates the fermentation, have on the final amount of glycerol formed (Radler and Schütz, 1982; Gardner *et al.*, 1993; Remize *et al.*, 2000).

In terms of the environmental factors that affect glycerol production, the traditional experimental approach of keeping the fermentation conditions constant and changing one factor at a time, has provided valuable data as to the influence that pH (Rankine and Bridson, 1971); fermentation temperature (Rankine and Bridson, 1971; Ough *et al.*, 1972; Gardner *et al.*, 1993); agitation or aeration (Gardner *et al.*, 1993); sulphur dioxide levels (Rankine and Bridson, 1971) and the nitrogen source in the culture medium or must (Omori *et al.*, 1995; Albers *et al.*, 1996) have on the formation of this compound. A recent review by Scanes *et al.* (1998) presents a detailed account of the environmental factors influencing the production of glycerol by yeasts.

It has frequently been reported that the glycerol concentrations in dry white table wines (*ca.* 5 - 8 g/L) are significantly lower than those most frequently reported for dry red table wines (*ca.* 8 - 12 g/L) (Mattick and Rice, 1970; Rankine and Bridson, 1971; Nieuwoudt *et al.*, 2002a). This observation is usually ascribed to the higher fermentation temperatures that are being used for the production of red wines, as opposed to those used for the production of white wines (Ribéreau-Gayon *et al.*, 2000a). It has been speculated that the metabolic pathway for glycerol production might be stimulated at higher temperatures due to the faster build-up of biomass at the higher temperatures (Rankine and Bridson, 1971), but to date several molecular aspects of this phenomenon remain unclear. Furthermore, the available experimental data are insufficient to establish the relationship between the glycerol concentration and the fermentation temperature *per se.* Upon an upward shift in fermentation temperature, complex indirect effects on the biochemical reactions of yeasts as well as the composition of the strain population in must have been reported (Torija *et al.*, 2002).

In recent years the use of immobilised cell systems in winemaking has received considerable interest, and active research on the various types of support systems used for immobilisation and the technological aspects of these fermentations is currently being undertaken (Kourkoutas *et al.,* 2002; Balli *et al.*, 2003). There is particular interest in using immobilised non-*Saccharomyces* yeasts in combination with *S. cerevisiae* for the production of wine (Ciani and Ferraro, 1998). In this respect *Candida stellata* offers an interesting option to manipulate the amount of glycerol formed during the fermentation process. The average glycerol concentration formed by *C. stellata* are much higher that those formed by *S. cerevisiae*, while the average concentrations of volatile acidity are lower than those formed by the wine yeast (see section 2.2.3), (Ciani and Ferraro, 1998).

2.3.2 THE MANIPULATION OF GLYCEROL LEVELS BY GENETIC AND MOLECULAR TECHNIQUES

In small-scale laboratory fermentations in must, the amount of glycerol formed by *S. cerevisiae* varies considerably, and concentrations ranging from 4 g/L – 11 g/L have been reported (Rankine and Bridson, 1971; Ough *et al.*, 1972; Prior *et al.*, 2000). Although environmental factors have a significant influence on the levels of glycerol formed by yeasts, evidence suggests that the variation is also due to genetic diversity amongst the strains (Rankine and Bridson, 1971). Accordingly, *S. cerevisiae* strains have been designated as "low" or "high" glycerol producers on the basis of characteristic amounts of glycerol formed under standard conditions (Prior *et al.*, 1999).

The formation of glycerol by wine yeasts is considered as a favourable attribute and it has been identified as one of the targets for their genetic improvement (Thornton, 1983; Pretorius, 2000). Here the long term aims include optimising the glycerol concentrations in wine and the development of a mechanism for redirecting the carbon flux away from ethanol towards glycerol, in order to decrease the ethanol concentrations formed in wine (Eglinton *et al.*, 2002).

Eustace and Thornton (1987) undertook a breeding strategy in order to select yeast strains with enhanced glycerol production. The parental strains were hybridised by spore-cell matings and the average glycerol concentrations formed by the hybrid strains (*ca.* 11 g/L) were much higher than the concentrations formed by either of the parental strains (ca.3 – 6 g/L). Prior *et al.* (1999) used a spore-spore mating of a commercial wine yeast and a yeast strain that was isolated from a natural fermentation. Several of the spore clones obtained in this study produced glycerol concentrations in excess of 11 g/L in small-scale laboratory fermentations. The sensory properties of Chardonnay wines that were produced under enological conditions with some of these hybrid strains were evaluated, and it was found that the formation of higher amounts of glycerol was accompanied by increases in the concentrations of acetic acid, 2,3,-butanediol, volatile acidity and acetaldehyde (Prior *et al.*, 2000). The overall quality and aroma of the experimental wines were rated lower than that of a control wine made with a commercial wine yeast.

There has been considerable interest in manipulating the glycerol levels formed by yeasts through the use of recombinant DNA techniques (Michnick *et al.*, 1997; Remize *et al.*, 1999; De Barros Lopes *et al.*, 2000; Eglinton *et al.*, 2002). In one study over-expression of the glycerol 3-phosphatase dehydrogenase gene of *S. cerevisiae (GPD2)*, led to a substantial increase in the glycerol concentration formed in Chardonnay wine produced by the recombinant strain (16.5 g/L), as opposed to the amount of glycerol formed by the parental strain (7.9 g/L). The increase in the glycerol concentration formed by the recombinant strain was accompanied by an increase in the acetic acid concentration (1.02 g/L for the recombinant strain, as opposed to 0.58 g/L for the parental strain) (De Barros Lopes *et al.*, 2000). It was of interest to note that the altered glycerol production of the recombinant strain reduced the ethanol concentration formed during the production of the wine by 6 g/L, which highlights the potential of this strategy for decreasing the amount of ethanol formed in wine. The increase in the acetic acid concentration was expected, since yeast glycerol metabolism serves, amongst other functions, as a redox sink for the surplus of NADH formed during assimilatory sugar metabolism. A stimulation of the glycerol formation pathway would therefore result in the formation of more oxidised metabolites such as acetic acid. Subsequent efforts were directed towards decreasing the acetic acid formation through deleting the aldehyde dehydrogenase gene (*ALD6*) which catalyses the conversion of acetaldehyde to acetic acid (Eglinton *et al.*, 2002).

2.4 THE RELATIONSHIP BETWEEN GLYCEROL AND WINE QUALITY

Glycerol has traditionally been associated with various organoleptic properties of wine. Terms that are most frequently used to describe these properties include "viscosity", "mouth-feel", "body" and sweetness (Hickinbotham and Ryan, 1948; Noble and Bursick, 1984). It has also been suggested that glycerol enhances the flavour of beverages such as wine, saké and shochu (Omori *et al.*, 1997). This could suggest that glycerol has an indirect effect on aroma and flavour components in these beverages. Since the early years of the 20^{th} century, winemakers and enologists have placed considerable emphasis on the positive contribution that glycerol is perceived to make towards wine quality. In general, higher glycerol concentrations than those normally found in wine were (and still are) considered to be necessary to further improve the quality of wine. Over the years, numerous research projects have been undertaken to manipulate the amounts of glycerol formed by the wine yeast during the fermentation process (see section 2.3), resulting in major advances towards our understanding of the fundamental aspects of glycerol metabolism by wine yeasts. Yet, only a limited number of studies have been undertaken to investigate the contribution of glycerol to wine quality.

The following sections present a brief historical overview that highlights some of the early developments pertaining to glycerol and wine quality (section 2.4.1), followed by an account of the experimental data that have been generated on the contribution of glycerol to viscosity and sweetness in wine (section 2.4.2) and the effect of glycerol on the volatility of aroma components in wine (section 2.4.3). Finally, the progress towards understanding the relationship between glycerol and wine quality is evaluated (section 2.4.4).

2.4.1 AN HISTORICAL OVERVIEW

Louis Pasteur reported on the formation of glycerol during alcoholic fermentation in 1858 and the impact that this finding would prove to have in a relatively short period of time on developments in the field of biotechnology, as well as in the field of enology, was considerable (Prescott and Dunn, 1959). In the period leading up to World War I, the demand for glycerol and acetone in the explosives industries of the world was greater than the chemical industries could supply and attention was focused on the production of glycerol via microbial fermentation. The development in the early years of the 20^{th} century of the yeast sulphite-steered glycerol fermentation process in Germany and the bacterial acetone-butanol fermentation process in

England are accredited for introducing the era of traditional microbial biotechnology (Demain, 2000).

In an early research paper on glycerol and wine by Hickinbotham and Ryan (1948), a detailed account of the research projects that were done from *ca*. 1900 to 1945 is given and the paper also offers interesting insights into the perspectives amongst enologists of that time regarding glycerol and wine quality. The effectiveness of glycerol in ameliorating the burning taste of wines containing too much alcohol, or wines that were made by the addition of spirit was apparently well known in these early years, and both in Europe and Australia early legislation was in place in attempts to regulate the addition of glycerol to wine. The early research projects were focused both on the development of analytical techniques to quantify glycerol in wine, as well as strategies to manipulate the fermentation conditions to favour increased production of glycerol by wine yeasts (Hickinbotham and Ryan, 1948). By 1911, more than 4000 German as well as several hundred other European wines were analysed for their glycerol content. On factors influencing glycerol production during fermentation, the summary contained in the article by Hickinbotham and Ryan was not too flattering and they wrote: "...it would serve no useful purpose to review the literature in detail, because the conclusions reached on almost every aspect of the problem, by a great number of eminent investigators, are extraordinarily confused and conflicting"! Notable at this stage was the absence of substantial, and in many cases *any* sensory data confirming or contradicting the general perceptions regarding glycerol and wine quality. It is of interest to note that the authors expressed some reservation with respect to the emphasis that was placed on glycerol and wine quality.

2.4.2 THE CONTRIBUTION OF GLYCEROL TO THE VISCOSITY AND SWEETNESS IN WINE

The sensory properties of any component in wine (or food) can not be understood without taking the physical and chemical properties of this component into account. Glycerol is a simple saturated alcohol and is a member of an important group of alcohols in wine, collectively referred to as the sugar alcohols, or polyols (Ribéreau-Gayon *et al.*, 2000b). The polyols are characterised by the presence of several hydroxyl groups in the same molecule. Overall, the glycerol molecule is uncharged, but the O-H bond in the hydroxyl group is highly polar due to the large difference in electron negativity between oxygen and hydrogen. Neighbouring glycerol molecules can therefore interact through hydrogen bonding, and this property has profound effects on the physical properties of the component (Dillard and Goldberg, 1972). In general, an increase in the number of hydroxyl groups in a molecule (and hence the degree of hydrogen bonding) leads to an increase in the boiling point (the boiling point of ethanol is 78°C while that of glycerol is 290°C). Hydrogen bonding also leads to increases in the viscosity, sweetness and solubility of the component in polar solvents, such as water or ethanol. Under the physiochemical conditions normally prevailing in wine, glycerol is amongst the less volatile components and has an extremely high odour perception threshold. It will therefore not be directly perceived in the aroma of wine.

Although few studies have been undertaken to investigate the influence of glycerol on the organoleptic properties of wine, one study made a systematic evaluation of the thresholds and minimum difference concentrations of various constituents, including ethanol, organic- and inorganic acids, sugars, some volatile components and glycerol in wine, using a triangular test procedure (Berg *et al.*, 1955). In excess of 2000 sets of triangles were tasted over a period of six months by two tasting panels (7 members each), one consisting of experienced tasters and the other of inexperienced tasters. Glycerol in water (threshold 3.8 g/L) was perceived to be sweeter than glucose (threshold 4.0 g/L), but not as sweet as fructose or sucrose (thresholds respectively 1.5 g/L and 3.0 g/L). Expanding these experiments to aqueous matrices containing other wine constituents, both ethanol (10% v/v) and acidity (pH 3.0 - 3.4) were found to increase the sweetness perception threshold for glycerol to at least 10 g/L (Hinreiner *et al.*, 1955a). Using the same test procedure, the minimum difference threshold for glycerol in dry white was found to be 9 g/L and 13 g/L in red wine (Hinreiner *et al.*, 1955b). It is of interest to note that for the majority of the constituents tested, the acuity of the inexperienced tasters was not significantly lower than that of the experienced panel. Furthermore, glycerol was one of the components for which the largest differences in individual response were observed.

In another project, the contribution of glycerol to the perceived sweetness and viscosity in a Thomson Seedless white wine was evaluated (Noble and Bursick, 1984) and it was found that the amount of glycerol required to perceptibly increase the sweetness of the base wine was 5.2 g/L. This value was approximately half the value reported by Hinreiner *et al.* (1955b). The effect of increased glycerol concentrations in the wine on physical viscosity (measured instrumentally as milliPascal-seconds using a capillary viscometer) was found to correlate linearly in the concentration range 2.5 - 30 g/L (Noble and Bursick, 1984). The viscosity minimum difference threshold that could be perceived by the panel members was evaluated through the addition of a tasteless xanthan gum to the wine, and was found to be 0.141 milliPascal-seconds. Through extrapolation it was therefore concluded that a minimum concentration of 25.8 g/L of glycerol would be required to perceptibly increase the viscosity of the wine.

2.4.3 THE EFFECT OF GLYCEROL ON THE PERCEIVED AROMA OF WINE

There is growing recognition that the volatility and hence the release of flavour components from wine can be influenced by interactions of the volatile components with other components in wine, such as polyphenols (Dufour and Bayonove, 1999), proteins and polysaccharides (Voilley *et al.*, 1991) and sugar, ethanol and lipids (Voilley and Lubbers, 1999). The nature of these interactions can be reversible van der Waals interactions, hydrophobic interactions and hydrogen bonding. Faint odour intensity in wine is usually interpreted as a defect and could implicate the fixation of aroma substances (Voilley *et al*., 1991). Due to the very low volatility of glycerol, it will not directly be perceived in the aroma of wine. To date, glycerol-aroma component interactions in wine have received very little attention. In one study a purge and trap analysis was used to determine the effect of glycerol concentration on the volatility rate constants of a selection of aroma components in a model wine and a white wine (Lubbers *et al*., 1994). No significant effect of glycerol over the range of 5 – 20 g/L was found and the authors concluded that the relationship that is often believed to exist between the amount of glycerol in wine and the changes in the perceived taste and/or aroma of the wine, is a misinterpretation.

2.4.4 WHY SO LITTLE PROGRESS AFTER 100 YEARS?

The interest in glycerol and its perceived contribution to wine quality has a long history. To date, however, very little progress has been made towards our understanding of the nature of this contribution. It is natural to speculate why such a situation has arisen, and a critical evaluation of the literature regarding glycerol and wine quality provides insight into this aspect. A detailed analysis of possible reasons for what appears to be misinterpretations regarding this topic is presented in the article *Glycerol and Wine Quality: Fact and Fiction* (Nieuwoudt *et al*., 2002b), which can be found in Appendix 1 of this dissertation.

2.5 ANALYTICAL TECHNIQUES FOR THE QUANTIFICATION OF GLYCEROL IN GRAPE JUICE, FERMENTING MUST AND WINE

The quantification of glycerol at various stages of the winemaking process provides useful information regarding issues that are directly or indirectly related to quality control. These issues include the evaluation of the sanitary state and overall quality of grapes (Zoecklein *et al*., 2000; Dubernet *et al*., 2001), evaluating the fermentative properties of wine yeasts (Barre *et al*., 1993; Dequin, 2001), monitoring the progress of the fermentation process (Compagnone *et al*., 1998; Esti *et al*., 2003) and providing a preliminary means for the detection of the illegal addition of glycerol to wine (Würdig and Woller, 1989). To date, glycerol determinations are seldom done on a routine basis in grape juice and wine, although the need for fast and accurate methods is clear. Several of the available analytical methods are not well suited for routine analysis, since they cannot be easily automated and have one or more drawbacks such as long assay times, high individual assay costs, requirements for sample handling (dilution, filtration, centrifugation), the need for highly skilled operators and the generation of toxic waste.

Methods that are currently being used for the quantification of glycerol in fermentation broths and wine include conventional chromatographic techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC) (see section 2.5.1), as well as spectrophotometric methods which include a colorimetric assay and enzymatic methods (see section 2.5.2.1). Recent advances towards *on line* and *at line* quantification of glycerol in industrial fermentation processes have been made through the development of biosensors, and several types of these sensors have been developed (see section 2.5.2.2). In the last two to three years, instrumentation based on Fourier transform infrared spectroscopy (FT-IR) has also had a major impact on the routine analysis of components (including glycerol) in grape juice, must under fermentation and wine (see section 2.5.2.3). The application of FT-IR for the quantification of glycerol in finished wine and in fermenting must was a focus point for two research projects presented in this dissertation and this section is therefore presented in some detail.

A few application studies that highlight the potential of some of the more recent analytical approaches for the quantification of glycerol are presented and discussed in the context of how the current analytical applications have evolved to meet the needs of the modern winemaking environment.

2.5.1 CHROMATOGRAPHIC METHODS

A procedure employing GC for the quantification of glycerol in wine has been described which involved the extraction of the glycerol component using 70-80% ethanol, as well as a derivatisation step (Avellini, 1977). Trimethylsilyl derivatives of glycerol were analysed by GC and a standard error in recovery of ± 0.02g / 100 mL was reported. It was estimated that an output of forty samples in a two-day period could be achieved with this application.

HPLC is extensively used for the simultaneous quantification of carbohydrates, alcohols and polyols in wine and in yeast fermentation broths. Sample preparation usually involves filtration or centrifugation, as well as a dilution step. Procedures can easily be automated and the run times typically range from *ca*. 30 to 60 minutes. Anion exchange liquid chromatography with pulsed amperometric detection (HPAE-PAD) was employed for the quantification of glycerol in wine, wort, beer and soft drinks (Klein and Leubolt, 1993). HPAE-PAD was also employed, using a Dionex DX-500 system (Sunnyvale, CA) and a CarboPac MA1 analytical column, for the quantification of glycerol (as well as several other alcohols and sugars) in fermentation broths of *S. cerevisiae* with detection limits of *ca*. 1 ng for the components tested (Hanko and Rohrer, 2000). Calull (1992) reported on the quantification of glycerol in grape juice as well as sweet wine, using ion-exchange chromatography and refractive index detection. In this study a solid-phase extraction step using a strong anion exchanger was included to avoid the interference of sugar. The accuracy of the results using this approach was in good agreement with results obtained by the conventional enzymatic method (see section 2.5.2.1).

2.5.2 NON-CHROMATOGRAPHIC METHODS

Non-chromatographic methods for the quantification of glycerol in fermentation broths and wine have the advantage that very little (if any) sample preparation is usually required. Quantification of components of interest can usually be done with a high degree of specificity in mixtures without any preliminary separation step. The assays are usually also rapid in terms of analysis time. These aspects make the application of some of the methods discussed below very attractive for the purposes of routine analysis, but also in the research environment in instances where large volumes of analytical work are required.

2.5.2.1 Colorimetric and enzymatic methods

A relatively fast colorimetric method for the estimation of glycerol in fermentation solutions has been described that involves the oxidation of glycerol by periodic acid to formaldehyde using an oxidation period of five minutes to minimise the interference from glucose. Formaldehyde is determined directly in the oxidation mixture in a colour reaction with chromotropic acid and absorbance is read at 540 nm (Lambert and Neish, 1950). This reaction occurs in the presence of excess quantities of sodium arsenite, and although the method is relatively fast, the generation of large quantities of toxic waste is a major disadvantage. Sugar concentrations in excess of 10 g/L in the fermentation broths can be a source of interference when using this procedure.

Glycerol is frequently quantified in wine and fermentation media by the use of an enzymatic method that is available as a kit from Roche. The method involves three successive enzymatic steps and the amount of NADH (which is stoichiometric to the amount of glycerol in the assay mixture) is measured by its absorption of light at 334 nm, 340 nm or 365 nm (Drawert and Kupfer, 1963). Sample preparation usually only involves a dilution step (if necessary). An output of 20-30 samples per hour can easily be achieved.

2.5.2.2 Biosensors

The past decade or two has seen major developments in the application of biosensors for the purpose of real-time *on line* monitoring of alcoholic fermentation in industrial-scale processes (for a recent review see Mello and Kubota, 2002). Currently, a wide array of biosensors are commercially available and numerous types of chemical components can be measured in a variety of foodstuffs and beverages The application of biosensors involves three general stages and these are (i) a signal recognition stage which frequently employs an enzyme as the recognition element; (ii) the transduction of the signal which can be via an electrochemical, optical or thermal route; and (iii) the processing of the signal against an established calibration. The monitoring of a red wine fermentation in wineries was achieved by the use of biosensors to quantify glycerol, glucose, fructose and ethanol (Compagnone *et al.*, 1998; Esti *et al.*, 2003). Platinum-based probes covered with immobilised glycerokinase and glycerol-3-phosphate oxidase enzymes were used as recognition elements for glycerol. Using a similar application, the glycerol concentrations in finished wine were quantified (Kiranas *et al.*, 1997) and the accuracy of the determinations was comparable to that obtained by the conventional enzymatic method. Some of the problem areas when using biosensors include chemical instability and a limited life span of the sensors and currently these aspects are being improved (Mello and Kubota, 2002).

2.5.3 INFRARED SPECTROSCOPY

Mid infrared - and near-infrared spectroscopy are not new applications in the field of analytical chemistry. In the last decade, applications of these technologies in the agricultural, pharmaceutical, environmental, petrochemical, medical and biological industries have increased at a staggering rate and the impetus for future developments is gaining momentum. It has aptly been said that at present "applications are running way ahead of theory" (Ian Murray, 11th International Conference on Near Infrared Spectroscopy, Córdoba, Spain, 2003). Recent improvements in instrumentation, together with the use of innovative software applications based on multivariate statistical methods have optimised these technologies, and the potential applications in viticulture and enology are numerous. These applications are both of a qualitative and quantitative nature and are thus ideally suited to meet some of the analytical challenges facing modern winemaking. Several of the qualitative applications for authenticity testing, will be discussed in section 2.6.

In the following sections, the application of FT-IR as an analytical tool in the routine wine laboratory is discussed. A brief overview of the basic theoretical principles pertaining to this technology is provided, and some of the main features of modern instrumentation are highlighted. Finally, applications for the purposes of quantitative analysis of components in wine, including glycerol, are presented.

2.5.3.1 Theoretical background

Infrared spectroscopy is based on the interaction of matter with light in the mid-infrared region of the electromagnetic region (Smith, 1999). This region is frequently divided into the near-, mid- and far-infrared regions (Table 3), and depending on the standpoint of the field of application or instrumentation, the division differs between different sources of reference (Pavia *et al*, 1999; Smith, 1999). Smith (1999) defines the near-infrared region as ranging from 14000 – 4000 cm^{-1} and the mid-IR region as ranging from 4000 – 400 cm^{-1}.

Table 3. Divisions in the infrared region of the electromagnetic spectrum (adapted from Pavia *et al.*, 2001)

Region	**Wavelength (λ) range, μm**	**Wavenumber ($\bar{\nu}$) range, cm^{-1}**	**Frequency (ν) range, Hz**
Near	0.78 to 2.5	12800 to 4000	3.8×10^{14} to 1.2×10^{14}
Mid	2.5 to 50	4000 to 200	1.2×10^{14} to 6.0×10^{12}
Far	50 to 1000	200 to 10	6.0×10^{12} to 3.0×10^{11}

The chemical bonds of infrared active groups such as C-C, C-H, O-H, C=O and N-H each have a different natural frequency of vibration and upon absorption of infrared radiation, the amplitude of the vibrational frequencies increases. The intensities of the measured frequencies are processed through a series of mathematical procedures, including Fourier transformation (see section 2.5.3.2) to an absorbance - or transmittance spectrum that is characteristic of the chemical bonds in a particular molecule. An infrared spectrum of a molecule therefore provides important information with respect to its chemical structure. The modes of vibration of the infrared active chemical bonds are typically of the *stretching* or *bending* type (Table 4).

Table 4. Vibrational modes of chemical bonds in the infrared region (adapted from Pavia *et al.*, 2001)

Bond	**Functional Group**	**Type of Vibration**	**Wavenumber (cm^{-1})**
C-H	Methilene	asymmetric bend	1465
		symmetric stretch	~2853
		asymmetric stretch	~2926
		scissoring bend	~1450
		rocking bend	~720
		wagging bend	~1250
		twisting bend	~1250
C=O	Aldehyde		1740-1720
	Ketone		1725-1705
	Carboxylic acid		1725-1700
	Ester		1750-1730
C-O	Alcohols, ethers, esters, carboxylic acids, anhydrides		1300-1000
O-H	Alcohols, phenols		
	Free		3650-3600
	H-bonded		3400-3200
	Carboxylic acids		3400-2400
N-H	Primary and secondary amines and amides		
	stretch		3500-3100
	bend		1640-1550

2.5.3.2 Modern instrumentation

Since the 1980's, instrumentation for the mid-infrared region has undergone major improvements, particularly in the development of the so-called *multiplex* instruments (Skoog *et al.*, 1997). The term *multiplex* in this context refers to instrumentation where all the components of a signal (for example the composite absorbance of all the components in a sample over the entire optical wavenumber range), are collected simultaneously at the detector. This is in contrast to instruments that employ filters or monochromators to select only a portion of the information in the spectrum. In order to determine the magnitude of each component represented in the original signal generated on a multiplex instrument, the information must first be *decoded* into its individual components.

Mostly, this decoding utilises a mathematical procedure known as Fourier transformation that was developed by a French mathematician Joseph Fourier (1768-1830). The essence of this transformation is that the basic input signal, which is recorded in a time-domain, is transformed by the Fourier principle, and plotted in a frequency-domain. Fourier transformation is a widely applied mathematical procedure and is not unique to infrared spectroscopy. The advantages related to the use of this approach include a very good signal-to-noise ratio and high resolving power. Furthermore, since all the information in the signal reaches the detector simultaneously, the data collection process can be completed within one second or less (Skoog *et al.*, 1997). Currently, FT-IR analytical instruments are available where the signal collection is completed in *ca*. 1 to 2 seconds, and the signal processing is completed in an analysis time of *ca*. 28 seconds, which results in a total assay time per sample of *ca*. 30 seconds (Foss Electric, Denmark; Skoog *et al.*, 1997). In modern FT-IR instrumentation, the Michelson interferometer is extensively used to modulate all the frequencies characteristic of this region of the electromagnetic spectrum. As a source of infrared heat, a Nernst glower or a tungsten filament is often used (Skoog *et al.*, 1997).

2.5.3.3 Quantification of components with FT-IR spectroscopy

The concept of calibration which is widely used in analytical chemistry, also applies to FT–IR spectroscopy, and in order to quantify a component of interest, a predetermined calibration for the component in the matrix in which the determinations will be done, is required. An FT-IR spectrum of grape juice or wine contains the collective information of all the infrared active components in the medium and as such presents a set of data of high complexity. In order to extract the relevant information for the purposes of quantifying a component(s) of interest, an extensive calibration process that involves multivariate statistical procedures such as principal component analysis (PCA), principal component regression (PCR) and partial least squares regression (PLS), is required (Eriksson *et al.*, 1999; Esbensen, 2000). Current instrumentation has optional software modules that contain ready-to-use calibrations for simultaneous determination of several components in a sample. One such an instrument that has recently been introduced onto the market is the WineScan FT 120 instrument (Foss Electric, Denmark). Commercial calibrations available with the instrument include those for quantifying ethanol, volatile acidity, total acidity, pH, malic acid, lactic acid, glucose, residual sugar, fructose and glycerol and the evaluation of the application of FT-IR for the routine analysis of wine has recently received much attention (Patz *et al.*, 1999; Dubernet and Dubernet, 2000; Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003).

Possible limitations in the use of this technology include the interference due to the absorbance of water, which decreases the accuracy of determination of some components, such as SO_2. In terms of concentration range, FT-IR is generally not considered to measure accurately below 0.1 - 0.2 g/L. The detection limit can be drastically improved when FT-IR instrumentation is used in conjunction with conventional analytical instruments such as HPLC and in a recent application, carbohydrates, alcohols (including glycerol) and organic acids in wine were quantified by HPLC-FTIR, enabling the quantification of glycerol in the range below 1 mg/L (Vonach *et al.*, 1998).

2.6 AUTHENTICATION OF THE SOURCE OF GLYCEROL IN WINE

2.6.1 INTRODUCTION

Authenticity is an important quality criterion for foodstuffs and beverages. In recent times, this criterion has become increasingly prominent as a result of greater consumer awareness and expectation of a safe product (Downey, 1998; Bisson *et al.*, 2002). Authenticity in wine means that the product is what it claims to be in terms of, amongst other factors, the grape cultivar, geographic origin, vintage, maturation period and regime. It also implies that the product conforms to official national and/or international guidelines regarding the source(s) and levels of the chemical components in the product. Wine is an easily adulterated product, due to its complex chemical basis, as well as the fact that it is produced throughout the world. Authorities consider that wine adulteration is widespread, and worldwide the enforcement of legislative measures to protect the product authenticity is stepped up (Arvanitoyannis *et al.*, 1999).

Authenticity testing in wine, and similarly for brandies, spirits and fermented fruit juices, is challenging, both as a result of the chemical complexity of these products, and due to the inherent variation that occurs naturally in these products. Furthermore, the practices used in adulteration are becoming increasingly sophisticated. The number of components that must be tested in order to ensure authenticity is also continuously increasing (Downey, 1998). To meet these challenges, major developments in analytical methods and instrumentation have occurred in the past few decades, and these include applications of mass spectrometry (MS) and high resolution nuclear magnetic resonance (NMR) spectroscopy (recently reviewed by Careri, *et al.*, 2002; Košir and Kidrič, 2002; Ogrinc *et al.*, 2003), as well as carbon dating to verify the vintage of wine (Martin *et al.*, 1998; Jones *et al.*, 2001). Recent developments have also seen several applications of Fourier transform near-infrared (FT-NIR) spectroscopy and Fourier transform mid-infrared (FT-IR) spectroscopy, coupled with multivariate data analysis (Downey, 1998; Palma and Barroso, 2002; Roussel *et al.*, 2003).

The adulteration of wine through the addition of industrial grade glycerol to the product is not new, and reports on this practice date back to the very early years of the 20th century (Hickinbotham and Ryan, 1948). In light of the current focus on wine authenticity, the efforts towards the detection and control of glycerol adulteration have also become an increasingly prominent issue. The next section provides a brief overview of the current international regulations regarding glycerol adulteration in wine, and the implementation of these regulations by some leading analytical laboratories. This is followed by a summary of the analytical strategies currently used for the detection of glycerol adulteration in wine.

2.6.2 GLYCEROL AND AUTHENTICITY TESTING IN WINE

The adulteration of wine through the addition of industrial grade glycerol has a long (and sometimes infamous) history. In a literature review on glycerol and wine covering the period from *ca*. 1900 to 1948, the extent of the problem as it manifested itself in Europe, California and Australia in the first half of the 20th century was discussed (Hickinbotham and Ryan, 1948). The major wine producing countries of that time had regulations in place to control the practice. According to this source, the addition of glycerol to Australian wine was taxed at a rate of five shillings per gallon in 1948, based on the Australian Excise Tariff regulations!

It is interesting to speculate on the reasons why winemakers considered it necessary to add glycerol to wine. The production of wine through the addition of industrial grade ethanol was apparently not too uncommon. In the early years of the 20th century, the ameliorating properties of glycerol in masking the burning taste of ethanol were also well recognised (Hickinbotham and Ryan, 1948). Winemakers and enologists also generally accepted that the addition of glycerol to wine would improve the organoleptic properties of wine. In addition, the perception that the extract content of wine provided a reliable indication of the body and fullness of the wine was also prevalent. Extract refers to the components in wine other than the volatile components, and in the sugar-free extract, the major components are glycerol and organic acids (Boulton *et al.*, 1996). The addition of glycerol to wine will therefore automatically result in an increase in the extract value. Nowadays, it is still perceived by some that high glycerol concentrations in wine, and/or high extract values can be associated with good mouth-feel properties and body in wine (Rankine and Bridson, 1971; Eustace and Thornton, 1987; Omori *et al.*, 1995; Ciani and Ferraro, 1998). Whether these perceptions can be used to explain the rationale for the addition of industrial grade glycerol to wine in modern times, however, remains speculative.

The addition of glycerol to wine is widely prohibited by national official policies and regulations of wine producing countries (e.g. O.I.V. Regulations, 2002; Government Gazette of the Republic of South Africa, Annexure 23410, 2002). The degree to which control is exerted, however, differs from country to country. Very strict quality control is found in Germany (personal communication, Dr. H. Otteneder, Landesuntersuchungsamt, Institut für Lebensmittelchemie, Trier, Germany, 2002) and France (personal communication, Matthieu Dubernet, Laboratoire d'Œnologie Dubernet, Narbonne, France, 2002). In California (personal communication, Gordon Burns, Enological Technical Services, Napa Valley, California, 2002) and South Africa (personal communication Wendy Jonker, South African Wine and Spirit Board, 2002), the situation is monitored and problems addressed as they appear. Wines destined for the export markets are normally subjected to intense scrutiny, since the authenticity is validated both in the country of origin, as well as in the country of destination. Against this background, the occurrence of glycerol adulteration does not seem to be an isolated problem, especially when the active research in the field of analytical methods for the detection of "marker" substances in industrial grade glycerol is taken into account (see section 2.6.2.2).

The strategies used for the detection of glycerol adulteration in wine can broadly be divided into two categories, namely those relying on determining the amount of glycerol in a wine followed by a comparison of the value with established data for an authentic product of the same type, and secondly, strategies aimed at the detection of unnatural "marker" compounds present in the adulterated product. The presence of such markers would then be used as the basis to suspect adulteration. The application of these two strategies is discussed in the next two sections.

2.6.2.1 Authenticity testing based on the glycerol levels in wine

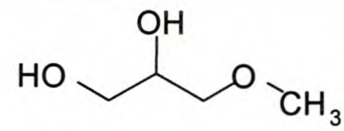
The stoichiometry of NADH metabolism in *S. cerevisiae* during fermentation determines that the ratio of glycerol: ethanol formed by the yeast remains fairly constant under controlled conditions (Oura, 1977). Based on these considerations, the so-called "glycerol factor" (calculated as: glycerol x 100/ethanol) is used to empirically calculate the glycerol: ethanol ratio in real wine fermentations (Würdig and Woller, 1989). Normally, it is assumed that the glycerol factor in ordinary table wines should be about 6. Research has shown that the glycerol factor for South African wines can be as low as 5 (Junge and Du Plessis, 1981). Currently, it is accepted that the glycerol content in wine lies between 6 to 10% of the ethanol content and certain tolerance levels are officially accepted to provide for the complexity of the factors that contribute to the final glycerol levels in wine (Würdig and Woller, 1989). These empirical values are being used especially in Germany where strict quality control is exerted. Wines, excluding botrytised wines, where the glycerol content is higher than 10% of the ethanol content, will be suspected of glycerol adulteration and automatically be subjected to further examination by regulatory authorities in order to validate product authenticity (personal communication, Dr. H. Otteneder, Landesuntersuchungsamt, Institut für Lebensmittelchemie, Trier, Germany, 2001). The addition of ethanol is suspected in wines where the glycerol content is below *ca.* 6% of the ethanol content. The balance of the fermentation products present in the sugar-free extract (of which glycerol is a major component), also receives considerable attention by regulatory bodies, especially those in Europe. Empirical values for the extract have been established and are used for comparative purposesto provide preliminary information on the authenticity of the fermentation products in wine (Boulton *et al.*, 1996).

Authenticity testing based on the quantity of glycerol in wine is considered as a preliminary means for the detection of the illegal addition of glycerol to wine and its interpretation is certainly not without problems. The use of empirical values can be problematic due to the complexity of the factors that determine the final concentrations of glycerol in wine (Scanes *et al.*, 1998). Furthermore, the variation introduced by some of the natural factors is sometimes comparable in magnitude to those expected from illegal additions. Despite these limitations, the quantification of glycerol in finished wine provides a powerful means of quality control both with respect to the authenticity of the fermentation process itself, as well as the source of glycerol in wine.

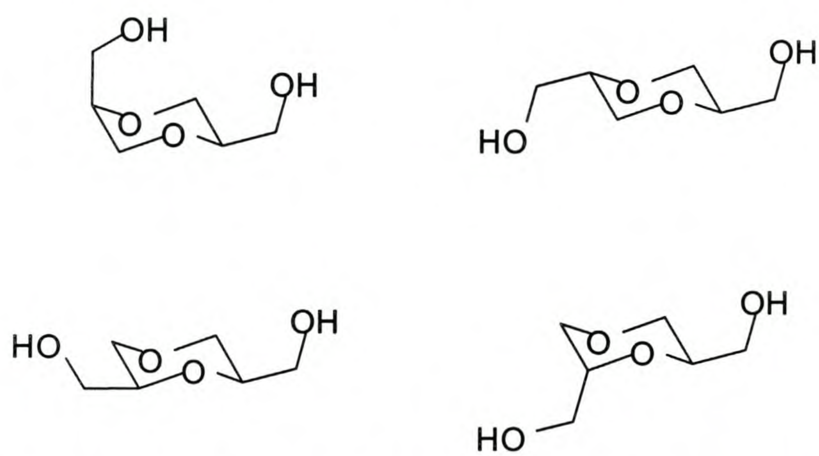
A recent software application, *GlycerolSoft,* has been developed to automate the transfer of analytical data generated with the enzymatic method for glycerol determinations in wine (Cordella *et al.*, 2003). This application, in the form of a Windows-based software package, eliminates the cumbersome process of establishing a new calibration for each set of determinations and at the same time, includes an updated file containing information regarding the glycerol content allowed in different foodstuffs. It therefore permits a conformity test of samples at the time of analysis to current international regulations.

2.6.2.2 Authenticity testing based on the identification of unnatural markers associated with the addition of glycerol to wine

Industrial grade glycerol is prepared by chemical processes from petrochemical feedstocks (such as polypropylene), fat (Wang *et al.*, 2001), and various plant oils, including palm oil (Sambanthamurthi *et al.*, 2000). During the purification process of glycerol, components characteristic of the purification process are formed, including 3-methoxy-1,2-propanediol (3-MPD) and cyclic diglycerols (CycD) (Figure 4). These substances are not natural components of grape juice and neither are they formed during the fermentation process, or added in the form of processing agents commonly used in winemaking. The detection of these substances in commercial wines is therefore interpreted as an indication of the addition of industrial grade glycerol to wine. Using GC-MS for the detection, Lampe *et al.* (1997) developed a method with detection limits of 0.1 - 3 mg/L for 3-MPD and 0.5 - 30 mg/L for CycD in wine. The application of this technology to 850 German and foreign wines, resulted in ~ca. 16% of the wines testing positively for the addition of industrial grade glycerol. It was of interest to note that the majority of the wines contained only 3-MPD, and very few contained both these markers.



3-Methoxy-propandiol



Isomers of cis-, trans-2, 5-Bis-(hydroxymethyl)-1, 4-dioxan

cis-, trans-2-Hydroxymethyl-6-hydroxy-1,4-dioxepane

Figure 4. Components that are frequently present in industrial grade glycerol (adapted from Lampe *et al.*, 1997).

A similar approach was used by Otteneder *et al.* (1999), and in this study the detection limits of the marker compounds was increased to 0.1 mg/L and lower. The application of this method to 190 wines resulted in the identification of three adulterated products. Bononi *et al.* (2001) reported further improvements of the same basic method.

Problems associated with the approach of using marker substances to identify the addition of industrial grade glycerol to wine, include (i) the availability of certified standards required for positive identification; (ii) the variation in the presence of the markers from one batch of glycerol to the next; and (iii) the potential limitations due to the high selectivity. It might indeed not be sufficient to rely on the presence of a few markers for the positive identification of adulteration and the combination of classical analytical techniques with less specific techniques such as FT-IR and FT-NIR spectroscopy might provide promising options. The processing of analytical data with multivariate statistical procedures such as principal component analysis (PCA), Soft Independent Modeling of Class Analogy (SIMCA), cluster linear analysis (CLA) and stepwise discriminant analysis (SDA) has successfully been used in studies on wine authenticity (Arvanitoyannis *et al.*, 1999).

2.7 PRESENT AND FUTURE PERSPECTIVES ON WINE QUALITY: STRIVING FOR AN INTEGRATED APPROACH

2.7.1 PRESENT AND FUTURE APPLICATIONS

In the field of analytical chemistry, major advances have been made towards increased sensitivity and hence lower detection and quantification limits for trace compounds in wine. In a recent application, stir bar sorptive extraction (SBSE) was coupled to gas chromatography / mass spectrometry (GC/MS) to analyse flavour components, off-flavour components, as well as agrochemicals in Cabernet Sauvignon wine (Hayasaka *et al.*, 2003). The recently developed SBSE (Hoffmann *et al.*, 2000) uses a stir bar that is incorporated into a glass tube and coated with polydimethylsiloxane (PDMS). The components in the liquid sample matrix (such as wine) are absorbed by the PDMS layer of the stir bar and can be analysed upon subsequent transfer to the GC/MS. Agrochemicals, which included compounds such as Chlorpyrifos Methyl, Atrazine and Carbaryl, could be quantified down to concentrations of 10 ng/L. The application of this method for the detection of 2,4,6-trichloroanisole (a compound associated with the formation of off-aromas in wine) resulted in a 10- to 100-fold improvement in the sensitivity of detection when compared to sensitivities that were reported using other methods. In the same study SBSE was also used for the flavour profiling of the Cabernet Sauvignon wines and more than 100 flavour constituents in the samples were detected.

For the purposes of routine analytical work, the recent technological developments were aimed at speed of analysis and automation. The optimisation of FT-IR based instrumentation such as the WineScan FT 120 instrument for this purpose has been discussed in section 2.5. A recent software application of the WineScan FT 120 instrument facilitates the objective monitoring of grape quality at harvest through the establishment of correlations, using artificial neural networks, between the analytical profile of a sample and the characteristic metabolic profiles of microorganisms associated with grape spoilage. The correlation is expressed as a numerical index value that correlates wih the degree of spoilage. This application was developed and patented by Laboratoire Dubernet, France, and awarded a gold medal for the most innovative solution for enology and viticulture for the year 2000 during the International VINITECH exhibition held at Bordeaux, France, 2000 (Anonymous, 2001).

For the purpose of *on line* monitoring of industrial scale fermentation processes, the application of electronic noses (Persaud and Pelos, 1982; Roussel *et al.*, 1999) and electronic tongues (Ivarsson *et al.*, 2001; Vlasov *et al.*, 2002) have recently seen major developments. Electronic noses utilise gas-sensor arrays to measure the volatiles present in the headspace of a closed container such as a fermentation tank or bioreactor. The technology was initially mostly applied for the detection of off-odours and the characterisation of the odours of particular foods and beverages. In a recent application focussing on wine quality, an electronic nose was used to discriminate between two red wines that had the same denomination (Gropello, Italy), but came from different vineyards (Di Natale *et al.*, 1996). The small, but significant differences between the two wines were explained in terms of soil characteristics and different agronomic features of the vineyards. In another application an electronic tongue, consisting of an array of potentiometric chemical sensors, was used to classify 56 Italian wines on the basis of their geographic origin and specific vineyard (Legin *et al.*, 2003). The application was further extended to flavour analysis of the wines, as well as the quantitative determination of sulphur dioxide, total polyphenols and glycerol, with an average error of prediction not exceeding 12% in comparison to the reference values.

Studies related to human sensory profiling include the conventional approach of descriptive profiling of products (including wine) that are widely used (Lawless, 1984; Heymann and Noble, 1989). Conventionally, a group of experts decides on descriptive terms to describe certain flavours and sensory properties of wine and then judges are trained to recognise and associate the correct term with a particular flavour or property. Recently, a novel approach in this respect was used by the so-called *free choice profiling* which was applied in the profiling of coffee, and here consumers were used to develop the descriptive language. This provided producers with an understanding of consumer associations and discriminations between products (Narain *et al.*, 2003). *Consumer profiling of liking* is also a recent development which is aimed at understanding the factors that influence human choice and product preference and in a recent application extensive analysis was done with Chardonnay wines (Hersleth, *et al.*, 2003).

There is also a growing recognition that the evolution of flavour in wine can change over time and that interactions (both physical and chemical) between the different components in wine can alter the final perceived flavour (Lubbers *et al.*, 1994; Dufour and Bayonove, 1999). Recent studies include investigations into the time-intensity characteristics of the contribution of ethanol on the perception of viscosity and density in white wine (Pickering *et al.*, 1998), time-intensity studies of astringent taste in white wine (Valentová *et al.*, 2002) and tannin-protein interactions (Edelmann and Lendl, 2002).

Recently, there are also increasing efforts towards data management, with particular reference to data contained in historic databases. For many topics that are currently being investigated in enology and viticulture, a wealth of data already exists, typically in written, anecdotal or electronic form. "Database mining" refers to the process of analysing data that are captured in databases, in order to extract factors ("inputs") that have a significant influence on the "output" of a particular application. In one such an application, outputs such as the duration of malolactic fermentation and the glycerol- and tartaric concentrations formed during winemaking were accurately predicted through the establishment of calibration models (Subramanian *et al.*, 2001).

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CHAPTER 3

RESEARCH RESULTS

Glycerol in South African table wines: An assessment of its relationship to wine quality

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RESEARCH RESULTS

Glycerol in South African Table Wines: An Assessment of its Relationship to Wine Quality

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ABSTRACT

Glycerol is an important by-product of glycolysis and is quantitatively one of the major components of wine. While the physicochemical and sensory characteristics of pure glycerol are well established, the impact of varying levels of glycerol on general wine quality remains a topic of debate. Previous reports have relied on limited numbers of either commercial or experimental wines to assess the role of glycerol, leading to contradictory conclusions. Here we report on a large-scale assessment of the relationship between glycerol concentration and wine quality, based on the analysis of a significant number of commercial South African table wines of adjudged quality. The mean glycerol concentrations of 237 dry red (10.49 g/L), 158 dry white (6.82 g/L), 22 off-dry white (6.55 g/L), 16 special late harvest (8.26 g/L) and 14 noble late harvest wines (15.55 g/L) were found to be associated with considerable variation within each respective style. The final glycerol concentrations were significantly associated with the wine style ($P<0.05$). Shiraz wines had a mean glycerol concentration (10.22 g/L) which was significantly lower ($P<0.05$) than that of Cabernet Sauvignon (10.81 g/L), Pinotage (10.46 g/L) and Merlot (10.62 g/L) wines. In both the dry white and off-dry white styles, the mean glycerol concentrations of Sauvignon blanc wines (6.31 g/L and 5.42 g/L, respectively) were significantly lower ($P<0.05$) than that of the Chardonnay wines (7.08 g/L and 7.03 g/L, respectively) and the Chenin blanc wines (6.81 g/L and 6.86 g/L, respectively). No significant association between the final glycerol concentrations in commercial wines and the vintage, geographic origin or yeast strain used in inoculated fermentations could be established ($P>0.05$). The mean glycerol concentrations for South African dry red wines were significantly higher than those of dry white and off-dry white wines. Wine quality could not be significantly associated with glycerol concentrations in the dry red wines ($P>0.05$). For the dry white, off-dry white and late harvest wines this association was significant ($P<0.05$), although the exact nature of the association

was somewhat different for the respective styles. Despite this positive statistical association, the observed differences between the mean glycerol concentrations of dry white and off-dry white wines of different quality ratings were too small to be of major practical value. The relationship between glycerol concentration and wine quality is reassessed on the basis of results obtained in this study as well as on recent reports in the literature.

3.1 INTRODUCTION

Research in modern enology is focused strongly on the improvement of wine quality through the enhancement of the sensory and flavour attributes of wine. From a scientific perspective, this requires the establishment of correlations between the individual components of the wine and its sensory characteristics. Wine, however, is a complex matrix consisting of several hundred components. These are thought to interact in a completely non-linear way to establish the aroma and flavour that are finally perceived (Voilley *et al.,* 1991; Lambrechts & Pretorius, 2000). As a result, the contribution of the majority of the individual components to wine quality, remains difficult to assess.

Glycerol (sometimes referred to as glycerine) is one of several polyols present in wine and the pure substance is a colourless, odourless, non-volatile sugar alcohol with a slightly sweet taste and a viscous nature. Quantitatively, glycerol is a major component of wine and the final levels in table wine are usually 7 to 10% of that of ethanol (Rankine & Bridson, 1971). The glycerol levels of wines from various origins, including several European countries, Australia, Argentina, California and New York State, have been reported in previous studies, and levels ranged from 1.36 to 14.7 g/L (Amerine, 1954; Mattick & Rice, 1970; Rankine & Bridson, 1971; Ough *et al.,* 1972). Exceptionally high glycerol levels of 14.6 to 24.7 g/L were reported for botrytised German Trockenbeerenauslese and French Sauternes wines (Amerine, 1954).

Glycerol found in wine is mainly formed as a by-product of glycolysis by wine yeasts. However, in the case of *Botrytis cinerea* infected grapes, significant amounts of glycerol can be found in grape must before fermentation, explaining the high levels of glycerol generally found in noble late harvest wines (Ribéreau-Gayon *et al.,* 2000a). In yeast, glycerol metabolism and glycerol itself play important roles in essential cellular processes, in particular, by maintaining the intracellular $NADH/NAD^+$ balance in conditions of low oxygen availability (Oura, 1977; Costenoble *et al.*, 2000) and by acting as a compatible solute for osmoregulation during hyperosmotic stress (Blomberg & Adler, 1989; Nevoigt & Stahl, 1997; Scanes *et al.*, 1998). Several parameters, including pH, temperature, the nitrogen source and the yeast strain used in inoculated fermentations, have been shown to influence the final glycerol levels in small-scale laboratory fermentations (Scanes *et al.*, 1998). The ripeness of grapes and microbial flora on the grape berries were also reported to affect glycerol levels (Ribéreau-Gayon *et al.,* 2000a).

A widely shared opinion between winemakers and other stakeholders in the wine industry suggests that glycerol contributes positively to wine quality, and several reports have reinforced this perception (Rankine & Bridson 1971; Eustace & Thornton, 1987; Omori *et al.*, 1995; Ciani & Ferraro, 1998). In addition to contributing to sweetness when present in quantities above its threshold taste level of 5.2 g/L in dry white wine (Hinreimer *et al.*, 1955), glycerol has been implicated in mouth-feel sensations by conferring "fullness" (also referred to as "viscosity" or "weight") to wine. Glycerol is also thought to improve the overall balance between alcoholic strength, acidity, astringency and sweetness, and hence is considered to confer a degree of roundness and smoothness on the palate (Eustace & Thornton, 1987; Ciani & Ferraro, 1998). Together with sugars, titratable acids, phenols, lactic acid and other minor components, glycerol is found in the total extract of wine and hence is associated with the characteristic full-bodied nature of wines with high extract values (Ribéreau-Gayon *et al.,* 2000b).

To date, no firm correlation has been established between glycerol and the adjudged wine quality, due to insufficient experimental data. Based on the widespread perception that the quality of wines could be improved by increasing the glycerol levels, several attempts aimed at establishing procedures to increase the final levels in wine have been undertaken. These include the manipulation of fermentation conditions (Radler & Schütz, 1982; Gardner *et al.*, 1993), the breeding of new wine yeast strains (Eustace & Thornton, 1987; Prior *et al.*, 1999; Rainieri *et al.*, 1999), the use of *Candida stellata* as fermentation starter cultures (Ciani & Ferraro, 1998) and re-directing carbon flux during wine yeast glycolysis using recombinant DNA techniques (Michnick *et al.*, 1997; Remize *et al.*, 1999; De Barros Lopes *et al.*, 2000). However, the impact of higher glycerol levels on the sensory evaluation of experimental wines has only been reported in a limited number of studies, and the wines containing increased glycerol levels were frequently judged less favourably than the control wines (De Barros Lopes *et al.*, 2000; Prior *et al.*, 2000). Furthermore, the validity of extrapolating the conclusions of studies involving small-scale experimental wines to large-scale, commercially produced wines, has not been established.

The relationship between glycerol concentrations and wine quality clearly needs to be reassessed. Here we report on the analysis of glycerol in a large number of commercial wines of adjudged quality. The data allow for the first time for statistically significant conclusions to be drawn, regarding: (i) the distribution of glycerol levels in South African (SA) table wines (about which very little information has been published); (ii) the relationship between the glycerol levels and wine style, geographic origin, vintage, cultivar and the yeast strain used; (iii) the relationship between glycerol levels and adjudged wine quality; and (iv) the possibility of using the data of the glycerol analysis as a predictor of wine quality.

3.2 MATERIALS AND METHODS

3.2.1 WINE SAMPLE COLLECTION

Samples of 447 commercial SA wines were collected. Of these, 414 wines were entered for the SA Veritas competitions of 1999 (101 wines) and 2000 (313 wines). Each wine was judged by 7 judges and rated out of a possible total of 20. All the Veritas competition wines used in this study were medal-awarded. The respective medals were allocated based on the following scoring system: Bronze, a median of 13 or 14; Silver, a median of 15 or 16, or a median of 14 and three judges score the wine 15 or more; Gold, a median of 17, or a median of 16 and three judges score the wine 17 or more; Double-gold, 17 points or more allocated by 5 of the 7 judges.

In addition, 33 wines (23 red, 2 dry white, 3 off-dry white, 2 special late harvest and 3 noble late harvest wines) that had not been entered for the Veritas competition were included. Where possible, samples from diverse winemaking regions in SA were selected. Wine samples were collected in sterile vials. Samples of the Veritas 1999 competition wines were obtained directly from wineries. The Veritas 2000 competition wines were sampled on the same day that the bottles were opened for judging. Commercial wines not entered for the Veritas competition were obtained from retail outlets and, in a limited number of instances, directly from the winemakers.

3.2.2 WINE SAMPLE STORAGE

Samples were stored at 4°C until analysed. Aliquots of a selection of samples were assayed for their glycerol content within 48 h of sampling. These values were used for reference purposes to monitor the effect of long-term storage of the samples on the glycerol concentrations.

3.2.3 DATA COLLECTION PROCEDURES

Data from the routine chemical analyses for ethanol, reducing sugar, titratable acidity and pH of the Veritas competition wines were obtained by means of a questionnaire sent to the winemakers of the respective wine cellars. Routine chemical analyses were conducted according to accepted reference methods (Amerine & Ough, 1980) and the values provided were those officially approved by the South African Wine and Spirit Board upon final certification of the wines. For a number of wines (n = 181), the information regarding the yeast strain used in inoculated fermentations was also obtained by means of the questionnaire.

3.2.4 GLYCEROL ANALYSES

Glycerol in wine was determined by the enzymatic method (Roche, kit no. 0148270). A total reaction volume of 100 µl was used in microtiter plates. Absorbance at

340 nm was read with a Universal Microplate Spectrophotometer (µQuant model, Bio-Tek Instruments, USA). The relative standard deviation (also referred to as the coefficient of variation, CV) of duplicate determinations was <3.5%. The accuracy of the enzymatic analyses was evaluated by means of a validation set of 35 wines of which the glycerol concentrations were also tested, with duplicate determinations, using high performance liquid chromatography (HPLC). A Dionex DX 500 system consisting of a Carbopac MA1 analytical column connected to a guard column (Dionex P/N 46122) was used, with 125 mM NaOH as eluent and a flow rate of 0.25 ml/min. For each sample in the validation set, the differences between mean values obtained using the enzymatic method and HPLC analyses were <0.2 g/L. For the purposes of the statistical analyses, only the data obtained with the enzymatic method were used.

3.2.5 STATISTICAL ANALYSES

Using the analytical data obtained by the enzymatic method, one-way analysis of variance of glycerol concentration was performed to compare the wine styles. For significance tests a critical level of 5% was used and 95% confidence intervals were calculated, using a General Linear Model output by the MINITAB program (MINITAB Reference Manual, 1995). To assess the association between glycerol concentration and wine style together with the Veritas rating, two-way analysis of variance, applying the same model, was used. A variance stabilising transformation was applied to the data prior to analysis, by taking the natural logarithms of the glycerol concentrations. The significance of the differences between the means of the $\log_e$(Glycerol concentration) values, was evaluated on the basis of Fisher intervals calculated by pair wise comparisons of the respective means (Snedecor, 1967). Discriminant analysis, using the reference data for concentrations of glycerol, ethanol, reducing sugar, titratable acidity and the pH levels, as predictors of wine quality, was done using a logistic regression model (Snedecor, 1967).

3.3 RESULTS AND DISCUSSION

3.3.1 RELATIONSHIP BETWEEN GLYCEROL LEVELS AND WINE STYLE

Large differences between the mean glycerol levels of dry red (10.49 g/L), white (dry and off-dry wines, 6.82 g/L and 6.55 g/L, respectively), special late harvest (8.26 g/L) and noble late harvest wines (15.55 g/L) were observed (Table 1). Notable was the wider range in glycerol levels, as reflected by the larger standard deviations, in the categories with greater means, and the application of a variance stabilising transformation was considered necessary. One-way analysis of variance, using $\log_e$(Glycerol concentration), showed a significant association between the mean glycerol concentrations and the wine style ($F = 285.82$; $P<0.005$, Table 1). Pair wise comparisons of the means, using $\log_e$(Glycerol concentration), showed that the difference between the means of the dry white and off-dry white wines was not significant. However, the differences between the means of the dry red wines and both the dry white and off-dry white wines, respectively, were significant. Similarly, the differences between the means of the special late harvest wines and the noble late harvest wines were significant. The differences in the means of both types of late harvest wines and the means of both the dry white and off-dry wines, respectively, were significant. No statistically significant association was found between the vintage and the glycerol levels (data not shown). For the purposes of the Veritas competition, the white cultivars Chardonnay, Sauvignon blanc and Chenin blanc are entered in various sub-classes. These sub-classes distinguish between wines that have been matured in wood, unwooded wines and delicate-styled or full-bodied wines. No significant differences in the mean glycerol concentrations of the respective sub-classes were found (results not shown).

Table 1. Glycerol levels in a selection of commercial South African table wines of various styles (vintage 1995 to 2000).

Style	No. wines	Glycerol g/L			$\log_e$(Glycerol concentration)	
		Mean	SD [(a)]	Range	Mean	SD [(a)]
Dry white (maximum sugar 4 g/L) [(b)]	158	6.82	0.91	5.21 – 9.36	1.911	0.130
Off-dry white (sugar 4.1-12 g/L) [(b)]	22	6.55	0.97	4.72 - 8.44	1.869	0.151
Dry red (maximum sugar 4 g/L) [(b)]	237	10.49	1.38	6.67 - 14.24	2.341	0.135
Special late harvest (maximum sugar 50 g/L) [(b)]	16	8.26	1.79	6.05 - 12.20	2.683	0.267
Noble late harvest (minimum sugar 50 g/L) [(b)]	14	15.55	3.39	9.81 - 20.21	2.091	0.205
F(4 , 442) = 285.82; P<0.0005						

[(a)] Standard deviation
[(b)] Specified according to the classification used by the South African National Wine Show Association

3.3.2 RELATIONSHIP BETWEEN GLYCEROL LEVELS AND GRAPE CULTIVAR

The relationships between the mean $\log_e$(Glycerol concentrations) and the Chardonnay, Chenin blanc and Sauvignon blanc cultivars within both the dry white ($F = 112.30$; $P<0.005$) and off-dry white wine styles ($F = 7.18$; $P = 0.006$) were significant (Table 2). In the case of the dry red wines, the P-value was quite small ($F = 2.48$; $P = 0.062$), but slightly greater than the critical value of 0.05. The relationship between the mean glycerol levels and the cultivars of the dry red style was therefore not significant. Pair wise comparisons of the differences between the means of the respective cultivars, showed that the Sauvignon blanc cultivar had a significantly lower mean glycerol level than those of either the Chardonnay or Chenin blanc cultivars in both the dry white and off-dry white styles (Table 2). In the dry red wines, Shiraz wines had a significantly lower mean glycerol level than the Cabernet Sauvignon, Pinotage or Merlot wines.

Taking the complexity of the factors that determine the final glycerol levels in wine into account, the data presented here are insufficient to establish the relationship between the glycerol levels and the wine cultivars *per se*. The association of lower glycerol levels with both Shiraz and Sauvignon blanc cultivars is nevertheless significant. In a set of 15 experimental Australian wines made from six grape cultivars, no clear relationship could be established between the cultivar and the glycerol content (Rankine & Bridson, 1971). Data on the relationships between glycerol levels and cultivars in commercial and experimental wines were found to be very limited in the literature, and to our knowledge, this research is one of the first large scale studies to report on this aspect.

Table 2. Glycerol levels in a selection of commercial South African table wines of various cultivars (vintage 1995-2000).

Style	No. wines	Glycerol g/L			$\log_e$(Glycerol concentration)	
		Mean	SD [(a)]	Range	Mean	SD [(a)]
Dry white						
Chardonnay	88	7.08	0.94	5.31 - 9.36	1.949	0.131
Sauvignon blanc	44	6.31	0.65	5.21 - 8.20	1.837	0.098
Chenin blanc	26	6.81	0.86	5.20 - 8.17	1.910	0.130
$F(2, 155) = 112.30$; $P<0.005$						
Off-dry white						
Chardonnay	4	7.03	0.75	6.39 - 7.86	1.946	0.107
Sauvignon blanc	5	5.42	0.51	4.72 - 6.10	1.686	0.095
Chenin blanc	10	6.85	0.92	5.18 - 8.44	1.917	0.136
$F(2, 16) = 7.18$; $P = 0.006$						
Dry red						
Pinotage	53	10.46	1.28	7.25 - 13.37	2.339	0.124
Merlot	24	10.62	1.46	7.47 - 14.24	2.354	0.135
Cabernet Sauvignon	79	10.81	1.24	7.47 - 14.00	2.374	0.115
Shiraz	58	10.22	1.51	6.67 - 13.65	2.313	0.154
$F(3, 210) = 2.48$; $P = 0.062$						

[(a)] Standard deviation

3.3.3 RELATIONSHIP BETWEEN YEAST STRAIN AND GLYCEROL LEVELS IN COMMERCIAL SA WINES

Although the impact of the yeast strain on final glycerol levels in laboratory fermentations and experimental wines has been documented in several studies (Scanes *et al.*, 1998; Prior *et al.*, 1999; Remize *et al.*, 2000), very little information has been published on possible relationships between the yeast strain used in inoculated industrial wine fermentations and the final glycerol levels in commercial wines. In this study, data on the yeast strains used for inoculation were obtained by means of a questionnaire sent to the respective winemakers of an arbitrary set (n = 450) of Veritas competition wines. A relatively large proportion of these wines (24%) were produced by blending separate lots of must fermented by different yeast strains, to obtain the required final style. Thirty different yeast strains were used in the production of the wines represented by this sample set, of which at least 20 different strains were used for the production of white wines (data not shown). These practices are in line with the worldwide trend towards increased diversity in white wine styles (Ribéreau-Gayon *et al.,* 2000b). Commercial yeast strains represented at the highest frequencies (in wines inoculated with a single yeast strain) were WE372 (30%), WE14 (5%), VIN13 (14%) and Lalvin D47 (6%).

Due to the somewhat uneven representation of some of the yeast strains (notably WE14 and Lalvin D47) in this sample set, only conditional conclusions could be drawn about the possible relationship between the inoculated yeast strain and the final glycerol levels in the commercial wines. Nevertheless, in the case of the wine yeasts WE14 and WE372 used for red wine production, no significant relationship between the yeast strain and the mean glycerol concentration, using $\log_e$(Glycerol concentrations), was found (F = 2.20; P = 0.14, Table 3). The difference between the mean levels associated with the yeast strains was also not significant. Similarly, for the wine yeasts VIN13 and Lalvin D47 used for white wine production, no significant relationship between the yeast strain and the mean glycerol concentrations associated with these yeast strains was found (F = 2.73; P = 0.11). The difference between the mean glycerol levels was also not significant. A striking observation, however, was the relatively large range of glycerol levels in wines produced by particularly WE372 (7.10 g/L to 14.24 g/L) and VIN13 (5.30 g/L to 9.32 g/L). In comparison, small-scale laboratory fermentations in synthetic must (20% glucose, 25°C), yielded final glycerol concentrations of 4.80 g/L ± 0.42 g/L (n = 3) for strain VIN13 and 5.11 g/L ± 0.39 g/L (n = 3) for strain WE372 (data not published). This observation highlights some of the possible difficulties in attempts to manipulate the final levels of glycerol in large-scale fermentations. It is also evident that careful consideration should be given when extrapolating data obtained from controlled laboratory fermentations to that of industrial fermentations. The fairly high upper glycerol levels reported in the fermentations represented in Table 3 should also be taken into consideration in studies aimed at the development of yeast strains that produce elevated levels of glycerol.

Table 3. Relationship between yeast strain and glycerol levels in commercial South African wines.

Wine style	Yeast strains	No Wines	Glycerol g/L			$\log_e$(Glycerol concentration)	
			Mean	SD [c]	Range	Mean	SD [c]
Dry red	WE372 [a]	56	10.45	1.36	7.10 - 14.24	2.338	0.131
	WE14 [a]	9	11.07	0.43	10.48 - 11.60	2.403	0.039
$F(1, 63) = 2.20$; $P = 0.14$							
Dry and off-dry white	Lalvin D47 [b]	10	7.36	0.71	6.28 - 8.38	1.991	0.098
	VIN13 [a]	26	6.81	1.04	5.30 - 9.32	1.907	0.148
$F(1, 34) = 2.73$; $P = 0.11$							

[a] Commercial active dry yeast produced by Anchor Yeast, Warren Chemicals, South Africa
[b] Commercial active dry yeast produced by Lallemand, Protea Chemicals, South Africa
[c] Standard deviation

3.3.4 RELATIONSHIP BETWEEN THE WINEMAKING REGION AND GLYCEROL LEVELS

Wine estates (52), private cellars (61) and co-operative cellars (28) were included in this selection. Results indicated that the winemaking region was not significantly associated with the mean $\log_e$(Glycerol concentrations) in either the dry and off-dry white wines ($F = 0.10$; $P = 0.96$, Table 4) or the dry red wines ($F = 0.59$; $P = 0.62$). Pair wise comparisons of the means showed no significant differences between the mean glycerol concentrations of the wines of the various winemaking regions for both the white wine styles and the dry red style. Very small sample sets of red and white table wines from diverse winemaking regions such as Cederberg, Benede-Oranje, Tulbagh, Overberg and Jacobsdal were also analysed for their glycerol levels (data not shown). Mean glycerol levels in wines from these areas, were similar to the values reported for the respective styles produced in the areas listed in Table 4.

3.3.5 RELATIONSHIP BETWEEN WINE QUALITY AND GLYCEROL LEVELS

Table 5 shows the range of the glycerol levels for the gold (including double gold), silver and bronze categories of the Veritas ratings within the major wine styles. Relatively few wines of the various wine styles received gold and double gold awards and the ratings of these two groups were combined. Two-way analysis of variance derived from a General Linear Model with interaction between the Veritas rating and the wine style, showed a significant relationship between the mean $\log_e$(Glycerol concentration) and both the Veritas rating and the wine style, but the interaction effect, however, was not significant (Table 6a). A repeat of the analysis using the same model with no interaction confirmed the significant relationship between glycerol levels and both wine style and Veritas rating (Table 6b). The significance of the differences between the means of $\log_e$(Glycerol concentrations) of the bronze-, silver- and gold ratings in the respective styles, was evaluated on the basis of t-statistics obtained through pair wise comparisons of the differences between means. The t-statistics were calculated as the ratios of difference/standard error with 407 degrees of freedom and expressed at level 0.05. These comparisons indicated that in the case of the dry white and off-dry white wines, the differences in mean glycerol levels between the bronze- and both the silver- and gold rated wines, were significant. The differences in mean values for the silver- and gold rated wines in these two wine styles were, however, not significant. For the dry red wines, the differences between mean glycerol levels of the bronze-, silver- and gold rated wines were not significant. The relationships between the Veritas rating and glycerol concentrations in the dry red and both the dry white and off-dry white wines are graphically presented in Figure 1. Error bars have been calculated so that the non-overlap of any two of them indicates statistical significance at level 0.05. The observation that there was no significant difference in glycerol levels between the wines with either a silver- or gold rating in the Sauvignon blanc, Chenin blanc or Chardonnay cultivars, does not support the widely held perception that higher levels of glycerol could improve the quality of these wines. In the case of the special late harvest and noble late harvest wines, the differences in means between bronze and silver rated wines and that between silver- and gold rated wines were in both cases significant. In view of the relatively high sugar concentrations of these wines, this result could imply that glycerol plays an indirect role in contributing to the overall quality of these wine styles.

Table 4. Distribution of glycerol levels in 1999 and 2000 Veritas competition wines from different winemaking regions.

Wine style (number of wines)	Glycerol g/L			$\log_e$(Glycerol concentration)	
	Mean	SD [(a)]	Range	Mean	SD [(a)]
Dry and off-dry white					
Robertson (n = 27)	6.78	0.86	5.42 - 8.80	1.907	0.124
Paarl/Franschoek/Wellington (n = 46)	6.23	0.62	4.82 - 6.91	1.909	0.148
Stellenbosch/Helderberg (n = 69)	6.86	0.86	5.21 - 8.90	1.918	0.127
Coastal (n = 14) [(b)]	6.92	0.89	5.42 - 8.23	1.927	0.133
$F(3, 152) = 0.10$; $P = 0.96$					
Dry red					
Robertson (n = 14)	10.89	0.89	9.84 - 12.67	2.385	0.080
Paarl/Franschoek/Wellington (n = 61)	10.48	1.39	6.98 - 14.24	2.340	0.135
Stellenbosch/Helderberg (n = 102)	10.43	1.38	6.62 - 13.65	2.335	0.137
Coastal (n = 10) [(b)]	10.46	1.34	8.87 - 13.64	2.340	0.120
$F(3, 183) = 0.59$; $P = 0.62$					

[(a)] Standard deviation
[(b)] Producing cellars in the Constantia, Tokai, Cape Point and Hermanus areas are included

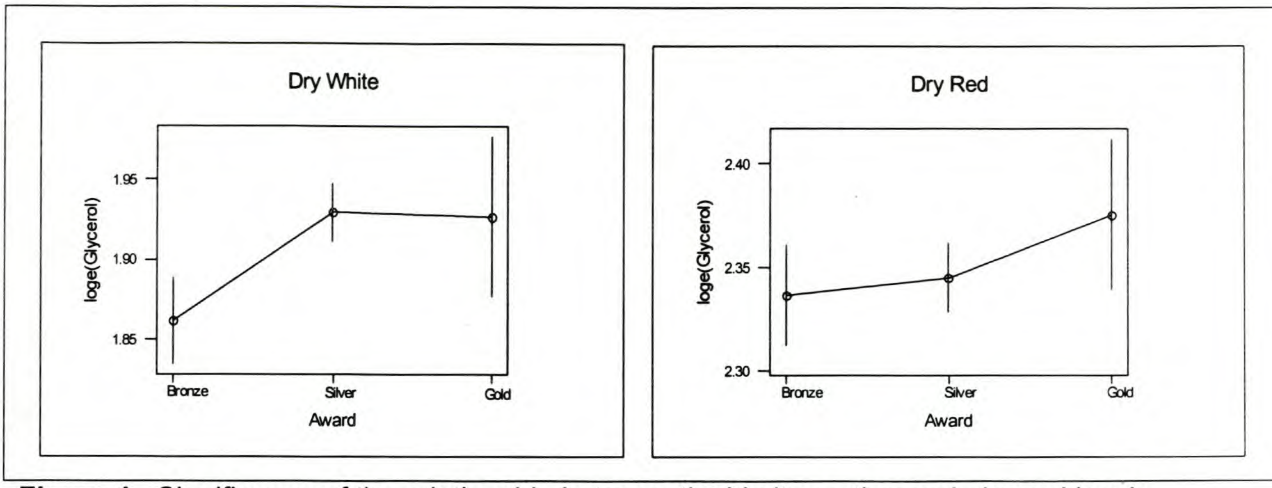

Figure 1. Significance of the relationship between the Veritas rating and glycerol levels.

Table 5. Relationship between glycerol concentration and adjudged wine quality in a selection of 1999 and 2000 commercial South African wines.

		No.	Glycerol g/L			$\log_e$(Glycerol g/L)	
Style	**Veritas rating**	**wines**	**Mean**	**SD [(a)]**	**Range**	**Mean**	**SD [(a)]**
Dry white	Bronze	44	6.47	0.72	5.21 - 8.17	1.879	0.111
	Silver	99	6.95	0.93	5.20 - 9.36	1.919	0.134
	Gold [(b)]	13	6.93	0.96	5.77 - 9.32	1.948	0.139
	Not entered	2	7.48	1.87	6.16 – 8.80	nd [(c)]	nd [(c)]
Off-dry white	Bronze	12	6.32	1.11	4.72 - 8.44	1.846	0.176
	Silver	6	6.78	0.85	5.46 - 7.86	1.886	0.125
	Gold [(b)]	1	7.24			1.915	
	Not entered	3	6.77	0.65	6.06 – 7.34	nd [(c)]	nd [(c)]
Dry red	Bronze	59	10.44	1.40	6.67 - 14.24	2.100	0.133
	Silver	129	10.53	1.40	6.67 - 13.84	2.140	0.133
	Gold [(b)]	26	10.82	1.15	8.04 - 13.47	2.169	0.106
	Not entered	23	9.90	1.47	6.78 – 12.65	nd [(c)]	nd [(c)]
Special late harvest	Bronze	7	7.64	0.85	6.08 - 8.48	2.644	0.111
	Silver	6	8.94	2.03	6.98 - 12.20	2.684	0.227
	Gold [(b)]	1	10.93 [b]			2.713	
	Not entered	2	6.40	0.50	6.05 – 6.76	nd [(c)]	nd [(c)]
Noble late harvest	Bronze	3	14.86	4.50	11.68 - 18.04	2.314	0.302
	Silver	4	15.62	2.14	13.39 - 18.43	2.354	0.137
	Gold [(b)]	4	16.37	4.57	9.81 - 20.21	2.383	0.279
	Not entered	3	14.84	4.25	10.11 – 18.32	nd [(c)]	nd [(c)]

[(a)] Standard deviation
[(b)] Gold- and double gold awarded wines are combined
[(c)] Not determined

In addition to the wines from the Veritas competition, 33 other commercial wines of ordinary quality were also tested for their glycerol content. Data obtained, using this sample set, were not subjected to statistical analysis due to the relatively small sample numbers and these wines were considered merely for comparative purposes. No major differences between the observed mean glycerol concentrations of the various styles represented by this group and those of the corresponding styles of the Veritas competition wines were found (Table 5).

Table 6a. Analysis of variance for $\log_e$(Glycerol concentration) with a General Linear Model with interaction between Veritas rating and wine style.

Effect	DF	Sequential SS	Adjusted SS	Adjusted MS	F	P
Veritas rating	2	0.911	0.162	0.081	4.22	0.015
Wine style	4	21.238	13.302	3.326	173.54	0.000
Veritas*Style	8	0.191	0.191	0.024	1.25	0.269
Error	399	7.646	7.646	0.019		
Total	413	29.986				

Table 6b. Analysis of variance for $\log_e$(Glycerol concentration) with a General Linear Model with no interaction between Veritas rating and wine style.

Effect	DF	Sequential SS	Adjusted SS	Adjusted MS	F	P
Veritas rating	2	0.911	0.195	0.098	5.07	0.007
Wine style	4	21.238	21.238	5.309	275.72	0.000
Error	407	7.837	7.837	0.019		
Total	413	29.986				

3.3.6 DISCRIMINANT ANALYSIS OF GLYCEROL AS PREDICTOR OF WINE QUALITY

The possibility of using the analytical data for glycerol and the reference values of the routine wine analyses to predict wine quality in the dry white style was tested. For this purpose a sample set of 101 wines was used, and the data for the gold and silver rated wines were pooled, based on the finding that the glycerol levels for these two groups did not differ significantly. The application of a mathematical regression, using the glycerol concentration in combination with the levels of either ethanol, titratable acidity, pH, or reducing sugar, to predict either a bronze or silver/gold Veritas rating, identified only glycerol in combination with pH as significant predictors of quality in the sample set used. The linear discriminant equation was determined as:

$$\text{Veritas award} = -1.8867 + 0.1105\text{Glycerol} + 0.5748\text{pH}$$

and this equation could be used to calculate the predicted Veritas award (either bronze or silver/gold) correctly, in 78 out of the 101 wines used for this analysis, by assigning a "cut-off" value of 0.7118 to "Veritas Rating". This function however, should be treated with some caution, since the same sample set was used for the determination as well as the validation of the equation. Furthermore, the equation has only been tested for the mentioned components in the concentration ranges specified in Table 7. Clearly, the use of an independent validation set, the inclusion of data for more chemical components, particularly the volatile components, as well as a wider concentration range for the components of interest, could add to the future applicability and usefulness of such a discriminant function.

Table 7. Chemical analyses of Veritas-awarded dry white SA wines.

Veritas rating	No. samples	Component	Mean g/L	Range g/L
Bronze	27	RS [(a)]	2.26 ± 0.69	1.20- 4.00
		pH	3.37 ± 0.16	3.11 - 3.62
		TA [(b)]	6.20 ± 0.78	4.90 - 8.10
Silver/Gold [(c)]	81	RS [(a)]	2.57 ± 0.79	1.20 - 4.00
		pH	3.41 ± 0.16	3.12 - 3.84
		TA [(b)]	6.27 ± 0.68	4.40 - 8.10

[(a)] Reducing sugar
[(b)] Titratable acidity
[(c)] Silver-, gold- and double gold rated wines are combined

3.4 CONCLUSIONS

In this study a large-scale investigation of the glycerol levels in Veritas-awarded commercial SA wines was undertaken. This approach facilitated the statistical analysis of the range of glycerol concentrations in wines of different styles, as well as an investigation into the possible relationships between cultivar, geographic origin, and the most commonly used yeast strains and the glycerol levels. All judgements on wine quality are necessarily subjective. The large number of wines submitted for the Veritas competition and the relatively constant and consistent tasting conditions, provided an opportunity to assess the possible relationships between glycerol concentration and wine quality, and to evaluate the commonly held perceptions regarding glycerol and wine quality against the industrial background of market ready wines.

Results obtained in this study indicated that the mean glycerol concentrations found for the red table wines were higher than those of the white table wines. A similar trend was found for the respective styles in wines from California (Ough *et al.*, 1972), New York State (Mattick & Rice, 1970) and Australia (Rankine & Bridson, 1971). Mean glycerol levels for SA red wines were comparable to red wines from New York State (9.4 g/L) and California (10.6 g/L), but were considerably higher than those reported for Australian red wines of the Claret type (7.7 g/L) and Burgundy type (8.0 g/L). In the case of white wines, the mean values for SA wines were lower than those reported for New York State (7.6 g/L) and German dry table wines

(7.7 g/L), but higher than those found in Australian Hock types (5.5 g/L) and Californian white table wines (4.8 g/L). The mean glycerol values found in SA white wines in an earlier limited study (Venter, 1955) were higher than the values reported here for the white wines (7.8 g/L versus 6.8 g/L). A similar situation was observed in Californian white wines (9.6 g/L in 1954 versus 4.8 g/L in 1972). In the SA Wine Industry, glycerol is not routinely determined for the analysis of commercial wines. Wines destined for the export market, however, are increasingly subjected to analysis additional to the standard requirements, including glycerol analysis. This is in line with the growing tendency for governments to adopt maximum and minimum acceptable limits for various constituents to ensure quality control. The data presented here could prove to be valuable in establishing the glycerol concentrations normally associated with the respective styles investigated.

Results obtained established statistically significant relationships between glycerol concentrations and adjudged wine quality for the dry white, off-dry white and special late harvest and noble late harvest wine styles. In both the dry white and off-dry white wines the differences between the mean glycerol concentrations of the wines with a silver rating and wines with a gold - or double –gold rating, were not significant. For these styles therefore, this result implies that the wine quality of the SA Chardonnay, Chenin blanc or Sauvignon blanc cultivars is unlikely to be improved by increased glycerol levels alone. The observed differences between the mean values of the different quality ratings, however, were small. This observation, together with the relative large range in glycerol levels found in most styles, could diminish the practical value of the statistical relationship and highlights the difficulties that could be encountered in attempts to control the final glycerol levels in large-scale industrial fermentations. Nevertheless, the sensory profile of each wine is unique and this therefore does not imply that an individual wine could not benefit from increased glycerol levels.

In reassessing the contribution of glycerol to wine quality, several recent reports in the literature on this topic should be mentioned. Some prominent enologists have expressed reservations on the emphasis that should be placed on the organoleptical role of glycerol in wine (Zoecklein *et al.*, 1995; Ribéreau-Gayon *et al.,* 2000a). Factors other than glycerol concentration have been implied in the mouth-feel properties of wine, including the ethanol concentration (Pickering *et al.*, 1998), barrel maturation, yeast autolysis, certain yeast cell wall mannoproteins (Ribéreau-Gayon *et al.,* 2000b), and the balanced sensory profile associated with certain yeasts (Delteil & Jarry, 1992). In relating the instrumental measurement of the contribution of glycerol to viscosity in wine, tasters reported that a minimum value of 25.8 g/L was necessary for perception (Noble & Bursick, 1984). The impact of ethanol concentration on the increase in viscosity, was however, not taken into account by these authors. In view of the fact that the glycerol content in wine is only 7% to 10% of that of ethanol in the dry and off-dry table wines (Rankine & Bridson, 1971), the impact of glycerol on perceived viscosity is probably negligible. It should also be borne in mind that thresholds are merely statistically determined endpoints that could be influenced by a variety of factors that cause fluctuations (Trant & Pangborn, 1983). Therefore, predictions made about threshold values for the levels of glycerol required for the optimum perceived contributions to the organoleptic characteristics in wine will be open for debate and should be retested at regular intervals.

Several aspects regarding the contribution of glycerol to wine quality must still be clarified and future work should include the establishment of a workable definition of "body" in wine (which to date does not exist) and the evaluation of glycerol as an impact factor in conferring a smooth mouth-feel. Furthermore, the establishment of the precise nature of the contribution of glycerol to wine quality through descriptive aroma analyses, as well as by means of an investigation into the possible indirect effects of glycerol on wine quality through physical/chemical interactions between glycerol and other flavour constituents in wine, require further investigation.

3.5 ACKNOWLEDGEMENTS

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CHAPTER 4

RESEARCH RESULTS

The effect of glycerol on the volatility of aroma compounds in a model wine and white wine as evaluated by headspace solid-phase microextraction gas chromatography

RESEARCH RESULTS

The effect of glycerol on the volatility of aroma compounds in a model wine and white wine as evaluated by headspace solid-phase microextraction gas chromatography

ABSTRACT

The effect of glycerol concentration on the volatility of aroma compounds in wine was investigated. Solid-phase microextraction, followed by gas chromatography was used to analyse the composition of the headspace, at equilibrium between the liquid phase and the gas phase of a model wine and a dry white wine that were spiked with different concentrations of glycerol. The aroma compounds used in this study included a selection of esters and higher alcohols commonly found in wine. The stepwise increments in the glycerol concentration over a range of 1-10 g/L in the model wine and in the white wine (which had a basal glycerol concentration of 5.4 g/L), were not accompanied by a proportional increase or decrease in the abundance of the aroma components in the headspace. However, results indicated that increasing glycerol concentrations had an effect on the volatility of the aroma compounds, and that the effect is of a complex non-linear nature. The white wine was also subjected to sensory analysis, and the results showed that glycerol concentrations over the range 5.4 – 15.4 g/L in the white wine, had no significant effect on the intensity of the perceived aroma, the overall quality of the wine or the smoothness of the wine. The effect of glycerol concentration on the perception of sweetness was proportionally correlated. The results obtained in this study do not support the general perception that the contribution of glycerol to wine quality is correlated to the concentration of glycerol in wine. However, the results presented here do not support previous reports that glycerol has no effect on the volatility of the aroma components in wine.

4.1 INTRODUCTION

There is growing evidence that interactions occur between aroma components and other components, such as proteins and polysaccharides (Voilley *et al.*, 1991; Lubbers *et al.*, 1994), ethanol, sugars, lipids, fining agents, yeast cell walls (Voilley and Lubbers, 1999) and polyphenols (Dufour and Bayonove, 1999) in wine. The interaction mechanisms are considered to include weak noncovalent bonds such as reversible van der Waals interactions, hydrophobic interactions and hydrogen bonding (Voilley *et al.*, 1991; Jung *et al.*, 2000). Such interactions can affect the volatility and solubility of the aroma components, or affect the release of aroma components from wine, and therefore affect the partitioning of these components between the liquid phase and the gas phase of a wine (Dufour and Bayonove, 1999). Changes in the aroma characteristics of a wine can affect the quality of the wine and an understanding of aroma-matrix interactions in wine is therefore important.

Glycerol is quantitatively a prominent component of wine and concentrations in white wine typically range from *ca.* 5 – 9 g/L, whereas concentrations ranging from 7 – 14 g/L, are common in red wines (Rankine and Bridson 1971; Noble and Bursick, 1984; Nieuwoudt *et al.*, 2002). Glycerol is mainly formed during alcoholic fermentation by the wine yeast *Saccharomyces cerevisiae* (Ribéreau-Gayon *et al.*, 2000). In the case of grapes infected by *Bortrytis cinerea*, significant amounts of glycerol are formed in the grape berries as a result of the metabolism of this fungus. A widely shared opinion amongst winemakers and enologists suggests that glycerol contributes positively to wine quality by imparting mouth-feel perceptions such as smoothness, viscosity and body to wine, and in general, high glycerol concentrations are associated with good quality wine (Rankine and Bridson 1971; Noble and Bursick, 1984; Eustace and Thornton, 1987; Ciani and Ferraro, 1998). To date, several strategies have been developed to increase the glycerol concentration formed by the wine yeast during alcoholic fermentation (Radler and Schütz, 1982; Gardner *et al.*, 1993; de Barros Lopes *et al.*, 2000; Prior *et al.*, 2000).

Under the physiochemical conditions normally prevailing in wine, glycerol is amongst the less volatile components and has an extremely high odour perception threshold. It would therefore not contribute directly to the aroma of wine. However, in beverages such as wine, sake and sochu (the latter being two fermented Japanese beverages), glycerol concentrations higher that those normally found in these beverages are thought to enhance the perception of flavour (Omori *et al.*, 1997). This could imply that glycerol has an effect on the volatility of aroma components in wine, and that such an effect is influenced by the glycerol concentration in wine. To date, glycerol-aroma interactions in wine have received very little attention. Lubbers *et al.* (2001) reported the use of a Purge and Trap analysis to study the effect of glycerol concentration on the volatility rate constants of a small number of aroma components in wine.

In this study an alternative approach was used to investigate the effect of glycerol concentration on the volatility of aroma components in wine. Headspace solid-phase microextraction (SPME) analysis, followed by gas chromatography, of a model wine and of a dry white wine that were spiked with known amounts of an external source of glycerol was used for this purpose. The white wine was also subjected to sensory analysis, in order to correlate the analytical data obtained by the headspace analysis with the sensorial perception of aroma in the white wine against a background of different concentrations of glycerol.

4.2 MATERIALS AND METHODS

4.2.1 CHEMICAL REAGENTS AND STOCK SOLUTIONS

The aroma standards (% purity) in the model wine were isoamyl acetate (99%), ethyl butyrate (99%), ethyl valerate (99%), ethyl lactate (98%), ethyl hexanoate (99+%), hexyl acetate (99+%), isoamyl alcohol, (99+%) and isobutanol (99+%). All the standards were obtained from Fluka, except for isoamyl acetate from Merck. Ethanol (absolute, 99.8%) and acetic acid (glacial, 99.99%) were obtained from Merck, and food grade glycerol (CAS No: 56-81-5; ECC No: 200-289-5; 99.81% purity) from Kimix, South Africa. Food grade glycerol is purified from raw glycerol of vegetable origin. The batch used in this study (batch No: 10118) was prepared from palm oil imported from Indonesia (Organic Chemical Corporation, Durban, South Africa).

A glycerol stock solution (100 g/L) was prepared with Milli-Q water and stored at ambient temperature (20 ± 2°C). For the purpose of the headspace SPME of the model wine, a global aroma stock solution containing all the aroma standards in a background matrix containing 26% v/v ethanol and 14 g/L acetic acid acid in Milli-Q water was used. The aroma compounds were added by weight, except hexyl acetate, ethyl hexanoate and ethyl butyrate that were added by volume, to obtain the concentrations listed in Table 1. The stock solution was stored at 4°C. Working solutions were prepared by diluting different amounts of the global stock solution with Milli-Q water to obtain the concentrations listed in Table 1 and these solutions were kept at ambient temperature (20 ± 2°C).

Table 1. Composition of the model wine used for headspace solid-phase microextraction analysis.

Component	Stock solution	Working solution	Final concentration
ethanol (% v/v)	26% v/v	26% v/v	13% v/v
ethyl butyrate (mg/L)	35.12	3.51	1.76
isobutanol (mg/L)	2153.00	215.30	107.65
isoamyl acetate (mg/L)	80.00	8.00	4.00
ethyl valerate (mg/L)	110.00	11.00	5.50
isoamyl alcohol (mg/L)	1677.00	167.70	83.85
ethyl hexanoate (mg/L)	10.15	1.02	0.51
hexyl acetate (mg/L)	17.52	1.75	0.88
ethyl lactate (mg/L)	2213.00	221.30	110.65
acetic acid (g/L)	1.40	1.40	0.70

4.2.2 WINE SAMPLES

A commercial dry white blended wine (Chenin blanc 85%; Sauvignon blanc 15%, alcohol 12.8% v/v; pH 3.2; glycerol 5.4 g/L; vintage 2001) was used for the headspace analysis as well as for the wine tasting experiments. For SPME, only one bottle was used for all the analyses, whereas 5 different bottles, all of the same batch were used for the sensory evaluation. Any variation between the different bottles was assumed to be negligible and hence would not significantly influence the outcome of the results. The basal concentration of glycerol in the white wine was 5.4 g/L, and the concentrations added were 0 g/L, 1.0 g/L, 5.0 g/L, 7.0 g/L and 10.0 g/L, respectively.

4.2.3 SAMPLE PREPARATION FOR HEADSPACE SOLID PHASE MICROEXTRACTION (SPME)

For each SPME analysis, 5 mL of the model solution or wine sample, plus 5 mL Milli-Q water, or appropriately diluted glycerol stock solution, was pipetted into a 15-mL glass vial and a small PTFE-coated magnetic stirrer bar was also placed in the vial. The vial was closed tightly with a PTFE-faced silicone septum cap. Subsequently the vial was placed on a magnetic stirrer that was operating at 1000 rpm and the SPME fibre was exposed to the headspace for 30 minutes at 20 ± 2°C. The pH of the sample was not adjusted. After completion of the sampling period, the fibre was removed from the sample vial, followed immediately by thermal desorption at 250°C in the injection port of the GC, using manual injection. Each model solution was analysed in triplicate by preparing a fresh sample for each individual analysis.

4.2.4 EQUIPMENT AND ANALYSIS CONDITIONS

4.2.4.1 SPME

A polydimethylsiloxane (PDMS) coated solid phase microextraction fibre (100 µm) (Supelco, Bellefonte, USA) was used. Only one fibre was used for the whole set of analyses, in order to eliminate small between-fibre variations in the sorption of analytes (Shirey, 1999; Mestres *et al.*, 2002). The fibre was conditioned prior to use at 250°C for 30 min. The fibre was re-conditioned between consecutive samples, by leaving the fibre in the injection port of a gas chromatograph for 5 min at 250°C, with a helium flow of approximately 50 mL/min through the injector liner.

4.2.4.2 Chromatography

Samples were analysed using a Hewlett-Packard 6890 Plus gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a split-splitless injector with electronic pneumatic control (EPC) and a flame ionisation detector (FID). A polyethylene glycol capillary column, ZB-Wax (30 mL x 0.25 mm i.d. x 0.5 µm f.t.) was used (Zebron, Phenomenex, USA). The carrier gas was helium at a flow rate of 1.8 mL/min and a pressure of 100 kPa, using the constant pressure mode. The splitflow was set to 18 mL/min resulting in a split ratio of 1/10. Operating conditions for the FID were as follows: H_2 at 40 mL/min, air at 450 mL/min and detector temperature at 250°C. Thermal desorption of the fibre in the injection port of the GC was done at 250°C for 30 seconds prior to starting the chromatographic process.

Desorption and injection was performed in the splitless mode with the splitless time set to 1 min. The temperature program for the GC oven was: 30°C, held for 1 min, raised at 2°C/min to 70°C, 20 min, 10°C/min to 200°C, 10 min, 20°C/min to 250°C, 1 min.

Components were identified by injection of standards and confirmed by retention indices available from the literature. In some instances gas chromatography – mass spectrometry (GC-MS) was also employed for further confirmation of peak identity. Quantitative data were obtained by calculating the absolute peak area.

4.2.5 SENSORY EVALUATION OF THE WINES

For the purpose of evaluating the effect of increasing glycerol concentrations on the perceived sensory properties of the commercial dry white wine, five bottles of wine were opened at the same time and of these, four bottles were spiked with undiluted glycerol to, respectively 1, 5, 7 and 10 g/L above the basal glycerol concentration (5.4 g/L). One bottle served as a control and this bottle was treated exactly the same as the experimental bottles, but no glycerol was added. The dilution error of the wines (through the addition of glycerol) was small, (0.67% and smaller, depending on the volume of glycerol added) and the assumption was made that this error would not affect the outcome of the sensory analysis. The bottles were closed, mixed very well by gentle swirling and stored at 8°C for 14 days prior to evaluation by a tasting panel. The wines were evaluated by a panel of seven trained judges and the judging was done according to standardised procedures (Swart *et al*, 2001). Wines were served at 15°C. A control wine (to which no additional glycerol was added) was evaluated first and thereafter, the judges used the control as a reference to evaluate all the other wines in random order per judge. Descriptive analyses for sweetness, smoothness and aroma as well as a quality rating were performed using an unstructured line scale of 10 cm to give an intensity rating. The lower end of the scale was marked "not noticeable" and the upper end "prominent". The quality scale was marked from "unacceptable" to "excellent". A critical level of 0.05 was used to denote significance.

4.2.6 ANALYSIS OF VARIANCE (ANOVA)

For the purpose of statistical analysis, the average values of triplicate determinations with the headspace SPME were used. A significance level of 5% was used in all cases. To assess the association between the treatment of the wines (in this case the increments in glycerol concentration) and the sensory evaluation of the wines, two-way ANOVA was done using Statistica release 6 (Microsoft Corporation, USA).

4.3 RESULTS AND DISCUSSION

4.3.1 SPME AND GC ANALYSIS OF THE MODEL WINES

The aroma compounds used for this analysis (Table 1) differ widely with respect to their odour perception threshold values in wine (Guth, 1997; Ferreira *et al.*, 2000). Furthermore they differ significantly in terms of polarity and hence solubility in aqueous solutions (Voilley and Lubbers, 1999), and therefore in their respective affinities for the PDMS coating of the SPME fibre. This selection of aroma compounds therefore provides an interesting background against which to monitor any changes that glycerol could have on their respective volatilities. In this study the aim was to analyse the headspace after equilibrium was reached between the liquid phase and the gas phase. In preliminary tests it was established that a period of 5 min, prior to SPME, with stirring at a constant speed of 1000 rpm at 20 ± 2°C, was sufficient to reach equilibrium (data not shown). The optimal time of exposure of the fibre to the headspace was also established in preliminary tests, and standardised to 30 min. Test runs were also done to establish the shortest period required for a complete desorption of analytes from the fibre at 250°C in the injection port of the GC, and a period of 30 seconds was found to be optimal. The between-run repeatability of the method was evaluated by triplicate independent analyses, and for all the aroma compounds evaluated, the relative standard deviation (RSD, calculated as standard deviation/mean of triplicate analyses) was 5% or lower (see Table 1 in Appendix 2). The reproducibility of the method was tested by triplicate analyses of a model solution on two consecutive days, and RSD values similar to the between-run repeatability were obtained (see Table 2 in Appendix 2). The RSD for acetic acid was much higher and varied between 10% and 20%. This is a result of the very high polarity of acetic acid and hence its very low affinity for the non-polar PDMS coating, resulting in very poor enrichment from the headspace.

A typical chromatogram obtained with SPME-GC analysis of the headspace in the model solution is shown in Figure 1 and for the wine sample in Figure 2. The chromatograph of the white wine samples showed a prominent peak at a retention time of 30.10 minutes, which was identified as diethyl succinate by analysing a pure standard of this component under exactly the same conditions as described for the model wine and white wine. For the purpose of investigating the possible effects of glycerol on the volatility of the aroma compounds, the abundance of the various components in the model solutions were compared. No internal standard (IS) were included for quantitative analyses, based on the assumption that glycerol, if it had an effect on the volatility of the aroma compounds, would also affect the volatility of the IS. The compensation by the IS for slight between-run variations in the chromatographic process would therefore also cancel the effect that glycerol might have on the volatility of the aroma compounds.

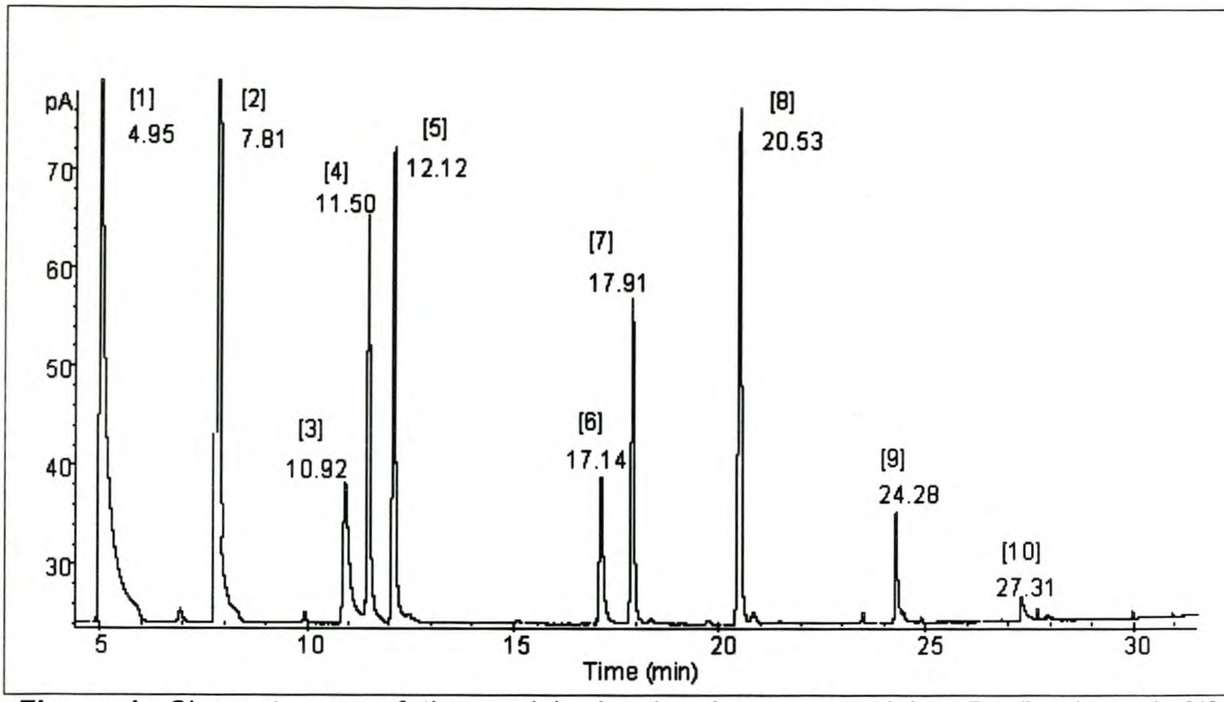

Figure 1. Chromatogram of the model wine headspace containing 5 g/L glycerol. [1]: ethanol; [2]: ethyl butyrate; [3]: isobutanol; [4]: isoamyl acetate; [5]:ethyl valerate; [6]: isoamyl alcohol; [7]: ethyl hexanoate; [8]: hexyl acetate; [9]: ethyl lactate; [10]: acetic acid.

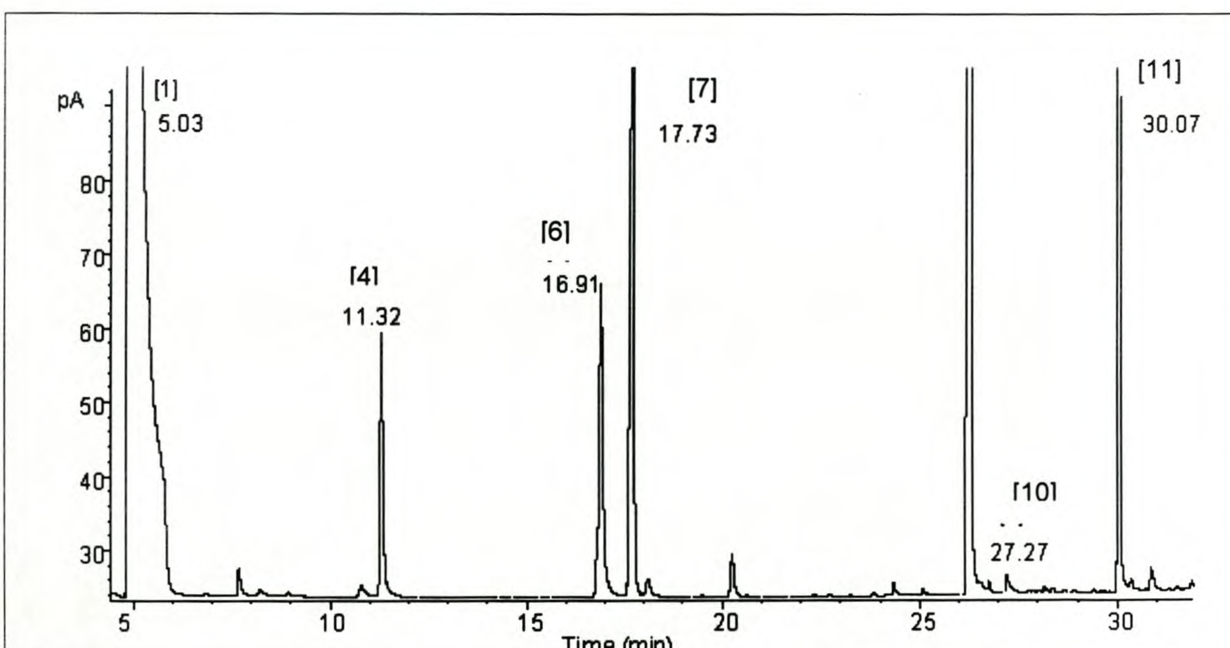

Figure 2. Chromatogram of the white wine headspace (glycerol 5.4 g/L glycerol). [1]: ethanol; [4]: isoamyl acetate; [6]: isoamyl alcohol; [7]: ethyl hexanoate [10]: acetic acid; [11]: diethyl succinate

Figure 3 shows a comparison of the abundance of the aroma components in the headspace at the different concentrations of glycerol in the model wine. Overall, the changes in the concentration of the volatile components in the headspace were small (as indicated by the % change for the respective components), except for hexyl acetate where a change of 28% was observed at a glycerol concentration of 5 g/L. A trend towards increased volatility of the aroma components over the glycerol concentration range investigated was observed for ethyl butyrate, isoamyl acetate, ethyl valerate, hexyl acetate and ethyl hexanoate. However, the effect of glycerol concentration on the volatility of the aroma components is clearly not of a straightforward linear nature.

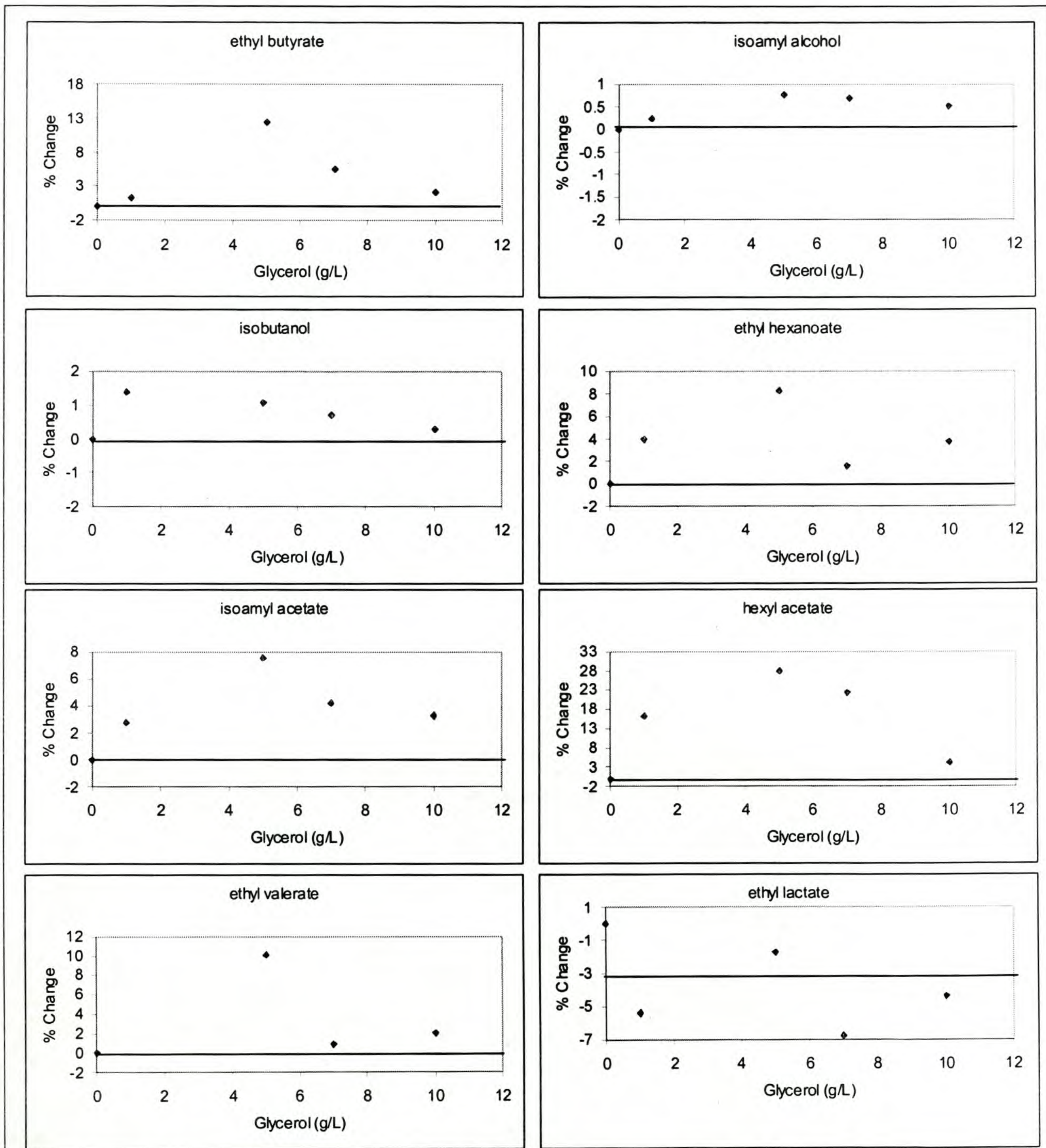

Figure 3. Effect of glycerol concentration on the volatility of aroma components in a model wine.

Overall, the trend in increasing glycerol concentration was not accompanied with a proportional, or inversely proportional trend in the abundance of the aroma compounds. The difference in the abundance of the aroma compounds associated with the lowest glycerol concentration and that associated with the highest glycerol concentration, was not significant at a critical level of 0.05 for all the aroma components used in this study.

The effect of glycerol concentration on the abundance of the aroma components that were identified in the white wine samples (Figure 4) showed that the average concentrations of isobutanol, ethyl valerate and isoamyl alcohol; associated with the lowest glycerol concentration, and those associated with the highest glycerol concentration, did not differ significantly. The stepwise increments in the glycerol concentration over a range of 1 – 10 g/L in the white wine, were not accompanied by a proportional increase or decrease in the abundance of the aroma components in the headspace. However, results indicated that increasing glycerol concentrations had an effect on the volatility of the aroma compounds, and that the effect is of a complex non-linear nature. These results therefore confirm the results obtained with the model wine.

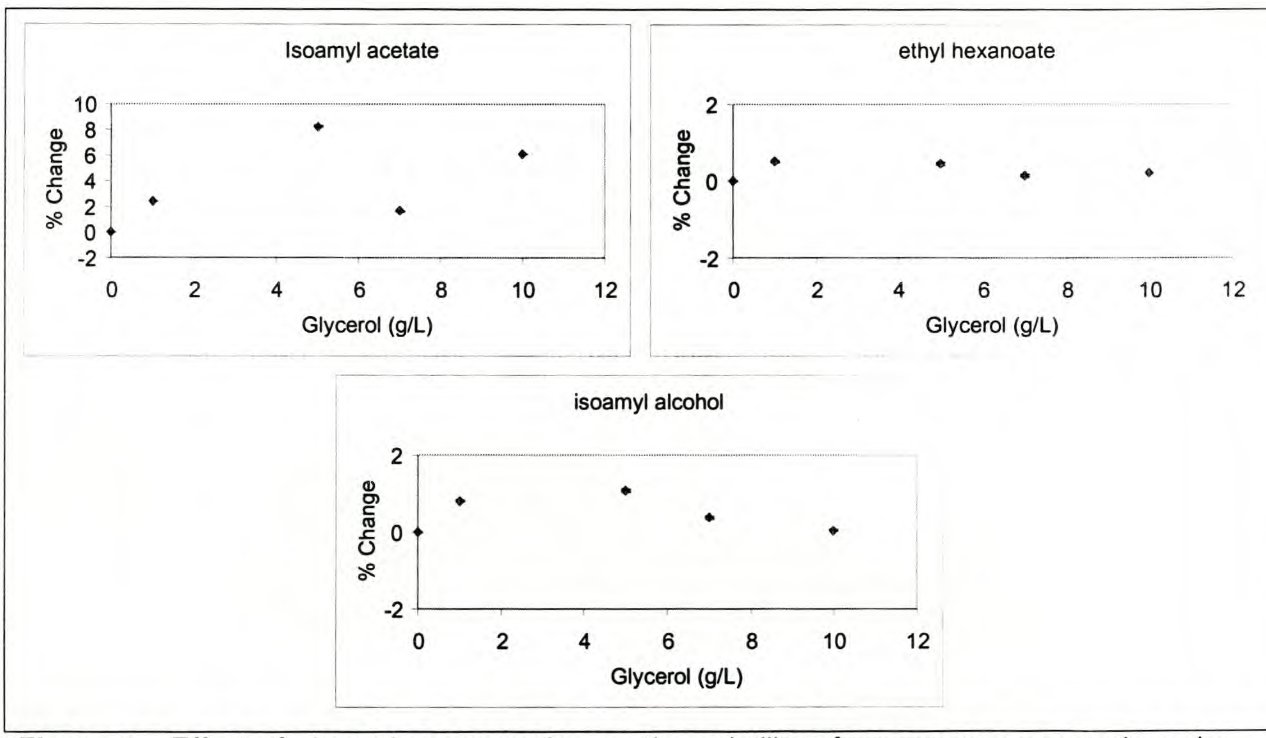

Figure 4. Effect of glycerol concentration on the volatility of aroma components in a dry white wine.

4.3.2 Sensory evaluation of the wines

The effect of glycerol on the sensory properties of a dry white wine was evaluated by spiking it with undiluted glycerol to increase the concentrations by respectively 0 g/L, 1.0 g/L, 5.0 g/L, 7.0 g/L and 10.01 g/L, above the basal glycerol concentration (5.4 g/L). The control (0 g/L glycerol added) was treated in exactly the same way as the experimental wines. The effect of the stepwise additions of glycerol on the sensory properties of the wine, as evaluated by a tasting panel, is presented in Figure 5. No major change in the aroma of the different wines was detected. For sweetness and smoothness, the tasters could detect differences between the different wines. The quality ratings for the different wines were also different. The ANOVA of the "treatment" of the wines (which in this case was the stepwise increases in glycerol concentration) on the sensory evaluation is summarised in Table 2. For smoothness ($P = 0.029$), aroma ($P = 0.044$) and quality ($P = 0.04$), there was a significant interaction between the "taster" and "glycerol concentration" and due to the large variation in the response of the tasters, the overall effects observed on the smoothness, aroma and quality of the wines (Figure 5), could not be ascribed to the increase in the glycerol concentration *per se*. It was of interest to note that the addition of glycerol over a concentration range of 1 g/L – 10 g/L above the basal glycerol concentration already present in the wine, had a significant effect on the increase in sweetness. This observation highlights the acuity of the tasting panel with respect to detecting, in a consistent manner, changes in the sensory properties of the wine. It can therefore also be speculated whether the descriptor "smoothness" in fact correctly describes the contribution of glycerol to the sensory properties of wine. It is clear that the different tasters interpret the effect "smoothness" differently.

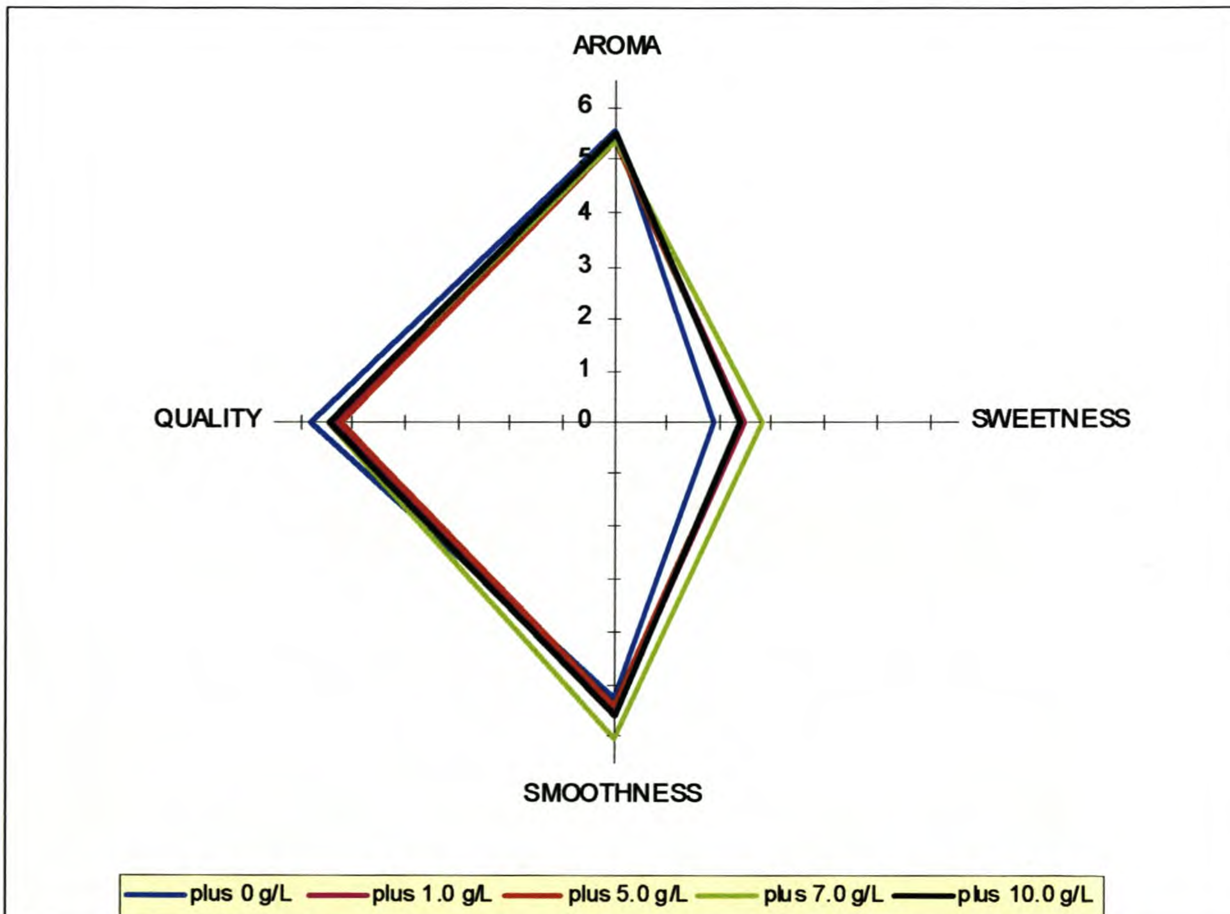

Figure 5. Sensory profile of wines to which glycerol was added in stepwise increments.

Lubbers *et al.* (2001) reported on the effect of glycerol concentration on the volatility rate constants of *n*-butanol, isoamyl alcohol, isoamyl acetate and ethyl hexanoate aroma components in wine, and reported that glycerol concentrations in the range of 5 g/L to 50 g/L did not modify the volatility rate constants of the four components tested. The authors concluded that glycerol did not have any effect on the volatility of the aroma components in wine. In this study stepwise increments in the glycerol concentration over a range of 1 – 15 g/L (above the basal concentration of 5.4 g/L already present in the wine) did have an effect on the volatility of the aroma compounds, and the effect seemed to be of a complex non-linear nature. This conclusion is therefore not in accordance to those reported previously (Lubbers *et al.*, 2001). In our work the focus was on the distribution of the aroma components between the liquid phase and the gas phase at equilibrium, and it is possible that the rate of transfer of the aroma components is independent of the distribution at equilibrium. Future work should include a closer investigation of the effect of glycerol on the volatile components, and more concentration intervals, over the concentration range tested in this study, should be included. The work should also be expanded to include more wine styles and more volatile components.

Table 2. Evaluation of the effect of increments in glycerol concentration on the sensory properties of the wine. Average values obtained from the triplicate score by 7 tasters are shown (see section 4.2.5 for details of the evaluation procedure).

Treatment	**Sensory property**			
Glycerol increment	**Aroma**	**Sweetness**	**Smoothness**	**Quality**
plus 0 g/L	5.59	1.90	5.22	5.79
plus 1.0 g/L	5.40	2.49	5.57	5.34
plus 5.0 g/L	5.35	2.42	5.42	5.16
plus 7.0 g/L	5.38	2.80	6.03	5.45
plus 10.0 g/L	5.47	2.42	5.60	5.43
average score	5.44	2.40	5.57	5.43
SD[a]	0.09	0.32	0.30	0.23
CV%[b]	1.7%	13.5%	5.3%	4.2%
P	0.0448	0.2381	0.0292	0.0455
$F_{(24, 70)}$	1.7014	1.2431	1.8088	1.6980

[a] Standard deviation; [b] Coefficient of variation; (calculated as standard deviation/average x 100).

4.4 CONCLUSIONS

It is generally perceived that glycerol contributes to the quality of wine, by imparting taste perceptions such as body, mouth-feel, smoothness and aroma intensity to wine. Generally, it is considered that this contribution is dependent on its concentration in wine. Glycerol has also been considered to enhance the flavour of wine, saké and shochu, and this perceived contribution is also considered to be dependent on its concentration in the beverages, thereby implicating that glycerol could have an effect on the volatility of the aroma components in wine, or affect the release of aroma components from wine. The results obtained in this study do not support the perceptions regarding the relationship between glycerol concentration and aroma intensity, smoothness or quality of wine. However, the results provide sufficient evidence that glycerol has an effect on the volatility of the aroma components studied, and that the effect is of a complex, non-linear nature. The results obtained in this study do not support the general perception that the contribution of glycerol to wine quality is correlated to its concentration in wine.

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CHAPTER 5

RESEARCH RESULTS

Principal Component Analysis applied to Fourier transform infrared spectroscopy for the design of glycerol calibration models in wine and for the detection and classification of outlier samples

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RESEARCH RESULTS

Principal Component Analysis applied to Fourier transform infrared spectroscopy for the design of glycerol calibration models in wine and for the detection and classification of outlier samples

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ABSTRACT

Principal component analysis (PCA) was used to identify the main sources of variation in the Fourier transform infrared (FT-IR) spectra of 329 wines of various styles. The implications of this variation (which included the sugar and ethanol content of the samples, as well as the stage of fermentation and the maturation period of the wines) for the design of calibration models with accurate predictive abilities were investigated using glycerol calibration in wine as a model system. PCA enabled the identification and interpretation of samples that were poorly predicted by the calibration models, as well as the detection of individual samples in the sample set that had atypical spectra, i.e. outlier samples. The Soft Independent Modeling of Class Analogy (SIMCA) approach was used to establish a model for the classification of the outlier samples. A glycerol calibration for dry wine with an improved predictive ability (SEP = 0.40 g/L) and a calibration for glycerol in sweet wine (SECV = 0.65 g/L) were established. This study demonstrates that a strategy which combines the careful design of calibration models to provide accurate predictions, with quality control measures that facilitate the early detection and interpretation of poorly predicted samples and outlier samples in a sample set, provides a powerful means of quality control which is necessary for the successful implementation of FT-IR in the routine wine analytical laboratory.

5.1 INTRODUCTION

The potential of Fourier transform infrared spectroscopy (FT-IR) as a powerful analytical tool in enology has been recognised for many years. The technology is based on the measurement of the frequencies of the vibrations of chemical bonds in functional groups such as C-C, C-H, O-H, C=O and N-H, upon absorption of radiation in the mid infrared (IR) region (Smith, 1999). The IR region is usually defined as ranging from 4000 – 400 cm^{-1}, or in terms of nanometers, from 2500 – 2.5 x 10^4 nm (Smith, 1999). The measured frequencies are processed through a series of mathematical procedures (which include Fourier transformation) to an absorbance spectrum, which in turn, is correlated to the actual concentrations of the relevant components in the sample matrix through a calibration process that involves multivariate statistical procedures such as principal component analysis (PCA), principal component regression (PCR) and partial least squares (PLS) regression (Eriksson *et al.*, 1999; Esbensen, 2000). Recent improvements in IR instrumentation (Skoog *et al.*, 1997) and the development of versatile and innovative software applications designed specifically for wine analysis (Foss Electric, Denmark) have optimised this technology. Currently, multi-component analytical instruments with impressive performance data in terms of accuracy, precision and speed of analysis are available. The evaluation of the application of FT-IR for the routine analysis of wine has recently received much attention (Patz *et al.*, 1999; Dubernet and Dubernet, 2000; Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003).

Glycerol (CH_2OH-CHOH-CH_2OH) is quantitatively a major component of wine and its determination at various stages of the winemaking process provides important information regarding issues that are directly or indirectly related to quality control (Würdig and Woller, 1989; Ribéreau-Gayon *et al.*, 2000; Zoecklein *et al.*, 2000). The application of FT-IR for quantifying glycerol in dry wine has been tested and standard error of prediction (SEP) values of 0.49 g/L (Patz *et al.*, 1999) and 1.32 g/L (Dubernet and Dubernet, 2000) have been reported. In addition, a ready-to-use calibration for glycerol in dry finished wine (SEP = 1.13 g/L) was recently made commercially available (Application Note 191, Foss Electric, Denmark, 2001). Based on theoretical considerations (and assuming a normal probability distribution) it should be kept in mind that the prediction error of only *ca*. 68% of the samples in a sample set will lie within ± one times the SEP value, whereas the remaining *ca*. 32% of the samples will have prediction errors that can be expected to be larger than this interval. The evaluation of calibration models based on regression statistics alone, therefore, often presents an over optimistic view of the predictive abilities of a model and provides limited information in terms of: (i) sample types for which the model would not be suitable; (ii) identifying poorly predicted samples in the sample set; and (iii) detecting and interpreting extreme deviating samples, i.e. the so-called "outlier samples" in the sample set, if present (Esbensen, 2000). These aspects are of particular importance in routine analysis of commercial wines, where wide variation in terms of style, vintage, cultivar, geographic origin, chemical composition, maturation periods and process technologies are encountered. It is to be expected that this variation will be reflected in the spectral properties of the samples and that some of these sources of variation will have major implications for the accuracy of prediction of calibration models. For the successful application of FT-IR in the analytical laboratory, accurate prediction data are required and a strategy aimed both at the development of robust calibration models encompassing this variation, as well as implementing quality control measures that enable the early detection and interpretation (where possible) of poorly predicted samples and outlier samples is therefore required.

In this study PCA was used as a tool to identify the main sources of variation between the FT-IR spectra of a large number of wines of various styles. The implications of this variation for the accuracy of prediction and hence the design of calibration sets, were evaluated using glycerol calibration in wine as a model system. The PCA results were also used to establish a classification model for the early detection and interpretation of outlier samples in the sample set by using the Soft Independent Modeling of Class Analogy (SIMCA) approach.

5.2 MATERIALS AND METHODS

5.2.1 WINE SAMPLES

The sample set consisted of bottled commercial South African red wines and white wines (n = 290) as well as young wines (both red and white, n = 39) that were close to the end of fermentation, but not yet bottled (Table 1). Collectively, the wines in the sample set represented more than 13 different cultivars and 22 different wine styles, as well as wide variation in terms of process technologies, geographic origin, vintage and maturation periods. The majority of the commercial wines (n = 263) were of vintages 1998 to 2002. A subset of red wines (n = 27) were of vintages older than 1998 and contained samples that had undergone three or more years maturation at the time of analysis.

5.2.2 FT-IR SPECTRAL MEASUREMENTS

Commercial wine samples were scanned upon reception without any further sample preparation. The young wines that appeared turbid were filtered with a Filtration Unit (type 79500, Foss Electric, Denmark) using filter paper circles graded at 20 – 25 µm and with diameter 185 mm (Schleicher & Schuell, reference number 10312714) in order to avoid disturbances in the optical path length of the cuvette. A WineScan FT 120 instrument (Foss Electric, Denmark) that employs a Michelson interferometer was used to generate the FT-IR spectra. Samples (7 ml) were pumped through the CaF_2-lined cuvette (optical path length 37 µm) which is housed in the heater unit of the instrument. The temperature of the samples was brought to exactly

40°C before analysis. Samples were scanned from 5011 – 929 cm^{-1} at 4 cm^{-1} intervals (i.e 1056 data points per spectrum), which includes a small section of the near IR region. The frequencies of the IR beam transmitted by a sample were recorded at the detector and used to generate an interferogram. The latter is calculated from a total of 10 scans before being processed by Fourier transformation and corrected for the background absorbance of water to generate a single beam transmittance spectrum. Two transmittance spectra for each sample were generated in order to calculate the absolute repeatability of the spectral measurements. The calculation of the absolute repeatability has been described (WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss Electric, Denmark, 2001). The transmittance spectra were finally converted into linearised absorbance spectra through a series of mathematical procedures. An aqueous solution of glycerol (10 g/L, analytical grade, BDH) was scanned under the same conditions as the wine samples.

Table 1. Wines used for FT-IR spectroscopy (n = 329).

Style / Description[a]	**Number of wines**
Dry white, maximum sugar 4 g/L[a]	
Chenin blanc, Sauvignon blanc, Chardonnay, blends, maximum 2 years aging, unwooded[b] and wooded[b] styles	86
Dry red, maximum sugar 4 g/L[a]	
Shiraz, Pinotage, Merlot, Cabernet Sauvignon, maximum 2 years aging	101
Various blends, 3 - 6 years aging	27
Single cultivars of Malbec, Pinot Noir, Petit Verdot	9
Off-dry white, sugar 4.1 - 12 g/L[a]	
Chenin blanc, Sauvignon blanc, unwooded[b] and wooded[b] styles	17
Sweet Wines (Semillon, Gewurztraminer, Weisser Riesling and blends)	
Special late harvest, maximum sugar content 50 g/L[a]	15
Noble late harvest, minimum sugar content 50 g/L[a]	28
Blanc De Noir, maximum sugar content 30 g/L[a]	
Pinotage, red muscadel	5
Low alcohol wines, alcohol less than 10% v/v, sugar content specified[a]	2
Chenin blanc and blend	
Young wines[c]	39
Total	329

[a]Specified according to the South African National Wine Show Association.
[b]Unwooded is defined as "no noticeable wood character"; wooded as "noticeable wood character".
[c]Young wines close to the end of fermentation, but not yet bottled.

5.2.3 MULTIVARIATE DATA ANALYSIS

5.2.3.1 Principal Component Analysis (PCA)

FT-IR spectra were exported to the Unscrambler Software (version 6.11, Camo ASA, Trondheim, Norway) for the purpose of PCA. Duplicate spectra were averaged and then autoscaled (mean centered and standardised). The complete data set, defined by the variables in the columns (in this study, 1056 wavenumbers) and the samples in the rows, was centered by column. With PCA the maximum directions of variation in a data set is modeled by projecting the objects (in this study, the FT-IR spectra) as a swarm of points in a space defined by principal components (PC's). Each PC is a linear function of a number of original variables, resulting in a reduction of the original number of variables. PC's describe, in decreasing order, the most variation amongst the objects, and since they are calculated to be orthogonal to one another, each PC can be interpreted independently. This permits an overview of the data structure by revealing relationships between the objects as well as the detection of deviating objects. In order to find these sources of variation, the original data matrix, defined by $X(n,m)$, is decomposed into the object space, the variable space and the error matrix. The latter represents the variation not explained by the extracted PC's and is dependent on the problem definition. The algorithm describing this decomposition is presented as:

$$X(n,m) = T(n,k)P(k,m)^T + E(n,m)$$

where X is the independent variable matrix, T the scores matrix, P the loadings matrix, E the error matrix, *n* the number of objects, *m* the number of variables and *k* the number of PC's used (Eriksson *et al.*, 1999; Esbensen, 2000).

5.2.3.2 Partial Least Squares Regression1 (PLS1)

PLS1 is a bilinear regression modeling method where the original x variables are projected onto a smaller number of PLS components (Esbensen, 2000). These components, also referred to as "latent variables" or "factors", are calculated according to the same mathematical procedures as PC's, but the data in the Y-matrix are incorporated in the calculation. The regression establishes the relationship between the X-matrix and the Y-matrix (in this study, the reference data for glycerol) with the objective to predict the y variables by using the most relevant PLS components. The relationship between the y and x variables can be described by the polynomial:

$$y = b_0 + b_1x_1 + b_2x_2 + b_nx_n$$

where y is the dependent variable, b_0 - b_n are the regression coefficients (b_0 is the intercept) and x_1-x_n represent the absorbance at the selected wavenumbers (see section 5.2.4).

5.2.3 3 Soft Independent Modeling of Class Analogy

Detection and classification of outlier samples were done using the Soft Independent Modeling of Class Analogy (SIMCA) application of the Unscrambler Software (version 6.11, Camo ASA, Trondheim, Norway). Two training sets, respectively, "typical dry red wine" (n = 10) and "typical dry white wine" (n = 10), were modeled using separate PCA models. For this application, a "cut-off" level of 11% v/v ethanol was used to differentiate the "typical" wines from the "atypical" wines and wines with an ethanol content below 11% v/v, were considered as "atypical". Class membership was defined at a significance level of 5%. A test set (n = 19) that included spectral outliers (as identified by PCA), as well as typical dry red and white wines was used to test the discrimination power of the classification model.

5.2.4 WAVENUMBER SELECTION

With the WineScan FT 120 instrument, a maximum of 15 "filters" (wavenumbers or small groups of wavenumbers) can be defined for calibration purposes. The wavenumbers at which the correlation between the measured absorbance and the corresponding reference values for glycerol (as determined with the enzymatic method, see section 5.2.6) was the highest, were selected by using the Advanced Performance Software Module version 2.1.0, which is an extension of the basic software of the WineScan FT 120 instrument. In order to exclude noise being introduced into the spectral data, only three regions, 964 - 1543 cm^{-1}, 1716 – 2732 cm^{-1} and 2434 – 2970 cm^{-1}, respectively, are made available for wavenumber selection. Possible overfitting of the calibration models (which introduces noise and uninformative variation into the calibration) was evaluated by deselecting the filters explaining a very low percentage of variation in a sample set in a stepwise manner, using the SEP values as guide. Here the objective was to find the lowest number of filters and the lowest possible SEP value for a particular calibration model.

5.2.5 EVALUATION OF THE PERFORMANCE OF CALIBRATION SETS

The statistical indicators for evaluating the performance of the calibration models were calculated using the Advanced Performance Module provided with the WineScan FT 120 instrument and included bias, SECV and SEP. Bias gives an indication of a systematic error in the predictive values (Esbensen, 2000) and it was calculated as the average of the residuals (residuals being the difference between the reference values and the predicted values). The accuracy of the predictive ability of the calibration model, relative to the reference data, was expressed as SECV when based on the calibration samples, and as SEP when based on independent validation sets. The calculations of these indicators are standard statistical procedures and several references describe these procedures (Eriksson *et al.*, 1999; Esbensen, 2000; WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss Electric, Denmark, 2001).

5.2.6 REFERENCE METHODS

5.2.6.1 Glycerol determinations

Glycerol was assayed with the Roche test kit "Glycerol UV method for the determination of glycerol in foodstuffs and other materials" (catalogue number 148270). The total assay volume was scaled down to 100 µl and duplicate determinations for each sample were carried out in microtiter plates (Sterilin, catalogue number 612F96) and readings taken at 340 nm using a µQuant spectrophotometer (Bio-Tek Instruments, USA). The accuracy of the reference method was expressed as the standard error of laboratory (SEL) and calculated as:

$$\text{SEL} = \sqrt{\frac{\sum (y_1 - y_2)^2}{2n}}$$

where y_1 and y_2 are the results of duplicate determinations and n is the number of samples.

5.2.6.2 Routine wine analysis

Reference data for ethanol, residual sugar (RS), titratable acidity (TA) and volatile acidity (VA) for the commercial wines were those officially approved by the South African Wine and Spirit Board upon certification of the wines. Corresponding data for the young wines were obtained by using the WineScan FT 120 instrument and the commercial calibrations for these components (Foss Electric, Denmark).

5.3 RESULTS AND DISCUSSION

5.3.1 ANALYSIS OF FT-IR SPECTRA

A FT-IR spectrum of a wine provides the collective absorbance of all the components present in the sample. The contribution of the absorbance of water to the FT-IR spectra was significant in two wavenumber regions, 3626 – 2970 cm^{-1} and 1716 - 1543 cm^{-1}, respectively (Figure 1a). These regions were typically broad and covered several hundreds of wavenumbers. The repeatability between duplicate FT-IR spectra was poor in these regions (data not shown) and these areas are known to contribute considerable noise in the spectra. The FT-IR spectrum of the aqueous glycerol solution showed prominent absorbance peaks in the 1600 – 929 cm^{-1} region. Some characteristic features of the FT-IR spectrum of glycerol that were observed (see insert Figure 1a) included the peak at 1462 – 1458 cm^{-1} (representing the H - C bend) and the peak at 1100 – 1075 cm^{-1} (representing the C – O stretch) (Pavia *et al.*, 2001). The major infrared band associated with the -OH group is located at 3350 ± 50 cm^{-1} (Pavia *et al.*, 2001). In the wine matrix, where water is abundant, the absorbance of the -OH group would not be expected to be very useful for the purposes of calibration. Visual inspection of the FT-IR spectrum of a dry white wine indicated that the region from 5011 – 3630 cm^{-1} showed little variation in absorbance, whereas prominent peaks were present in the region from 1800 - 929 cm^{-1} (Figure 1a). The information in the latter region, referred to as the "fingerprint" area, is particularly useful in molecular absorption spectroscopy, since many different IR bands, including those corresponding to the vibrations of the C-O, C-C, C-H and C-N bonds occur in this region (Smith, 1999).

Distinct variation between the FT-IR spectra of wines of various styles was observed in the region from 1800 – 929 cm^{-1} as illustrated by the spectra of the dry red, low alcohol and noble late harvest wines (Figure 1b). Notable was the prominent peaks at 1647 – 1640 cm^{-1} which correspond to the area where the carbohydrates absorb (Coimbra *et al.*, 1998). Collectively, more than 85% of the variation in the glycerol content of the wine samples could be correlated to the absorbance at the filters shown in Figure 1b. The most prominent filter was at 1462 – 1458 cm^{-1} (corresponding to the H – C bend), which explained 62% of the total variation in the glycerol content of the sample set. The filters at 1060 – 1057 cm^{-1} and 1084 cm^{-1} respectively, (each explaining 2% of the variation) corresponded to the C – O stretch (Pavia *et al.*, 2001).

5.3.2 PCA MODELING OF WINE SAMPLES

In the explorative stages of PCA, the complete data matrix, which included all the samples and all the wavenumbers, was modeled. The score plot of PC1 versus PC2 showed a distinct clustering of the samples that was related to wine style, as well as individual deviating samples and groups of deviating samples (Figure 2a). Group A consisted exclusively of two unwooded dry white wine styles. The cluster locating near the origin of the model (group B) consisted of the dry red, dry white, off-dry white and young unbottled wines. Most of the young wines located at the extreme periphery of group B and tended to separate towards the positive end of PC2. Group C located towards the positive end of PC1 as well as towards the negative end of PC2 and consisted exclusively of the special late harvest and noble late harvest wines. Six samples were located far away from the other samples (groups D1, D2 and D3) and clearly had spectra deviating considerably from the rest. An analysis of the residual X-variance and leverage of these deviating spectra showed a significant influence on the PCA model (results not shown) and these samples were therefore considered to be true outliers. PC1 explained a relatively low level of variation in the sample set (49%), which was not unexpected, since all the wavenumbers were included in the modeling and background noise due to the absorbance of water would therefore also be included. The PC1 loadings plot showed high loadings for wavenumbers where water is known to absorb (regions B, Figure 2b). Prominent loadings were also observed in region C (1500 – 1000 cm^{-1}) pointing to the contribution of the absorbance of several of the group wavenumbers of ethanol, glycerol, the sugars and organic acids which are known to absorb in this region (Smith, 1999). Region A showed very low loadings and confirmed the earlier interpretation that this region provided very little useful information and could contribute to noise in the spectra.

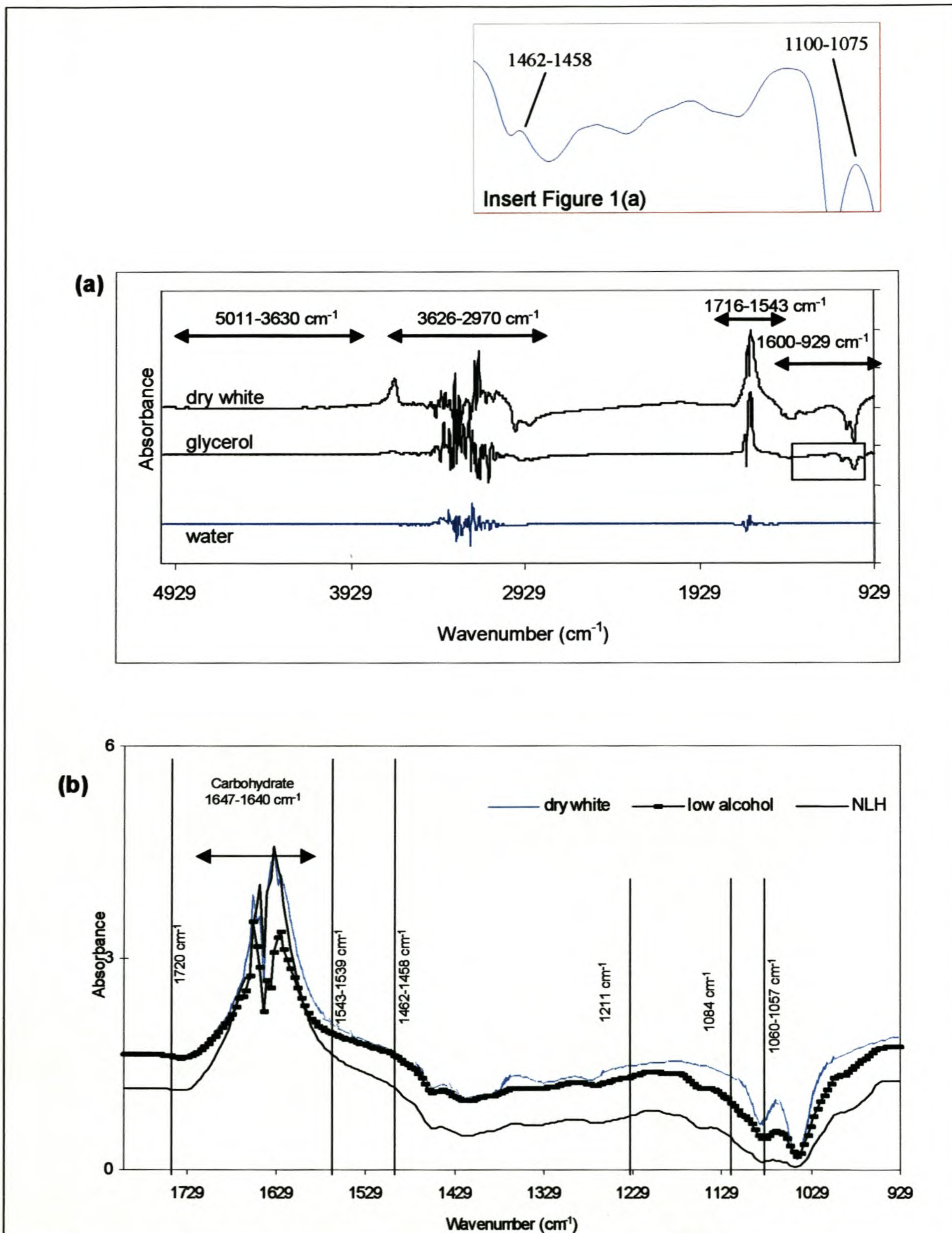

Figure 1. (a) FT-IR spectra of water, glycerol (10 g/L) and a dry white wine in the region 5011-929 cm^{-1}. Spectra were offset for clarity. (b) Spectral variation between dry red, low alcohol and noble late harvest wines in the region 1800-929 cm^{-1}. Vertical lines indicate the areas, where collectively, more than 85% of the variation in the glycerol content of the samples was explained.

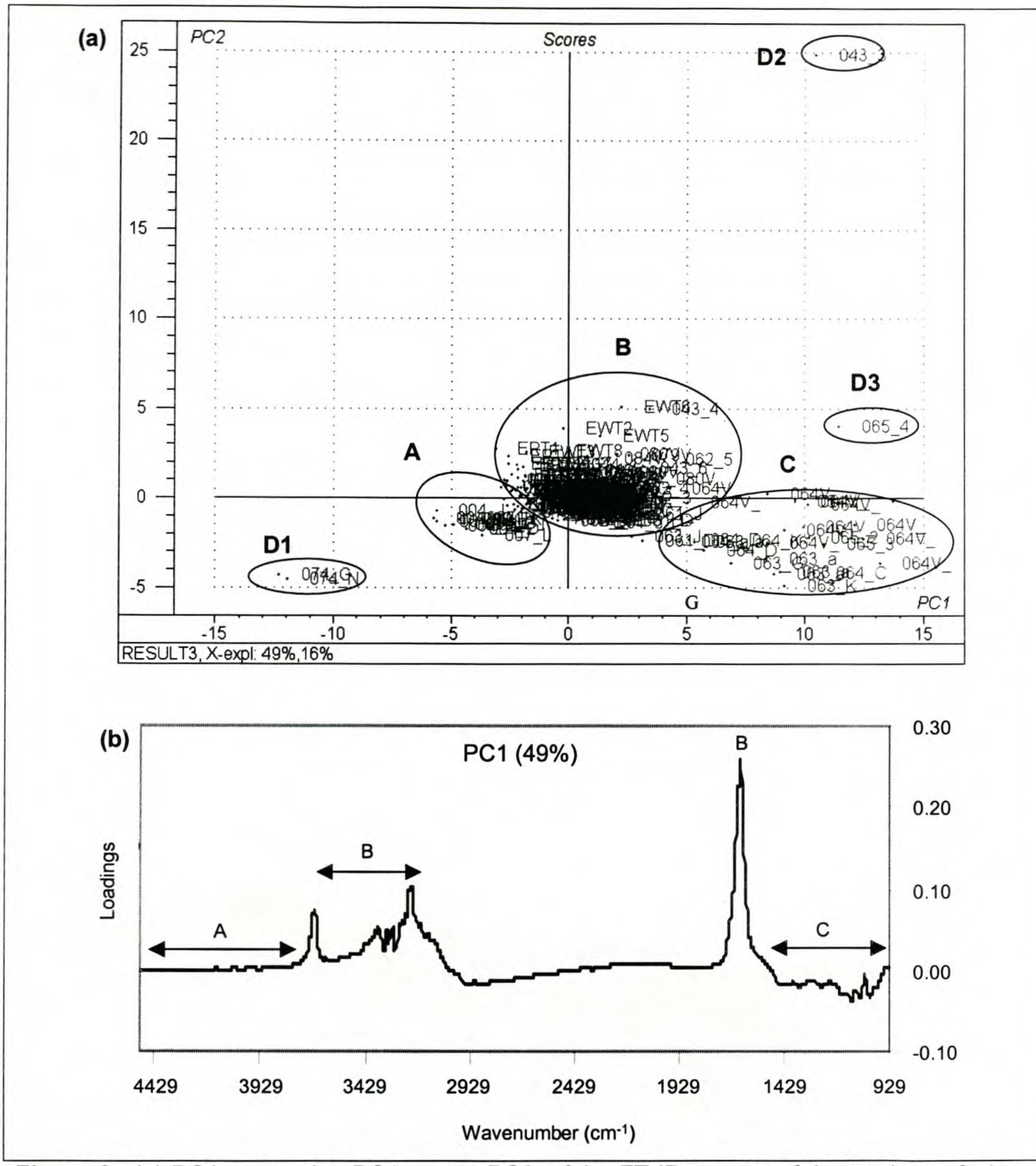

Figure 2. (a) PCA score plot, PC1 versus PC2, of the FT-IR spectra of the total set of wines (n = 329) and all the wavenumbers included. The model was centered and the axes cross each other at the origin. (b) PC1 loadings plot. See text (see section 5.3.2) for the interpretation of the symbols.

Subsequent PCA was done with the outlier samples in groups D (n= 6) removed from the original sample set. The two regions where water absorbs strongly (3626 - 2970 cm^{-1} and 1716 – 1543 cm^{-1}, respectively) and the region showing little useful information (5011 – 3630 cm^{-1}) were also deselected. Where appropriate, the interpretation of the score plots was based on the concentration ranges of the major chemical components of the wine samples (Table 2). PC1 (explaining 96% of the variation) seemed to distinguish between samples based on sugar content (Figure 3a). Samples in group A, which consisted almost exclusively of noble late harvest wines (RS levels ranging from 82 to 147 g/L; average 130.2 g/L, Table 2), appeared as a highly diverse and scattered group. Samples in group B consisted of special late harvest wines (RS levels ranging from 31 - 47 g/L, average 43.1 g/L). Group C consisted of commercial dry red and white wines, off-dry white wines and the young wines (RS levels collectively ranging from 0.5 – 13 g/L). Some of the young wines and some of the older red wines that have undergone more than three years maturation located on the extreme periphery of group C, with the young wines locating towards the negative end of PC2 and the older red wines towards the positive end of PC2 (Figure 3a). Samples in Group D represented the low alcohol wines (less than 8% v/v ethanol), and PC2, explaining 3% of the remaining variation, seemed to distinguish between samples based on the ethanol content. These results confirmed the earlier observation that both the RS and ethanol levels were major sources of variation between the different styles and also suggested that the late harvest wines as well as the low alcohol wines would have to be treated as separate groups in the design of calibration models for glycerol. The PC1 loadings plot (Figure 3b), showed particularly high loadings for several wavenumbers in the region from 1150 – 950 cm^{-1}, which are known to be associated with the absorbance of the carbohydrates (Coimbra *et al.*, 1998). The PC1 loadings plot confirmed the interpretation of the score plot in Figure 3a, but also pointed to the need to extract the most pertinent wavenumbers related to the variation in the glycerol content for the purpose of calibration. The separation of the samples based on the sugar content was not surprising since the largest variation in the chemical composition between the different wine styles was seen in the RS levels, both in terms of the range in concentrations and the standard deviation within each style (Table 2).

Table 2. Component range of calibration samples.[a]

Style	Glycerol (g/L)	Ethanol (% v/v)	Residual sugar (g/L)	Volatile acidity (g/L)[b]	Titratable acidity (g/L)[c]
Dry white	6.81 ± 0.82	12.47 ± 0.71	2.34 ± 1.10	0.36 ± 0.13	6.01 ± 0.59
Off-dry white	6.58 ± 0.77	12.48 ± 0.88	5.94 ± 1.99	0.28 ± 0.10	6.05 ± 0.46
Low alcohol	3.47 ± 0.53	7.28 ± 0.31	2.04 ± 0.05	0.21 ± 0.06	5.43 ± 0.31
Dry red	10.61 ± 1.08	12.93 ± 0.73	1.75 ± 0.69	0.54 ± 0.14	5.88 ± 0.36
SLH[e]	6.61 ± 0.87	11.62 ± 0.49	43.14 ± 8.12	0.33 ± 0.12	5.64 ± 0.50
NLH[f]	15.02 ± 3.82	12.93 ± 1.35	130.19 ± 24.95	0.87 ± 0.27	7.13 ± 0.67
Blanc De Noir	5.60 ± 0.46	12.39 ± 0.80	14.90 ±9.79	na[d]	5.86 ± 0.95
Young red wines	9.86 ± 0.93	11.01 ± 0.58	4.93 ± 1.17	0.40 ± 0.13	4.77 ± 0.53
Young white wines	6.43 ± 0.57	11.08 ± 1.30	4.70 ± 1.34	0.37 ± 0.17	5.61 ± 0.56

[a]Values given are average ± standard deviation.
[b]Expressed as g/L acetic acid.
[c]Expressed as g/L tartaric acid.
[d]Not available.
[e]Special late harvest wines.
[f]Noble late harvest wines.

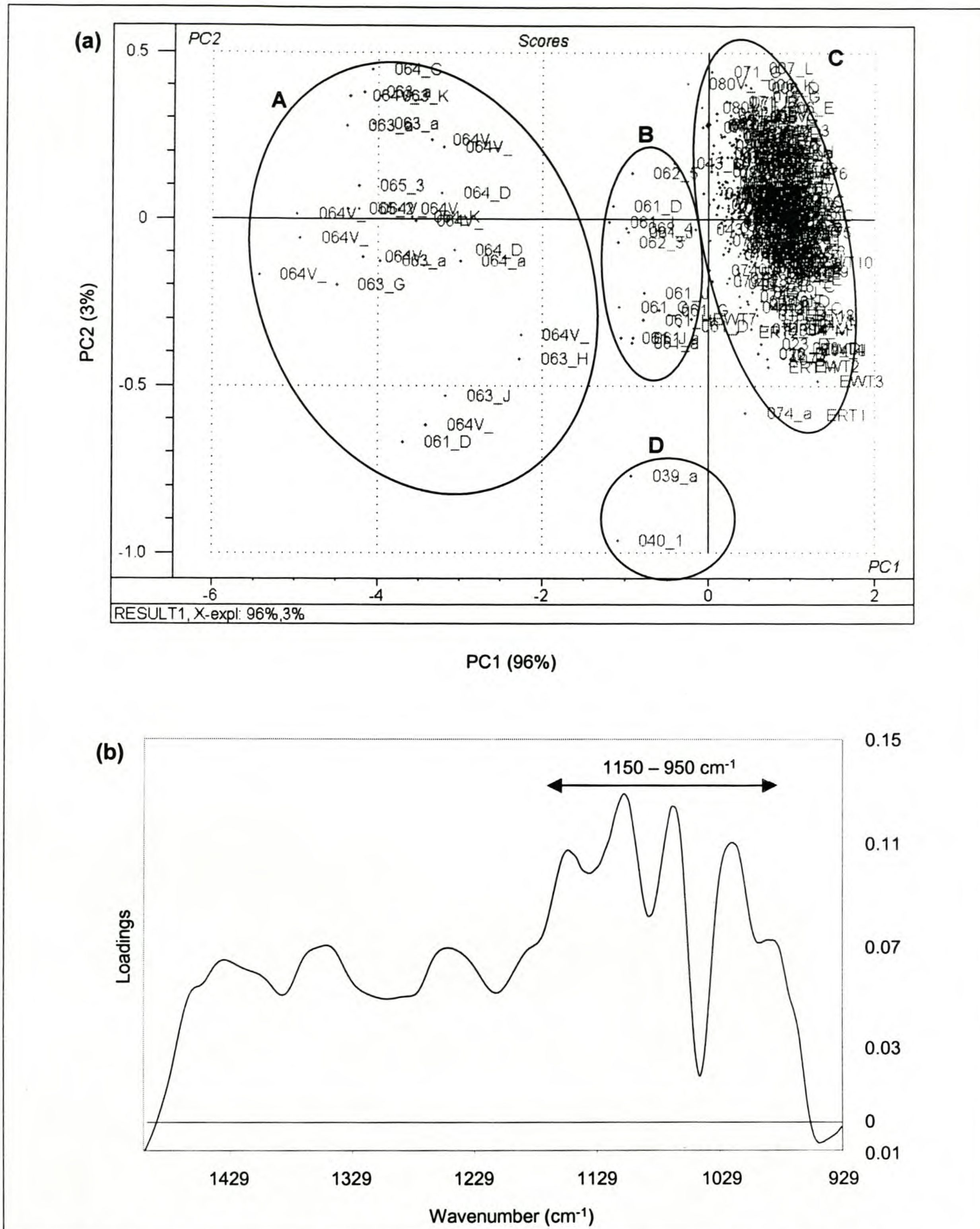

Figure 3. (a) PCA score plot, PC1 versus PC2, of the wine spectra, with the outliers (n = 6) removed and with the wavenumber regions 5011-2970 and 1716-1543 cm^{-1} removed. The model was centered and the axes cross each other at the origin. (b) PC1 loadings plot. See text (section 5.3.2) for the interpretation of the symbols.

In order to model the relationships between the wine samples on the basis of their glycerol content, PCA was done using only the 15 filters that collectively explained more than 98% of the variation in the glycerol content of the samples. The separation of samples in the score plot of PC1 vs PC2 (Figure 4) could be interpreted as follows: (i) the samples clearly separated on the basis of their glycerol content and samples with the highest glycerol levels located towards the negative end of PC1; (ii) the red and white wines separated in two groups, although with overlap between the two groups; (iii) the older red wines that have undergone more than three years of maturation as well as some of the young unbottled wines appeared on the "extreme" periphery of the clusters shown in Figure 4 and seemed to be spectrally different from the rest of the samples. These results provided preliminary evidence that both the maturation period of the wines, as well as the stage of fermentation should be taken into consideration in the design of calibration sets and that these sources of variation could have implications for the accuracy of prediction.

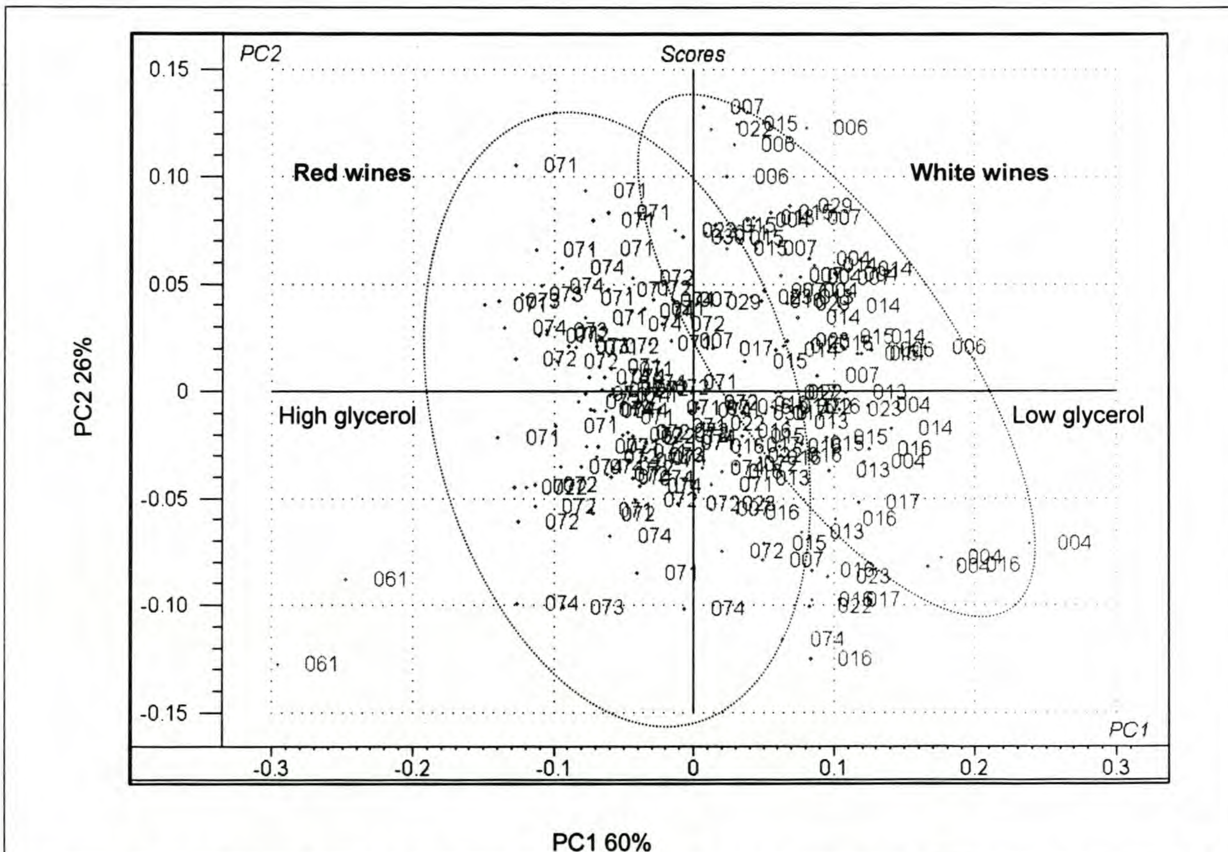

Figure 4. PCA score plot, PC1 versus PC2, of the FT-IR spectra of dry wines (n = 284; RS levels < 30 g/L). The fifteen most pertinent wavenumbers, explaining more than 98% of the variation in the glycerol content of the samples were used for the modeling.

5.3.3 DESIGN OF CALIBRATION SETS FOR GLYCEROL IN WINE

The clustering of the wine samples (as observed with PCA) clearly indicated that the original sample set had to be subdivided in appropriate subsets, in order to establish calibration models with accurate prediction abilities for the various groups of wine styles. The strategy in the design of calibration sets was aimed at low SECV values, but at the same time keeping the number of calibrations as small as possible. In the exploratory stages of calibration a "cut-off" level of *ca*. 8% v/v ethanol was used to differentiate the "low ethanol" wines from the wines with higher ethanol levels and a RS level of *ca*. 30 g/L to differentiate between "dry" and "sweet" wines. For the design of the calibration sets, the "low ethanol" wines were treated as a separate group. The dry wine calibration set contained the dry red, dry white, off-dry white, Blanc De Noir and the young wine styles, while the sweet wine calibration set consisted of the special late harvest and noble late harvest styles (Table 1).

5.3.3.1 Glycerol calibration for dry wine

No significant gain in terms of accuracy for glycerol prediction was obtained by separating the red and white wines into different calibration sets, whereas the inclusion of the young wines in the original calibration model, resulted in an increase in the SECV (from 0.38 g/L to 0.52 g/L). Based on these results the red and white wines were not separated for the final design of the calibration set, but the young wines were removed from the original sample set and treated as a separate entity. Decisions regarding the design of calibration sets, should, however, clearly be made in the context of a particular application. For the quantification of ethanol for instance, where very high levels of accuracy are required, preliminary results indicated that the separation of white wines and red wines into different calibration sets improved the accuracy (data not shown). The final calibration set for dry wine (n = 135) spanned the glycerol concentration range in the original sample set and contained the "extreme" spectral members of each subset (as revealed by PCA modeling), as well as samples that were unusual in terms of their geographic origin. Some of the older red wines were also included as calibration samples.

Fifteen filters were selected that collectively explained more than 98% of the accumulated variation in the glycerol content of the calibration samples. The SECV for the calibration set was 0.38 g/L (Table 3). An independent sample set (n = 98) was used to test the predictive accuracy of the calibration model. These samples were selected to span the glycerol concentration range over which predictions in future samples had to be done. The SEP value (0.40 g/L, Table 3) was in agreement with the error for the reference method for glycerol, SEL = 0.30 g/L. SEP values of 1.32 g/L (Dubernet and Dubernet, 2000) and 1.13 g/L (Foss Electric, 2001) have been reported for the quantification of glycerol in dry wine. In comparison, the glycerol prediction with the calibration established in this study showed an improvement in accuracy. Patz *et al.* (1999) reported SEP values of 0.49 g/L and 0.32 g/L, depending on the sample set used for the validation.

The error of prediction for glycerol in the red wines that have undergone more than 3 years aging, was in excess of 0.6 g/L for some wines and these samples were clearly predicted less accurately by the model. This result was not surprising in view of the tendency of these samples to locate towards the extreme periphery of the dry wine cluster in the PCA score plot (Figure 4). Complex changes occur in the chemistry of red wines during aging, particularly due to the polymerisation and condensation of the tannins and recent research on wines that have been subjected to different aging regimes, showed changes in the chemical composition of tannins that were reflected in the FT-IR spectra of the samples (Edelmann and Lendl, 2002).

Table 3. Regression statistics for glycerol calibration and validation in dry wine.

(a) Calibration set		**(b) Validation set**	
Number of factors	7	Bias	0.02
Number of filters	15	r	0.96
Number of samples	135	Number of samples	98
SECV[a] (g/L)	0.38	SEP[a] (g/L)	0.40
AR[a]	0.08	AR[a]	0.08
Glycerol range (g/L)[b]	4.74 – 14.00	Glycerol range (g/L)[b]	5.46 –13.40
Average glycerol (g/L)[b]	8.71	Average glycerol (g/L)[b]	8.92

[a]Abbreviations used: SECV, standard error of cross validation; SEP, standard error of prediction; AR, absolute repeatability.
[b]As determined by the reference method.

5.3.3 2 Glycerol determination in low alcohol wines

Based on the PCA results, a "cut-off" level of *ca.* 8% v/v ethanol was used to differentiate the "low ethanol" wines from wines with higher ethanol levels. The prediction of the glycerol content in this wine style was not satisfactory using the calibration established for the dry wines (SEP > 2 % v/v). Due to the very small sample size (n = 2) it was not possible to develop a separate glycerol calibration for this group of wines. Future efforts will be directed towards the enlargement of the sample set and more work is required to fully characterise these wines in terms of their spectral properties in the IR range. Very little information on the application of FT-IR for the analysis of low alcohol wines has been reported in the literature, although Patz *et al.* (1999) reported analysing wines with ethanol concentrations of 8.5% v/v. No information was provided on the specific prediction error for these samples.

5.3.3.3 Glycerol calibration for sweet wines

Due to the large spectral variation in the sweet wines (as observed by PCA) and the relatively small sample size (n = 43, Table 1), the full sample set was used for calibration purposes. This produced a SECV of 0.65 g/L (Table 4), although the model has not been validated on an independent sample set. In a recent study where near infrared reflectance spectroscopy was used for the simultaneous determination of ethanol, glycerol, glucose and fructose in botrytised sweet wines, the highest error in the prediction results (17%) was found for the estimation of glycerol (Garcia-Jares and Médina, 1997).

5.3.3.4 Glycerol determination in young wines

The young wines were treated as a separate validation set of the glycerol calibration established for the dry wines and this resulted in a SEP of 0.85 g/L. A better fit of the data set was obtained by adjusting the intercept of the original calibration, and using this strategy, a SEP of 0.43 g/L was obtained. The average levels of the components listed in Table 2 did not appear to be significantly different between the young wines and finished dry red or white wines, but it is to be expected that the stage of the fermentation would have a major influence on the spectral properties of the samples. In this respect, the CO_2 levels as well as the stage of the malolactic fermentation have been shown to influence the accuracy of quantification of various components using FT-IR spectroscopy (Dubernet and Dubernet, 2000). This is, however, clearly a situation that should be evaluated for each specific sample set and the results presented here, merely serve the purpose of illustrating that the prediction accuracies for the young wines need monitoring and may, in some instances require additional validation.

Table 4. Regression statistics for glycerol calibration in sweet wine.

Number of factors	9
Number of filters	15
SECV[a] (g/L)	0.65
AR[a]	0.09
Number of samples	43
Glycerol range (g/L)[b]	4.74 – 14.00
Average glycerol (g/L)[b]	8.71

[a]Abbreviations used: SECV, standard error of cross validation; AR, absolute repeatability.
[b]As determined by the reference method.

5.3.4 INTERPRETATION AND CLASSIFICATION OF OUTLIER SAMPLES

In the exploratory stages of PCA all the wavenumbers were included in the modeling *en lieu* of selectivity and 6 extreme outlier samples were identified. These samples were poorly described by the PCA model and did not appear to belong to any of the major groups shown in Figure 2a. The glycerol estimations for these samples, using the calibrations established in this study, were also poor and the SEP values were in excess of 0.8 g/L. In this study several strategies were used to interpret the outlier status of these samples, including: i) a comparison of the component ranges of the outlier samples to that of similar samples in the sample set; ii) an examination of the statistics (mean, maximum, minimum and SD) of the raw spectra over the entire wavenumber range; and iii) visual inspection of the raw spectra. The component ranges of the outlier samples, as compared to that of similar types of wines, did not reveal any obvious unusual features. The outlier status of two of these samples could be ascribed to poor repeatability (as judged by a large SD between the absorbance at some wavenumbers in duplicate scans) that could be an indication of poor sample quality or inhomogeneity in the sample. In the other instances, however, the spectra of the outlier samples were markedly different from those of similar wines (data not shown) and the atypical nature of the spectra was confirmed with repeated scanning.

For the early detection and classification of the spectral outliers, the SIMCA application of the Unscrambler Software was used to make two disjoint PCA class-models, respectively, for "typical white wine" and "typical red wine". The class-membership of a test set containing outliers as well as dry white and dry red wines, assumed to be typical, was tested at a significance level of 5%. The classification results are graphically presented in Figure 5, where the area below the horizontal line delimits membership of "typical white wines" and the area to the left of the vertical line delimits membership of "typical red wines". The area near the origin of the plot delimits samples showing membership to both models (Esbensen, 2000). Results showed a 100% non-membership to both models for the outlier samples. The typical dry white wines in the test set correctly classified as "typical" (as can be seen from their position below the horizontal line). A few of the red wines, assumed to be typical, falsely classified as outlier samples, which points to the limits of the discriminating power of the model, but also to the complexity of the red wines. Future work would be aimed at enlarging the database of deviating spectra and to establish a discriminatory PCA calibration on the WineScan FT 120 instrument, in order to provide a conformity test at the time of analysis, as well as a warning of suspected outlier samples. Such samples should then automatically be tested with appropriate reference methods and subjected to further investigation for the purposes of quality control. Recently, a quality assurance software module was made commercially available and this application can be used as a basis from which to develop customised calibration models for the purpose of quality control (In Focus, 2003).

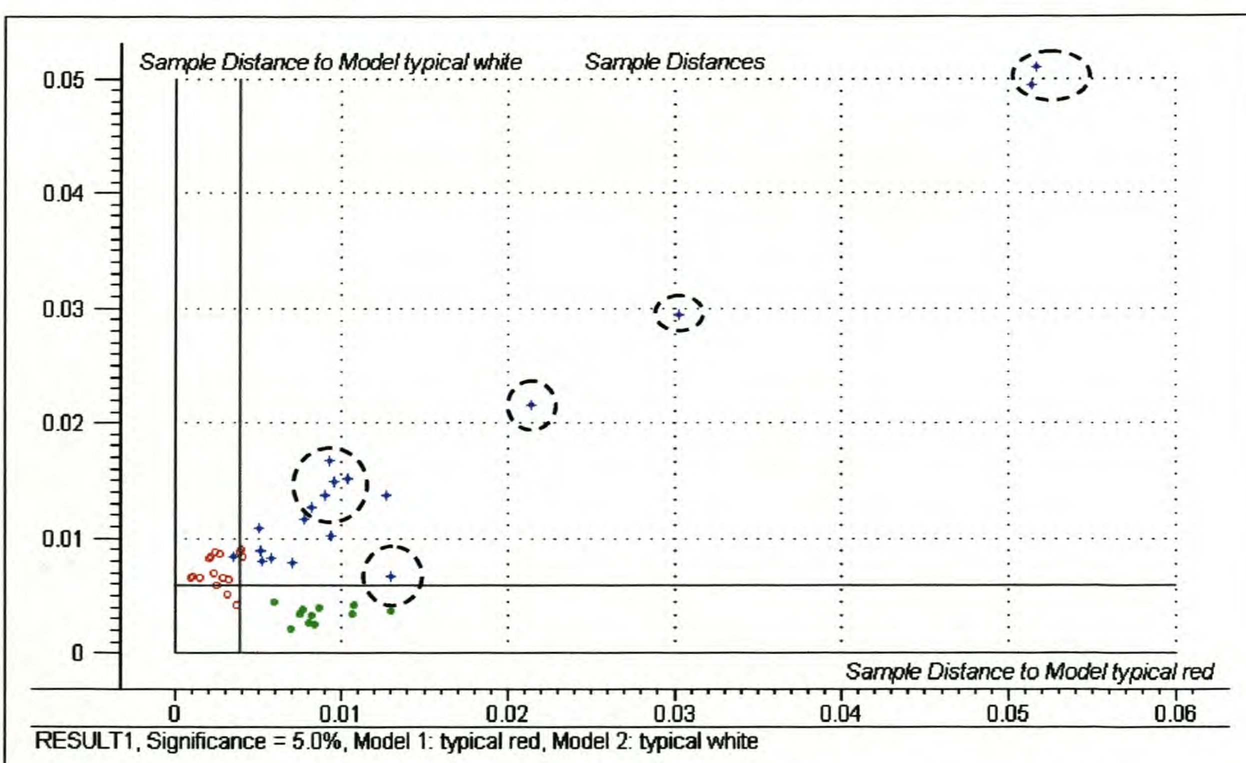

Figure 5. Coomans plot showing the distances of the outlier samples (circled with dashed lines) to the selected models for "typical red wine" and "typical white wine", respectively.

From a spectroscopic perspective, wine is a challenging matrix both in terms of its chemical complexity as well as in the variation introduced in the spectra by factors such as style, process technology, cultivars and geographic origin. For the purposes of quality control in the analytical laboratory, the outlier samples are important and require special attention. Due to the atypical nature of the spectral properties of these samples, it is to be expected that the accuracy of prediction in these samples will be unsatisfactory. Furthermore, if the cause for the outlier status of these samples could be interpreted, the appropriate action to be taken and the decisions on how to handle similar future samples would be more informed.

5.4 CONCLUSIONS

This study has shown that PCA provides a powerful tool to identify the major sources of variation in the FT-IR spectra of wine samples. The sources of variation identified in this study included the sugar and ethanol contents of the samples, the stage of the fermentation process and the maturation period. The implications of this variation for the accuracy of prediction of calibration models were evaluated using glycerol calibration as a model system, and clearly showed that calibration samples have to be carefully selected in order to design calibration models that find a balance between robustness and accuracy of prediction. PCA of the FT-IR spectra also facilitated the early detection and classification of poorly predicted samples, as well as a small number of extreme outlier samples in the sample set. It is our opinion that the successful implementation of FT-IR for the routine analysis of wine requires an approach that combines the development of robust calibration models, together with the implementation of quality control measures (such as PCA calibrations or SIMCA models) to enable the early detection and interpretation of poorly predicted samples and outlier samples. The latter aspect clearly also involves data management, specifically in terms of the interpretation of the reasons for the poor predictions or outlier status of deviating samples.

5.5 ACKNOWLEGDEMENTS

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CHAPTER 6

RESEARCH RESULTS

The application of Fourier transform infrared spectroscopy as a tool for the rapid screening of the fermentation profiles of wine yeasts

RESEARCH RESULTS

The application of Fourier transform infrared spectroscopy as a tool for the rapid screening of the fermentation profiles of wine yeasts

ABSTRACT

The aim of this work was to develop a rapid screening method for the evaluation of the fermentation profiles of wine yeasts using Fourier transform infrared spectroscopy (FT-IR). The yeast strains used included commercial wine yeasts frequently used in winemaking in South Africa, as well as a selection of hybrid yeasts that had been developed in a yeast strain development programme aimed at increasing glycerol levels. Calibration models were developed and optimised for the quantification of volatile acidity, glycerol, ethanol, reducing sugar and glucose in small-scale fermentations in Chenin blanc juice and in a synthetic must. The predictive accuracy for the quantification of volatile acidity in both Chenin blanc and the synthetic must was excellent, and the standard error of prediction (SEP) values were 0.03 g/L and 0.04 g/L, respectively. The quantification of ethanol (SEP = 0.32% v/v and 0.31% v/v, respectively), glycerol (SEP = 0.38 g/L and 0.32 g/L, respectively), residual sugar in Chenin blanc must (SEP = 0.56 g/L) and glucose in the synthetic must (SEP = 0.39 g/L), was in agreement with the accuracy obtained by the respective reference methods used for the quantification of these components. Principal component factor analysis of the analytical data obtained with FT-IR was used to provide an overview of the relationships between the strains with respect to their fermentation profiles. This study illustrates the potential of FT-IR as a tool to rapidly screen wine yeasts for their fermentative properties and to speed up the screening and evaluation processes in the initial stages of yeast strain development programmes.

6.1 INTRODUCTION

Yeast strain development programmes are ongoing processes in all major wine industries. These programmes often involve the screening of large numbers of natural yeast isolates in order to select favourable variants within a population of wine yeast strains, or alternatively, the evaluation of variants of established wine yeasts that have been optimised for specific properties (Pretorius, 2000; Dequin, 2001). Projects of this nature typically involve large volumes of analytical work, and successful outcomes therefore require considerable time investments. Yeast development programmes have recently been under pressure to accelerate in order to meet the current market-orientated transformation of the international wine industry (Pretorius and Bauer, 2002). It is clear that technological innovations that can speed up the yeast strain evaluation process, at least in the initial stages of strain selection, would be of major advantage.

The potential of Fourier transform infrared spectroscopy (FT-IR) as an analytical tool in enology has been recognised for many years. This technology is based on the measurement of the absorption of radiation in the mid infrared (IR) region (4000 - 400 cm^{-1} or in terms of nanometers, from 2500-2.5 x 10^4 nm) by molecules that contain chemical bonds such as C-C, C-H, O-H, C=O and N-H (Smith, 1999). Recent improvements in FT-IR instrumentation, combined with innovative and versatile software applications, have optimised the application of this technology in enology, and the WineScan FT 120 spectrometer (Foss Electric, Denmark) currently enables the simultaneous quantification of several components in a sample, in an assay time of less than 30 seconds. The concept of *calibration* which is widely used in analytical chemistry, also applies to FT–IR spectroscopy, and in order to predict the concentration of a component of interest, a predetermined calibration for the component is required. Due to the complexity of the information contained in the FT-IR spectra, an extensive calibration process that involves multivariate statistical procedures such as principal component analysis (PCA), principal component regression (PCR) and partial least squares regression (PLS), is required (Eriksson *et al.*, 1999; Esbensen, 2000). Optional software modules are available with the WineScan FT 120 instrument, and these include commercial ready-to-use calibrations of which the slope and/or intercept can be adjusted to provide a better fit to a specific set of data, as well as an advanced performance module which facilitates the creation of new calibrations. To date, the application of FT-IR spectroscopy as an analytical tool in enology has largely been focussed on the routine analysis of wine (Patz *et al.*, 1999; Dubernet and Dubernet, 2000; Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003).

This study reports on the application of FT-IR spectroscopy as a tool to rapidly screen the fermentation profiles of a selection of *S. cerevisiae* yeast isolates that have been developed as part of a yeast strain development programme aimed at the selection of strains with increased glycerol production (Prior *et al.*, 1999). The yeast strains were developed though a selective breeding programme and some of the hybrid yeast strains have been studied in greater detail with respect to their fermentation profiles in Chardonnay juice (Prior *et al.*, 2000). The results obtained in trial fermentations showed that the formation of increased levels of glycerol by the hybrid strains was accompanied by increases in the levels of secondary metabolites, notably acetic acid, acetaldehyde, succinic acid and 2,3 – butanediol, in a response to the altered carbon flux in the modified yeasts (Prior *et al.*, 2000). The potential negative implications of these increases, particularly that of acetic acid, for the quality of the wine, necessitates extensive analysis and evaluation of the fermentation profiles of the modified yeasts.

In this study the WineScan FT 120 instrument (Foss Electric, Denmark) was used to develop a rapid screening method for the quantification of glycerol, ethanol, volatile acidity (VA), reducing sugar (RS), and glucose in small-scale laboratory fermentations of Chenin blanc grape juice, as well as a synthetic must that is frequently used for the initial evaluation of wine yeast strains (Radler and Schütz, 1982). The fermentation profiles of the hybrid yeast strains were compared to those of a selection of commercial wine yeast strains frequently used in South African winemaking. A new calibration for the determination of glycerol in each matrix was created. Commercial calibrations (Foss Electric, Denmark) were used for the quantification of VA, ethanol, glucose and RS, after being validated for accuracy of prediction, and adjusted where necessary.

6.2 MATERIALS AND METHODS

6.2.1 YEAST ISOLATES AND FERMENTATION CONDITIONS

The yeast strains used in this study are listed in Table 1. The isolates were stored at – 80°C and subcultured on YPD agar (BioLab) prior to being used for fermentation studies. Chenin blanc juice (pH 3.34, 20.7 °Balling) and a synthetic must YEPD (20% glucose, 2% bacteriological peptone, 0.2% yeast extract, 0.1% K_2HPO_4, pH 3.20, Radler and Schütz, 1982) were used for the fermentations. Chenin blanc juice was treated with Velcorin^R (2 - methyl dicarbonate, Bayer) at a final concentration of 0.2 mL/L. The sterility of the treated juice was verified through plate counts prior to inoculation.

Table 1. Yeast strains used for small-scale fermentations.

Yeast strain	**Source/Reference**
Commercial wine yeasts	
WE14 (*S. cerevisiae*)	commercial wine yeast[a]
WE372 (*S. cerevisiae*)	commercial wine yeast[a]
VIN13 (*S. cerevisiae*)	commercial wine yeast[a]
VIN7 (*S. cerevisiae*)	commercial wine yeast[a]
NT116 (*S. cerevisiae*)	commercial wine yeast[a]
NT50 (*S. cerevisiae*)	commercial wine yeast[a]
NT112 (*S. cerevisiae*)	commercial wine yeast[a]
NT7 (*S. cerevisiae*)	commercial wine yeast[a]
N96 (*S. bayanus*)	commercial wine yeast[a]
Enoferm Bordeaux Red (*S. cerevisiae*)	commercial wine yeast[b]
Lalvin D47 (*S. cerevisiae*)	commercial wine yeast[b]
228 (*S. cerevisiae*)	commercial wine yeast[b]
Lalvin 71B (*S. cerevisiae*)	commercial wine yeast[b]
Strains used in breeding experiments: Parental strains	
Premier Cuvée UCB4 (*S. cerevisiae*)	Prior *et al.*, 1999, 2000
Prise de Mousse UCB2 (*S. cerevisiae*)	Prior *et al.*, 1999, 2000
Hybrids obtained through breeding	
S.cerevisiae XPB3 range (n=19)	Prior *et al.*, 1999, 2000

[a]Anchor Yeast, Warren Chemicals, South Africa; [b]Lallemand Protea Chemicals, South Africa.

Overnight cultures of the yeast strains in YPD broth (BioLab) were prepared at 30°C and used to inoculate aliquots of the fermentation media (130 mL in 150 mL bottles) at an inoculum level of 1 to 6x10^6 c.f.u./mL. Triplicate independent fermentations (20°C ± 2°C) were conducted and the progress of the fermentations was monitored on a daily basis through weight loss. The time course for glycerol production in Chenin blanc must and YEPD was monitored with a test set of fermentations inoculated with *S. cerevisiae* VIN13. For this purpose, the fermentation bottles were sampled aseptically on a daily basis and the samples were stored at - 20°C until analysed.

6.2.2 REFERENCE METHODS

Glycerol was assayed with the enzymatic method (Roche, catalogue number 148270), but the total assay volume was scaled down to 100 µL. Assays were done in microtiter plates (Sterilin, catalogue number 612F96) and absorbance readings taken at 340 nm using a µQuant spectrophotometer (Bio-Tek Instruments, USA). Enzymatic methods were also used for ethanol (Roche, catalogue number 0176290) and glucose (Roche, catalogue number 716251) determinations. The total volume for the glucose assay was scaled down to 1 mL and absorbance readings for glucose and ethanol were taken at 340 nm using an Ultraspec 2000 UV/Visible spectrophotometer (Pharmacia Bio-Tek Instruments, Cambridge, England). RS was determined with the Rebelein method and VA with steam distillation (Ough and Amerine, 1988). All the assays were done in duplicate. The accuracy of the reference method was expressed as the standard error of laboratory (SEL) and calculated as:

$$\text{SEL} = \sqrt{\frac{\sum (y_1 - y_2)^2}{2n}}$$

where y_1 and y_2 are the results of duplicate determinations and n is the number of samples.

6.2.3 FT-IR SPECTROSCOPY AND WAVENUMBER SELECTION

FT-IR spectra of the fermentation broths were generated in the wavenumber region 5011 – 929 cm^{-1}. The fermentation broths were degassed by filtration before the analysis, using the Filtration Unit (type 79500, Foss Electric, Denmark) with filter paper circles graded at 20 – 25 µm and with diameter 185 mm (Schleicher & Schuell, reference number 10312714). The multivariate statistical procedures, including principal component analysis (PCA), principal component regression (PCR) and partial least squares (PLS), that are required for the establishment of new calibrations and/or the validation of existing calibrations, have been described (Eriksson *et al.*, 1999; Esbensen, 2000), and the Advanced Performance Software Module of the WineScan FT 120 instrument (WineScan FT 120 Type 77110 and

77310 Reference Manual, Foss Electric, Denmark, 2001) were used for these calculations.

6.2.4 STATISTICAL PROCEDURES

6.2.4.1 Evaluation of the accuracy of prediction of the calibration models

The statistical indicators for evaluating the accuracy of the predictive abilities of the calibration models included bias, the standard error of cross validation (SECV), when based on the calibration sample sets, and the standard error or prediction (SEP), when based on independent validation sample sets. These indicators were calculated using the Advanced Performance Module of the WineScan FT 120 instrument according to the following equations:

$$\text{bias} = \frac{1}{n}\sum_{i=1}^{n}\left(y_i - \hat{y}_i\right); \quad \text{SECV / SEP} = \sqrt{\frac{\sum_{i=1}^{n}\left(y_i - \hat{y}_i - Bias\right)^2}{n-1}}$$

where y_i is the reference value for the i^{th} sample; $\hat{y}_i$ is the predicted value for the i^{th} sample and n is the number of samples (WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss Electric, Denmark, 2001).

6.2.4.2 Analysis of variance (ANOVA) and principal component factor analysis

The fermentation data obtained with the WineScan FT120 instrument were used for all the statistical calculations. The data obtained with the triplicate independent fermentations were averaged, and the mean values were used for the statistical calculations. A significance level of 5% was used in all cases and the calculations were done using Statistica release 6 (Microsoft Corporation, USA). For the purposes of ANOVA and principal component factor analysis, the yeast isolates were classified into two groups. One group (referred to as "hybrid strains"), consisted of the segregant strains obtained through the breeding experiments and the second group consisted of the commercial strains which included the two parental strains used in the breeding experiments (referred to as "commercial wine strains", see Table 1). One-way ANOVA of glycerol, ethanol, VA, RS (in the case of fermentations done in Chenin blanc juice) and glucose (in the case of fermentations done in YEPD) was performed to compare the two groups of yeasts.

To investigate the internal correlation-structure between the variables VA, RS, ethanol and glycerol, principal component factor analysis was used to determine the common factors responsible for the correlation-structure (Johnson and Wichern, 1992). Two common factors were extracted from the data obtained from fermentations in juice, and these, collectively explained 81.85% of the variation in the data set. In YEPD, 79.04% of the total variation in the fermentation data was explained by the two extracted factors. A Varimax rotation was used to determine the factor loadings.

6.3 RESULTS AND DISCUSSION

6.3.1 SMALL-SCALE FERMENTATIONS AND TIME COURSE FOR GLYCEROL PRODUCTION

Chenin blanc juice and YEPD synthetic must (20% glucose, pH 3.20) were used for small-scale fermentations at 20°C ± 2°C. CO_2 production was monitored on a daily basis through weight loss for all the strains used. On average, the maximum weight loss occurred from day 1 to 3 and the fermentations were usually completed (as judged by weight loss) within 16 days. The time course for glycerol production by *S. cerevisiae* VIN13 is shown in Figure 1 and represents a typical pattern observed for the yeast strains in both must and YEPD under the conditions used in this study. The steepest increase in glycerol concentration in the fermentation broths was associated with the early stages of the fermentation (days 1 to 5) and after ~12 days the levels stabilised. Similar results for the production of glycerol in small-scale fermentations have been reported (Radler and Schütz, 1982). This pattern of glycerol production under fermentative conditions was expected in terms of the regulatory role in $NADH/NAD^+$ redox balancing that has been ascribed for glycerol formation by *S. cerevisiae* during the early stages of fermentative metabolism (Bakker *et al.*, 2001). For the purposes of comparison, a 16-day fermentation period was used, after which the samples were directly analysed with the WineScan FT 120 instrument.

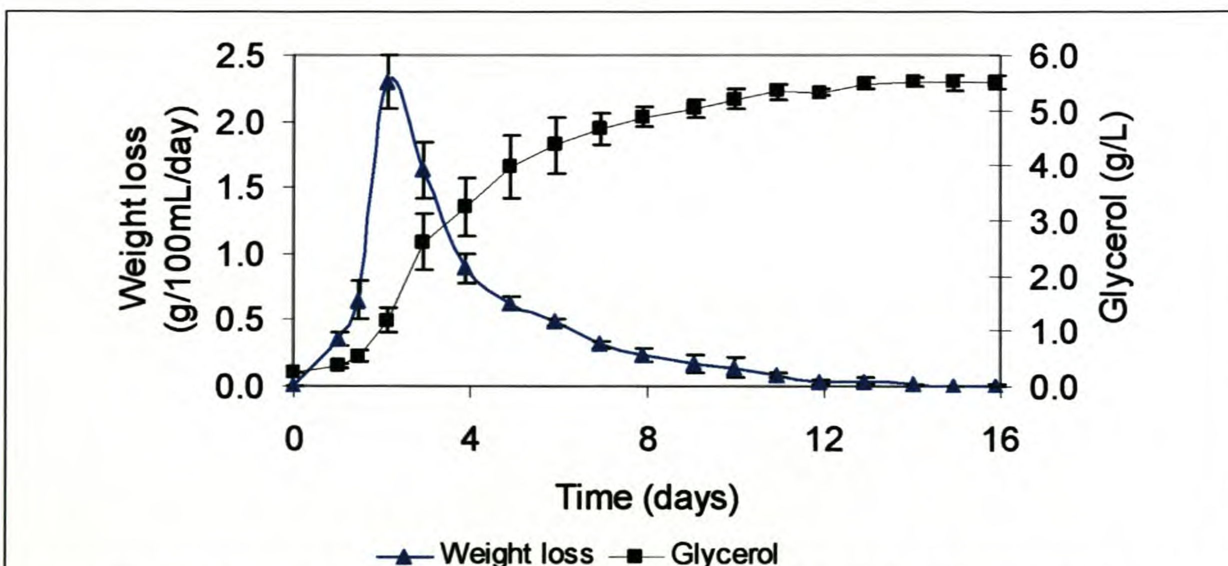

Figure 1. Time course of glycerol production by *S cerevisiae* VIN13 in Chenin blanc must at 20°C ± 2°C. Error bars indicate standard deviations of independent triplicate fermentations. Where error bars are not visible, the width of the error bar is smaller than the width of the marker.

6.3.2 FT-IR SPECTROSCOPY

Major changes occur in the chemical composition of fermentation broths during the course of fermentation, including the depletion of sugar and the formation of several novel components. A comparison of the FT-IR spectra at the start and at the end of the fermentation clearly reflected these changes (Figure 2). The spectra represent the collective information of all the IR active components in the medium, including yeast cells in the case of the fermented broths. The peaks in the wavenumber regions 3626 -2970 cm^{-1} and 1716 – 1543 cm^{-1}, respectively, can be ascribed to the absorbance of water (Smith, 1999). The absorbance in the region 1800 – 929 cm^{-1} corresponds to the vibrations of several chemical bonds, including the C-O, C-C and C-H bonds, and is therefore particularly rich in information with respect to the organic components such as the alcohols, sugars and organic acids present in the sample. As would be expected, the most pertinent wavenumbers for the purposes of establishing glycerol calibration, were extracted from the 1800 – 929 cm^{-1} region of the IR spectrum (data not shown).

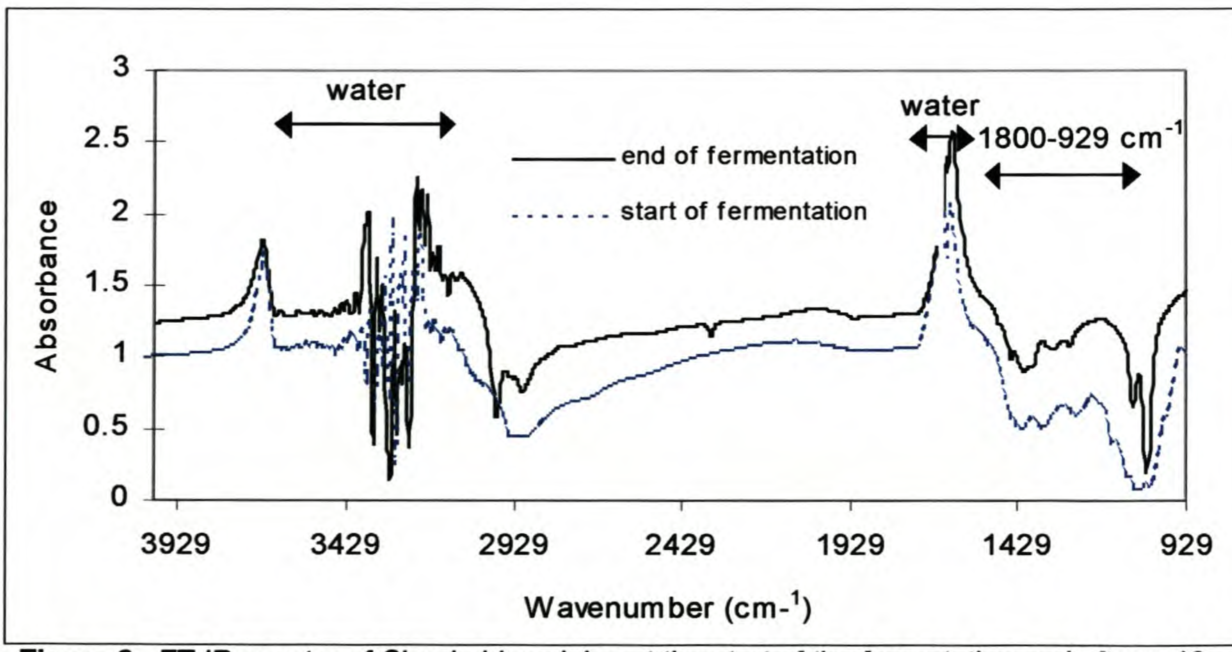

Figure 2. FT-IR spectra of Chenin blanc juice at the start of the fementation and after a 16 - day fermentation period at 20°C ± 2°C by *S. cerevisiae* VIN13. Spectra have been corrected for the absorbance of water.

6.3.3 QUANTIFICATION OF VA, ETHANOL, RS, GLUCOSE AND GLYCEROL

6.3.3.1 Validation of the commercial calibrations

Table 2 summarises the validation statistics for the quantification of VA, ethanol, RS and glucose, using the ready-to-use commercial calibrations (Foss Electric, Denmark). Preliminary results for the quantification of glucose in YEPD were not satisfactory (SEP = 1.54 g/L, mean bias = 1.06 g/L). An independent validation sample set was therefore established and used to adjust the slope and intercept of the regression line of the commercial glucose calibration, to provide a better fit to the data. The samples in the independent validation set were selected on the basis of their respective glucose levels (as determined with the reference method) in order to span the complete glucose concentration range over which predictions in future samples had to be done (*ca.* 0 – 9 g/L, see Table 2).

Table 2. Validation statistics for the estimation of volatile acidity, ethanol, reducing sugar and glucose in small-scale yeast fermentations in Chenin blanc must and a synthetic must (YEPD) using FT-IR spectroscopy.

Fermentation medium/parameter	Volatile acidity (g/L acetic acid)	Ethanol (% v/v)	Reducing sugar (g/L)	Glucose (g/L)
Chenin blanc				
number of samples	19	15	15	
concentration range[a]	0.19 - 0.85	9.50 – 12.96	0.1 – 5.10	
average ± SD[ab]	0.65 ± 0.28	11.51 ± 1.63	2.57 ± 2.09	
SEP[c]	0.03	0.32	0.56	
r[d]	0.98	0.99	0.97	
mean bias	0.001	0.10	0.15	
SEL[e]	0.02	0.29	0.20	
Synthetic must (YEPD)				
number of samples	14	14		32
concentration range[a]	0.24 – 1.098	7.69 – 12.36		0 - 8.62
average ± SD[ab]	0.58 ± 0.43	10.83 ± 2.52		4.68 ±-3.29
SEP[c]	0.04	0.31		0.39
r[d]	0.98	0.98		0.99
mean bias	0.006	-0.082		0.09
SEL[e]	0.02	0.20		0.15

[a]As determined with the reference method; [b]Standard deviation; [c]Standard error of prediction;.
[d] Correlation coefficient; [e]Standard error of laboratory.

The commercial calibrations for VA, ethanol, and RS did not require any adjustments for quantification purposes in either Chenin blanc or YEPD, and the SEP values presented in Table 2 were those obtained with the unadjusted commercial calibrations. In the interpretation of these results, it should be kept in mind that the commercial calibrations were established for a background matrix of fermented grape juice, and it was therefore not surprising that some calibration models will have to be adjusted to measure against a different background matrix. YEPD is a complex matrix with many undefined components being introduced into the medium through the yeast extract and bacteriological peptone components, (see Materials and Methods, section 2.1 for the composition of YEPD), and it is to be expected that this source of variation could interfere with the accuracy of prediction using FT-IR spectroscopy. The observation that the calibration of glucose, but not that of VA, ethanol or RS, needed adjustment, could be an indication that the absorbance at some of the wavenumbers selected for the glucose calibration, also included absorbance due to interfering substances present in the matrix. The potential source of error in the prediction data due to the so-called "matrix effect" has been discussed in detail (Esbensen, 2000; Smith, 1999).

From the regression results presented in Table 2, it is clear that excellent predictive accuracies were obtained for the quantification of VA in Chenin blanc and YEPD (SEP = 0.03 and SEP = 0.04, respectively), over the concentration ranges tested. The predictive errors for ethanol (SEP = 0.32 for Chenin blanc, and SEP = 0.31 for YEPD) were in agreement with the respective SEL values. Here it should be kept in mind that FT-IR is an indirect method based on the reference values, and the predictive errors (SEP) can never be smaller than those obtained for the reference methods (SEL). The largest predictive errors were found for the quantification of RS (SEP = 0.56 for Chenin blanc, and SEP = 0.39 for YEPD). Overall, for the purposes of screening, the accuracy of prediction for VA, ethanol, RS and glucose (over the concentration ranges tested) was considered satisfactory.

6.3.3.2 Glycerol calibrations

Calibrations for the determination of glycerol in Chenin blanc and YEPD were established and the regression statistics are summarised in Table 3. Samples for the respective calibration sets were selected to cover the range in glycerol concentrations expected in future samples.

Table 3. Calibration and validation statistics for the estimination of glycerol in Chenin blanc must and synthetic must (YEPD) using FT-IR spectroscopy.

Chenin blanc			
calibration set		validation set	
number of factors	4	bias	0.13
number of filters	15	r[a]	0.96
SECV[b]	0.24 g/L	SEP[c]	0.38 g/L
number of samples	35	number of samples	19
glycerol range	3.43 – 20.65 g/L	glycerol range	5.46 – 14.40 g/L
average glycerol	7.77 g/L	average glycerol	7.92 g/L
SEL[d]	0.19 g/L		
Synthetic must (YEPD)			
calibration set		validation set	
number of factors	7	bias	0.19
number of filters	15	r[a]	0.95
SECV[b]	0.32 g/L	SEP[c]	0.32 g/L
number of samples	41	number of samples	18
glycerol range	4.12 - 19.87 g/L	glycerol range	4.34 - 16.29 g/L
average glycerol	8.56 g/L	average glycerol	6.98 g/L
SEL[d]	0.17 g/L		

[a]Correlation coefficient; [b]Standard error of cross validation; [c]Standard error of prediction; [d]Standard error of laboratory.

The prediction error based on the calibration set (SECV = 0.24) was in agreement with the laboratory error (SEL). The SECV was also in agreement with the prediction error based on independent validation set (SEP = 0.38), which provided an indication of the robustness of the glycerol calibration. The samples for the independent validation set were selected to cover the glycerol concentration range over which future predictions had to be done (*ca.* 3 – 20 g/L). It was of interest to note that the highest accuracy (as judged by the lowest SEP values) was obtained by creating a separate glycerol calibration for each matrix, as opposed to adjusting the slope and/or intercept of one calibration, and furthermore, that the wavenumbers selected for the quantification of glycerol in the two matrices were not similar, although some overlap was observed (data not shown). The accuracy of prediction obtained with the established glycerol calibrations in Chenin blanc and YEPD (over the concentration ranges tested) was considered satisfactory for screening purposes.

6.3.4 FERMENTATION PROFILES OF THE COMMERCIAL WINE YEASTS AND THE HYBRID *S. CEREVISIAE* STRAINS

The analytical data obtained with FT-IR spectroscopy and the established calibrations are shown in Tables 4 and 5, respectively. For both the commercial strains (Table 4) and the hybrid strains (Table 5), the average VA levels formed in Chenin blanc must were very similar to those formed in YEPD, whereas the average ethanol and glycerol levels formed in Chenin blanc must were slightly higher than those formed in YEPD. In all the samples analysed, the RS consisted mostly of fructose, while the glucose component in the juice was almost completely exhausted (data not shown). This result was not unexpected in light of the glucophilic nature of *S. cerevisiae* (Bisson, 1993). Wide variations between the different hybrid strains were observed for the VA and glycerol levels, and the average levels for these two components were also much higher for the hybrid strains (in both Chenin blanc juice and YEPD) than for the corresponding levels of the commercial strains.

One-way ANOVA of glycerol, ethanol, VA, RS and glucose concentrations in Chenin blanc juice showed that as groups, the commercial strains and the hybrid strains differed significantly with respect to the average VA, ethanol and glycerol levels. A similar analysis in YEPD showed that the two groups differed significantly in the average VA and glycerol levels, but the differences between the average ethanol and glucose levels, were not significant. For each of the yeast strains tested, the average VA, glycerol and ethanol levels formed in the two respective matrices differed, although the general tendencies stayed the same. In general, the tendencies described here were similar to that reported earlier (Prior *et al.*, 1999).

It should be kept in mind though, that several factors influence the final levels of glycerol formed in industrial-scale fermentations (Scanes *et al.*, 1998), and it has been pointed out that care should be taken when extrapolating fermentation data obtained from controlled laboratory fermentations to those of industrial fermentations (Nieuwoudt *et al.*, 2002).

Table 4. Fermentation products of commercial wine yeasts. Average values of triplicate fermentations (± standard deviation) in Chenin blanc must and YEPD in small-scale fermentations conducted at 20 ± 2^{0}C are presented.

Yeast strain	**Volatile acidity (g/L acetic acid)**		**Glucose / RS (g/L)**		**Ethanol (% v/v)**		**Glycerol (g/L)**	
Commercial strains[a]	**Must**	**YEPD**	**Must**	**YEPD**	**Must**	**YEPD**	**Must**	**YEPD**
228	0.29 (0.03)	0.28 (0.05)	3.82 (0.98)	0.56 (0.29)	11.12 (0.35)	10.84 (0.49)	6.45 (0.35)	5.22 (0.23)
VIN7	0.23 (0.02)	0.42 (0.02)	2.73 (0.71)	0	11.09 (0.38)	10.70 (0.73)	6.11 (0.11)	4.88 (0.30)
N96	0.31 (0.03)	0.25 (0.07)	1.72 (0.62)	1.10 (0.21)	11.37 (0.22)	10.50 (0.54)	5.65 (0.21)	4.42 (0.39)
NT116	0.33 (0.01)	0.29 (0.03)	2.54 (0.38)	0.81 (0.53)	10.91 (0.31)	10.76 (0.58)	7.43 (0.25)	5.20 (0.15)
NT50	0.42 (0.02)	0.27 (0.05)	1.22 (0.19)	0.90 (0.09)	11.70 (0.25)	10.11 (0.09)	6.95 (0.43)	5.72 (0.80)
71B	0.36 (0.04)	0.38 (0.03)	2.49 (1.02)	0	10.64 (0.09)	11.08 (0.42)	5.86 (0.18)	4.63 (0.56)
VIN13	0.29 (0.03)	0.56 (0.05)	1.45 (0.85)	0.21 (0.58)	10.47 (0.17)	11.03 (0.60)	6.76 (0.43)	5.53 (0.09)
NT7	0.28 (0.01)	0.35 (0.03)	2.17 (1.15)	1.42 (0.37)	11.51 (0.14)	10.89 (0.25)	6.01 (0.08)	4.78 (0.60)
WE14	0.42(0.03)	0.25 (0.04)	2.81 (0.76)	0	11.27 (0.08)	10.21 (0.32)	5.00 (0.07)	3.7 (0.73)
Bred[a]	0.26 (0.04)	0.47 (0.04)	1.80 (0.42)	1.09 (0.09)	11.92 (0.51)	11.01 (0.60)	6.56 (0.61)	5.03 (0.56)
WE372	0.30 (0.01)	0.38 (0.03)	2.82 (0.55)	0.98 (0.15)	11.87 (0.46)	10.69 (0.41)	7.77 (0.27)	5.54 (0.26)
D47	0.37 (0.02)	0.19 (0.02)	3.14 (0.39)	0	11.59 (0.38)	11.00 (0.16)	7.42 (0.09)	4.76 (0.45)
Ba25	0.31 (0.02)	0.33 (0.04)	2.31 (0.19)	0	9.9 (0.39)	10.77 (0.43)	4.09 (0.27)	4.59 (0.37)
UCB4	0.27 (0.02)	0.49 (0.03)	2.47 (0.54)	0	9.87 (0.53)	10.36 (0.50)	4.42 (0.22)	4.17 (0.27)
average ± SD	0.32 ± 0.06	0.35 ± 0.11	2.39 ± 0.69	0.54 ± 0.53	11.09 ± 0.66	10.71 ± 0.31	6.18 ± 1.11	4.87 ± 0.55
range	0.23 – 0.42	0.19 - 0.56	1.22 - 3.82	0.0 – 1.42	9.87 - 11.92	10.11 - 11.08	4.09 - 7.77	3.77 - 5.72

[a]Abbreviated strain designations are given, see Table 1 for full details; [b]Bordeaux Red

Table 5. Fermentation products of hybrid *S. cerevisiae* wine yeasts. Average values of triplicate fermentations (± standard deviation) in Chenin blanc must and YEPD in small-scale fermentations conducted at 20 ± 2^{0}C are presented.

Yeast strain	Volatile acidity (g/L acetic acid)		Glucose / RS (g/L)		Ethanol (% v/v)		Glycerol (g/L)	
Hybrid strains	Must	YEPD	Must	YEPD	Must	YEPD	Must	YEPD
XPB3-1A	0.51 (0.03)	0.41 (0.04)	3.68 (1.05)	0	10.53 (0.64)	10.29 (0.38)	7.55 (0.32)	7.66 (0.18)
XPB3-2A	0.54 (0.01)	0.69 (0.05)	3.19 (0.47)	0	10.43 (0.31)	10.64 (0.10)	8.81 (0.130)	8.56 (0.35)
XPB3-3A	0.49 (0.02)	0.32 (0.02)	1.08 (0.91)	0.40 (0.23)	11.10 (0.37)	10.96 (0.39)	9.55 (0.63)	7.87 (0.34)
XPB3-5A	0.83 (0.03)	0.58 (0.03)	1.19 (0.38)	0	10.42 0.09)	10.03 (0.27)	13.01 (0.18)_	11.90 (0.40)
XPB3-1B	0.42 (0.04)	0.71 (0.04)	3.41 (0.15)	1.89 (0.98)	10.45 (0.32)	10.31 (0.26)	7.27 (0.28)	7.52 (0.66)
XPB3-2B	0.88 (0.03)	0.67 (0.06)	2.45 (0.66)	0.37 (0.64)	10.16 (0.59)	10.68 (0.12)	15.13 (0.09)	15.61 (0.70)
XPB3-3B	0.41 (0.02)	0.53 (0.09)	2.39 (1.32)	0	9.98 (0.17)	10.57 (0.46)	7.52 (0.51)	7.90 (0.40)
XPB3-4B	0.52 (0.01)	0.55 (0.06)	1.18 (0.47)	0	10.67 (0.09)	10.51 (0.39)	9.94 (0.14)	9.28 (0.62)
XPB3-5B	0.98 (0.02)	0.81 (0.08)	1.32 (0.61)	1.45 (0.93)	10.17 (0.75)	9.72 (0.21)	19.47 (0.57)	17.43 (1.03)
XPB3-1C	0.62 (0.03)	0.72 (0.06)	1.47 (0.38)	0.51 (0.29)	10.21 (0.41)	10.76 (0.21)	11.29 (0.29)	9.98 (1.07)
XPB3-2C	0.71 (0.04)	0.76 (0.01)	1.09 (0.15)	0.53 (0.91)	10.09 (0.58)	10.44 (0.86)	14.03 (0.42)	13.14 (0.52)
XPB3-3C	0.41 (0.02)	0.58 (0.06)	1.54 (0.48)	0	10.44 (0.23)	10.97 (0.36)	7.93 (0.24)	7.22 (0.41)
XPB3-4C	0.63 (0.05)	0.61 (0.02)	1.88 (0.29)	1.97 (0.12)	10.17 (0.29)	9.91 (1.02)	16.97 (0.21)	17.49 (0.23)
XPB3-5C	0.57 (0.01)	0.66 (0.01)	3.27 (0.91)	0.17 (0.29)	10.60 (0.05)	10.90 (0.58)	11.21 (0.39)	10.74 (0.41)
XPB3-1D	0.70 (0.03)	0.71 (0.01)	3.55 (0.56)	0	10.77 (0.13)	10.51 (0.30)	13.90 (0.62)	14.09 (0.72)
XPB3-2D	0.49 (0.04)	0.58 (0.03)	2.29 (0.27)	0	10.58 (0.21)	10.46 (0.07)	9.16 (0.43)	8.78 (0.43)
XPB3-3D	0.54 (0.05)	0.60 (0.01)	2.95 (0.18)	0.41 (0.85)	9.93 (0.48)	9.85 (0.38)	9.21 (0.27)	8.81 (0.35)
XPB3-4D	0.76 (0.06)	0.61 (0.05)	1.82 (0.40)	0.17 (0.30)	10.14 (0.19)	10.49 (0.25)	14.61 (0.09)	15.40 (0.37)
XPB3-5D	0.61 (0.04)	0.45 (0.06)	1.90 (0.98)	0	10.76 (0.53)	10.91 (0.90)	9.23 (0.51)	9.38 (0.35)
average ± SD	0.61 ± 0.16	0.61 ± 0.12	2.19 ± 0.91	0.41 ± 0.63	10.40 ± 0.31	10.47 ± 0.38	11.36 ± 3.51	10.99 ± 3.48
range	0.41 - 0.98	0.32 - 0.81	1.08 - 3.68	0 - 1.98	9.93 - 11.10	9.72 - 10.97	7.27 – 19.47	7.22 - 17.49

Table 6. Principal component factor loadings for the variables VA, ethanol, glycerol, RS and glucose in Chenin blanc and synthetic must. A significance level of 0.05 was used.

	Chenin blanc		YEPD	
Variable	Factor 1	Factor 2	Factor 1	Factor 2
VA[a]	0.914539	0.250581	-0.902835	0.064995
ethanol	-0.754769	0.189499	0.470118	-0.633481
glycerol	0.870349	0.314330	-0.926118	0.133475
RS[b]/glucose	-0.089769	-0.951315	0.037056	0.918283
Explained variance	79.04%		81.85%	

[a]Volatile acidity; [b]Residual sugar.

6.3.5 PRINCIPAL COMPONENT FACTOR ANALYSIS

Principal component factor analysis of the fermentation data was performed in order to model the relationships between the yeasts with respect to their fermentation profiles. Two factors were extracted and the loadings plot, factor 1 vs factor 2, for the fermentation data obtained in Chenin blanc is shown in Figure 3. Factor 1 could be interpreted as a combination of the variance in the VA, ethanol and glycerol levels (judged by the high factor loadings for these three variables (Table 6), and Factor 2 could be interpreted in terms the RS levels. The loadings plot of the principal component factor analysis (Figure 3) showed a clear separation between the commercial strains and the hybrid strains, with some overlap between the two groups at the centre of the plot. Strains XPB3-5B and XPB3-2B appeared as extreme members of the hybrid group. These results are not surprising when interpreted in terms of the fermentation data of these strains (Table 4 and Table 5), which showed that strains XPB3-5B and XPB3-2B had the highest levels of glycerol, but also very high levels of VA (0.8 g/L). These strains would therefore not be considered as the best candidates for further development as wine yeasts. Strains XPB3-3A. XPB3-4B, XPB3-3C, and XPB3-2D and XPB3-1B all produced glycerol at higher levels than the average for the commercial strains, as well as VA levels below 0.5 g/L, and also located close to the commercial strains in the loadings plot. These strains (amongst several other possible candidates) could therefore be considered as promising candidates to be used in successive rounds of breeding, or alternatively, be considered for manipulation using recombinant DNA techniques. The loadings plot for the fermentation profiles in YEPD (Figure 4) provided similar results and once again identified XPB3-3A and XPB3-3C as possible candidates.

6.4 CONCLUSIONS

The results presented in this study show that FT-IR spectroscopy, can provide a powerful tool to rapidly evaluate the fermentation profiles of yeast strains in small-scale laboratory fermentations and the data presented here should be seen in the context of the potential of the application, rather than as a result. In this study, 33 yeast isolates were screened (triplicate fermentations) and one worker can easily complete this volume of work in *ca.* 3 hours after the completion of the fermentation. It was necessary to optimise some of the existing commercial calibrations to obtain accurate prediction data in the synthetic must, because the commercial calibrations were initially developed to quantify in the background matrix of grape juice. In the research environment new yeast isolates are frequently evaluated in synthetic must in the initial stages of strain development programmes, and the work presented here illustrates the potential to expand the calibrations to measure in different background matrices. The work also illustrates the potential of increasing the number of components assayed, by adding more calibrations for components of interest (like glycerol in this study) to the basic software package. A validation process is, however, required to establish the accuracy of prediction. In this way the scope of the fermentation profile can be enlarged, without increasing the analysis time. Principal component factor analysis of the fermentation data demonstrates that this application may prove to be a valuable tool in assisting in the interpretation of the analytical data and hence also in the strain evaluation process.

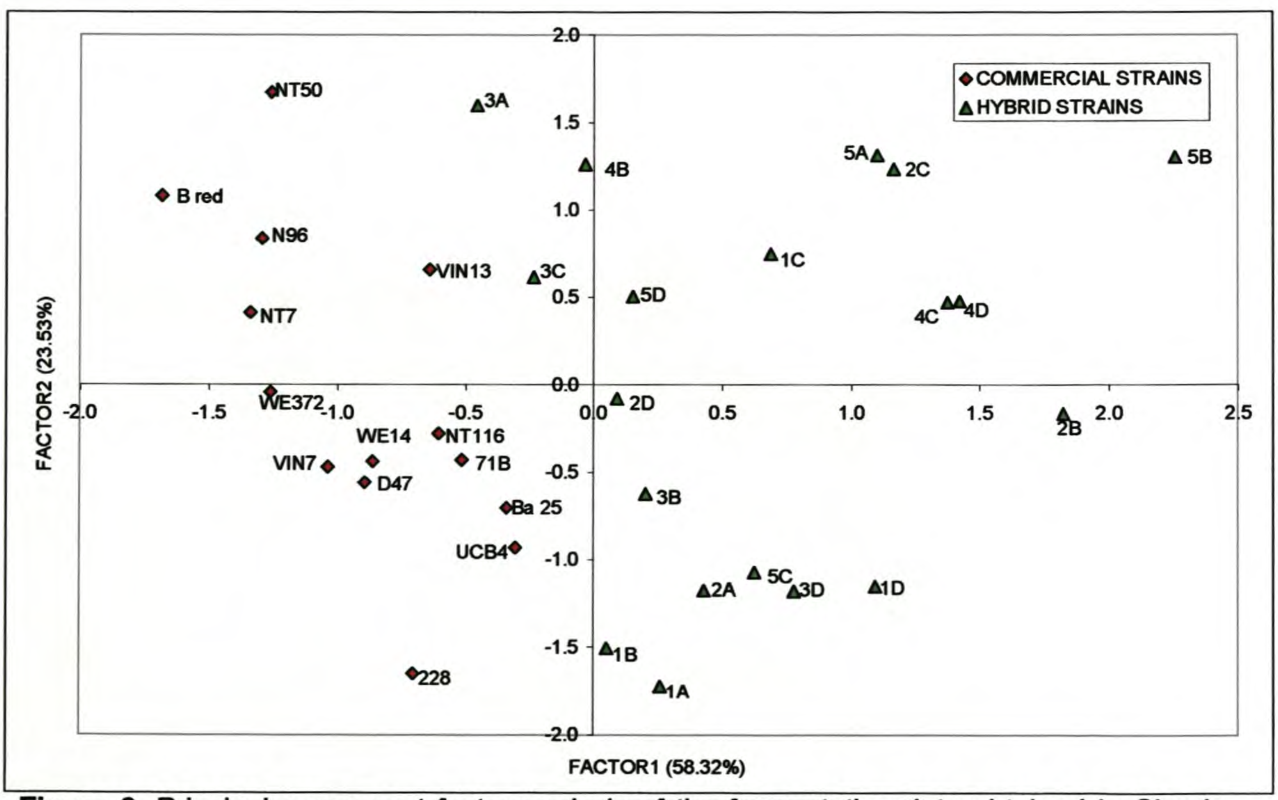

Figure 3. Principal component factor analysis of the fermentation data obtained in Chenin blanc. The prefix XPB3 of the hybrid strains is omitted for clarity (see Table 1).

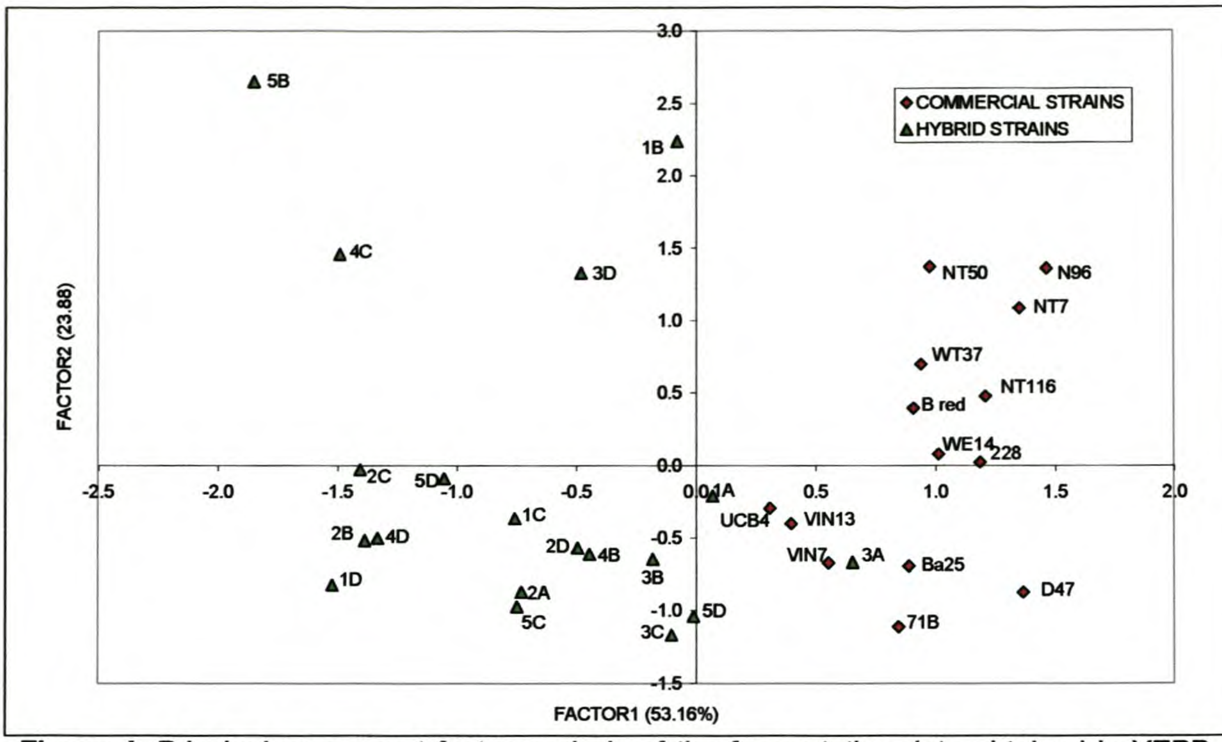

Figure 4. Principal component factor analysis of the fermentation data obtained in YEPD. The prefix XPB3 of the hybrid strains is omitted for clarity (see Table 1).

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CHAPTER 7

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

7.1 INTRODUCTION

Glycerol has been positively associated with the quality of wine since the early years of the 20th century. It has been widely accepted by enologists and winemakers that a correlation between glycerol levels and the quality of wine exists, at least for certain wine styles. As a result, numerous research projects were undertaken that focussed on glycerol production by wine yeast strains, and a limited number of investigations were also done into the relationship between glycerol and wine quality. To a large extent, these perceptions dominated the interpretation of the relevance of glycerol for wine quality and the value of glycerol as an indicator for quality control purposes (when considered in the context of wine spoilage) remained largely unexploited.

Over many years, a large historical set of data containing information relevant to glycerol and wine quality has been established, but to date, several aspects regarding the relationship between glycerol and wine quality have remained unclear. The limited progress that was made in this respect offers valuable lessons to be learnt in terms of research approaches and clearly points to the need for regular and critical evaluation of the directions and strategies used in addressing a complex issue such as wine quality.

Wine producers of the 21st century are facing major challenges in order to be successful in the modern international wine marketplace (Bauer and Pretorius, 2000 ; Bisson *et al.*, 2002). Leading enologists and market analysts have identified the critical success factors for sustainable profitability and these include consumer satisfaction and the sustained production of wine of consistent and good quality. At the same time the stringent international requirements for product and process authenticity have to be met. For the scientist the goal is to convert the challenges into practical, workable technological solutions for the whole wine value chain.

In the past (and to a certain extent still at present), the various scientific disciplines active in research fields related to viticulture and enology, tended to function in isolation of one another. The result was that the relationships between complementary sets of data were not always established and progress was in some instances delayed. This problem, which is particularly evident in the somewhat artificial separation that exists between the fields of enology and analytical chemistry, was recently addressed through the launch of the society *In Vino Analytica Scientia* (IVAS) in 1997 (Eveleigh, 2002). IVAS is organised by the Ecole Européenne de Chimie Analytique under auspices of the Office International et du Vin and the Divisions of Analytical Chemistry and Food Chemistry of the Federation of European Chemical Societies. IVAS is intended in bringing together researchers from related disciplines, particularly enology and analytical chemistry, and the society strives to stimulate amongst scientists, an awareness of the need of using an integrative approach to deal with some of the challenges of modern enology.

The results obtained in this study provide valuable information with respect to the glycerol levels in South African table wines and have shown that several of the perceptions regarding glycerol and wine quality are not supported by scientific data. It would indeed seem that too much emphasis is sometimes placed on the supposed contribution of glycerol to wine quality. The information gained from the South African winemakers, highlighted the fact that the "mouth-feel" property of some wine styles is considered as an area where the quality of wine can be improved. It is well known that several factors other than glycerol, such as the mannoprotein component of yeast cell walls and the fermentation profiles of certain wine yeast strains, are associated with the mouth-feel of wine (Delteil and Jarry, 1992). Data obtained from research projects focusing on these factors will provide a valuable point of reference against which to "weight' the impact of glycerol as a contributor to the mouth-feel properties of wine.

Strong indications were found that glycerol does affect the volatility of some aroma components in wine, but the nature of this effect appears to be complex and non-linear. In this respect, the work done in this study serves as a starting point and future work should include a detailed investigation of glycerol-volatile component interactions, using several different wine styles, more volatile components and smaller glycerol concentration intervals, in an attempt to establish an "optimal" concentration for glycerol in a particular wine style. In addition, aspects such as the influence of temperature and alcoholic strength, in combination with the glycerol concentration, on the volatility of the aroma components should also be investigated. It would also be interesting to undertake a project where the effect of increasing glycerol concentrations in wine is evaluated by a panel of non-expert wine tasters, with the aim to obtain a full range of sensory descriptors associated with glycerol in wine, as well as to evaluate the profile of liking by the panel, of such wines.

The work presented in this dissertation also contributed towards establishing fast and accurate analytical techniques, using Fourier transform spectroscopy, for the quantification of glycerol in finished wine. This opens up several future possibilities to manage and implement the analytical data generated, for the purposes of quality control in wine. Furthermore, the techniques that were developed and optimised can be used as a basis from which to expand the scope of the yeast fermentation profile screen, by adding new calibrations for parameters of interest and by including more background matrices in which future evaluations can be done, to the existing calibrations. The development of such methods will certainly contribute towards speeding up of the initial screening and evaluation process of yeast isolates in strain development programmes.

Future work regarding glycerol and quality control, should include the development of quality control calibrations to be used in conjunction with the FT-IR spectroscopy. Here, the most recent addition software application that has been designed for the WineScan FT 120 instrument, offers very powerful potential applications, through the provision of so-called "open equations". With this application, mathematical equations that are needed for the development of quality control calibrations, can be developed and customised to suit the needs of a particular quality control problem. This application will be officially launched in 2004 (personal communication, Torben Selberg, Production and Technical Manager - Wine, Foss Electric, Hillerød, Denmark).

In terms of using glycerol for authenticity testing, the South African wine industry can benefit from the development of new and/or the improvement of existing technologies available for the verification of the source of glycerol in wine. In this respect, analytical approaches aiming at increased sensitivity of detection, through for instance the use of the newly developed stir bar sorptive technique (Sandra *et al.*, 2001), together with the processing of the data using multivariate statistical procedures, should be a focus point. For this application to be of real value for the wine industry, the ultimate goal should be to develop the analytical technique to such an extent that it can be used for routine purposes.

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Appendix 1

GLYCEROL AND WINE QUALITY: FACT AND FICTION

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APPENDIX

GLYCEROL AND WINE QUALITY: FACT AND FICTION

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ABSTRACT

For more than 100 years, glycerol has been thought to contribute positively to wine quality. To date, the contribution of glycerol to wine quality remains unclear. Here we present the results of a comprehensive assessment of the relationship between glycerol and wine quality, based on the input of a specialist panel of SA winemakers and the quantitative data on the glycerol content of a statistically significant number of SA wines. In the dry white and dry red wine styles, the perceived strong correlation between glycerol concentration and wine quality was not obvious from the analytical data. At the concentrations normally found in the wine styles investigated in this study, glycerol may contribute to overall quality. The combined approach used here leads to a critical evaluation of commonly expressed opinions regarding glycerol and wine quality.

1. INTRODUCTION

Glycerol is predominant amongst several polyols commonly found in wine which include erythritol, arabitol, mannitol, sorbitol, meso-inositol and 2,3 butanediol. After ethanol and carbon dioxide, glycerol is the most abundant product of yeast fermentation (Ribéreau-Gayon *et al.*, 2000). Several parameters have been shown to influence the final glycerol levels in wine (Scanes *et al.*, 1998). These include the ripeness of grapes, the microbial flora on grape berries and cellar equipment, as well as the pH, fermentation temperature, the nitrogen source and the yeast strain. Glycerol is typically found at concentrations of 4 -10 g/L in dry wine and in the case of the noble late harvest wines, levels in excess of 20 g/L are not uncommon (Ribéreau-Gayon *et al.*, 2000). In the latter case, grape berries infected by *Botrytis cinerea* already contain significant amounts of glycerol as a result of the metabolism of the fungus, which explains the high glycerol levels commonly found in this wine style.

It is frequently suggested by winemakers, enologists and wine writers that glycerol contributes positively to wine quality. The perceived contribution is usually defined in terms of mouth-feel properties and is thought to be strongly dependent on the glycerol concentration in the wine. In general, higher glycerol levels are considered to improve wine quality. To date, the opinions regarding the relationship between glycerol and wine quality appear to be based on anecdotal and empirical evidence. In some instances, clear anomalies exist between the perceptions and actual data that have been obtained through experimental work.

In this article the relationship between glycerol and wine quality is critically reassessed in the context of modern South African (SA) wine styles and cultivars. For this purpose two sets of data were used: a) a quantitative database containing the analytical data of the glycerol levels of some 450 commercial SA wines of which *ca.* 90% received Veritas awards (1999 and 2000 Veritas competitions) and were therefore of adjudged quality (Nieuwoudt *et al.,* 2002) and b) a qualitative database containing the opinions of an expert panel of 15 individuals, on various aspects regarding glycerol and wine quality (this article). These aspects were communicated by means of a short questionnaire. The panel consisted of individuals involved with the training of winemakers, experts in the field of wine chemistry, and leading SA winemakers. All members have previously been involved in the official judging of wine quality. The winemakers on the panel are specialist producers of SA wines and are actively involved with the international marketing of SA export wines. The panel members, several of whom have expressed particular interest in glycerol in wine, were encouraged to express their own personal opinions gained through practical experience. The producing cellars represented diverse geographical winemaking regions of SA, and included Paarl, Robertson, Worcester, Cederberg, the Cape Peninsula, Stellenbosch and Hermanus.

2. SOME NEW PERSPECTIVES

The results of the expert opinions on the range of questions submitted to the panel are presented in the following sections.

On the perceived importance of glycerol for wine quality and the nature of its contribution the questions were:

1. Is glycerol in your opinion important for wine quality?

2. How does glycerol add to wine quality in your opinion?

The majority (80%) of the panel members were of the opinion that glycerol is important for wine quality. The perceived contribution was generally defined in terms of mouth-feel and texture properties. Glycerol was perceived to ensure consistency in style and to confer "suppleness" to wine and a "roundness" and "smoothness" on the palate. In addition, glycerol was thought to confer "fullness" (also referred to as "viscosity" or "weight" by the panel members) to wine and to lessen the perception of acidity, particularly in dry white wines. Several panel members were of the opinion that glycerol contributes to the complexity of wine and the length of the finish. Its contribution to sweetness was considered significant, but only when present at high concentrations.

The input of the panel members provided valuable insights with respect to the attributes that are sought in wine. The key issue here is clearly whether these attributes can be positively linked to glycerol. The concept of mouth-feel is one of the most complex, but also least understood sensory attributes of wine (Pickering *et al.,* 1998). The absence of reliable sensory data not only complicates the formulation of a workable definition for mouth-feel, but also the identification of the component(s) in wine responsible for the perceived attributes. No positive relationship between glycerol ***per se*** and the mouth-feel attributes of wine has yet been established and several factors other than glycerol have been implicated in mouth-feel. These include the ethanol concentration, the yeast cell wall mannoproteins, barrel maturation, yeast autolysis and the yeast strain used in the fermentation (Ribéreau-Gayon *et al*., 2000; Deltail & Jarry, 1992). Furthermore, at the concentrations at which glycerol is normally found in wine, the impact that it could have on the viscosity of wine would probably not be perceived by even the most experienced tasters (Noble & Bursick, 1984). Against this background it is quite possible that the perceived contribution of glycerol to mouth-feel can easily be over-emphasised.

On particular wine styles and optimal glycerol concentration the questions were:

1) Which wines do you think would benefit from glycerol?
2) What would you say is the optimal level of glycerol in any wine of your choice?

The panel members suggested that several white wine styles could benefit from glycerol. These were Chardonnay (especially barrel fermented wines), the fuller-styled Chenin blanc and Sauvignon blanc wines, as well as Semillon and Viognier. The optimal glycerol concentrations suggested ranged from 5 g/L – 7 g/L for the white wines, although a few members felt that much higher concentrations of 10 g/L – 15 g/L would be favourable. Glycerol was considered to be important for the quality of the noble late harvest wines. Several panel members were of the opinion that the red cultivars, particularly Cabernet Sauvignon and Pinot Noir would also benefit from glycerol and optimal concentrations of 5 g/L – 8 g/L were suggested. In general, the opinion was that the quality of the delicate-styled wines would be negatively affected and the fuller-styled wines (red and white) would be positively affected by glycerol. "Putting a figure to the optimal concentration of glycerol in a particular wine style however, does not make much sense" according to Manuel Bolliger, previously from Cape Point Vineyards, and in his opinion, "optimal is the quantity derived from physiologically ripe fruit".

In our assessment of the glycerol levels in SA wines, no major differences between mean glycerol levels of wines of different quality ratings were observed for the dry white and dry red styles, respectively (Table 1). The glycerol concentrations in the noble late harvest wines were, however, significantly associated with quality.

Unfortunately, no analytical data were available for Semillon, Viognier and Pinot Noir, and these cultivars should be included in future studies. The perception that there is a strong positive correlation between glycerol concentration and the quality of the dry white and dry red wines is therefore not supported by the analytical data. Within the concentration ranges reported, no major differences between mean glycerol levels of the delicate-styled and the fuller-styled Chardonnay wines were found (Table 2). The perception that glycerol has a negative impact on wine quality in the delicate-styled wines, was therefore also not supported by the analytical data obtained in this study.

Table 1. Glycerol levels in a selection of South African wines.

Cultivar/Style	Perceived Optimal Glycerol Concentration [a]	Actual Glycerol Concentration g/L [b]				
		Veritas award	No. wines	Mean	SD [c]	Range
Chardonnay	5 - 7 g/L, alternatively 10 - 15 g/L	gold [d]	10	7.11	1.02	5.77 - 9.32
		silver	66	7.12	0.94	5.31 - 9.36
		bronze	11	6.65	0.66	5.47 - 7.75
Sauvignon blanc	5 - 6 g/L	gold [d]	3	6.31	0.29	6.01 - 6.58
		silver	15	6.35	0.82	5.42 - 8.20
		bronze	25	6.28	0.59	5.21 - 7.90
Chenin blanc	5 - 7 g/L, alternatively 10 - 15 g/L	gold [d]	0	na [e]	na [e]	na [e]
		silver	18	6.80	0.81	5.20 - 7.88
		bronze	8	6.83	1.04	5.72 - 8.17
Dry red [g]	8 - 10 g/L, alternatively 13 -15 g/L	gold [d]	26	10.82	1.15	8.04 - 13.47
		silver	129	10.53	1.40	6.67 - 13.84
		bronze	59	10.44	1.40	6.67 - 14.24
Noble late harvest	ND [f]	gold [d]	4	16.37	4.57	9.81 - 20.21
		silver	4	15.62	2.14	13.39 - 18.43
		bronze	3	14.86	4.50	11.68 - 18.04

[a] Cumulative opinions of the panel members, see text
[b] Quantitative data obtained from commercial SA wines, see text
[c] Standard deviation
[d] Gold and double-gold ratings are combined
[e] Not applicable
[f] No Data available
[g] Pinotage, Merlot, Shiraz, Cabernet Sauvignon cultivars

Table 2. Glycerol levels in a selection of commercial Chardonnay table wines (vintage 1998 to 1999.

Style	Veritas rating	No. wines	Glycerol g/L		
			Mean	SD [a]	Range
Delicate style	**All**	**37**	**7.17**	**1.04**	**5.47 - 9.19**
	gold [b]	4	6.87	0.80	5.77 - 7.90
	silver	25	7.15	0.89	5.96 - 9.19
	bronze	8	6.75	0.72	5.47 - 7.75
Full style	**All**	**28**	**7.28**	**0.93**	**5.52 - 9.36**
	gold [b]	2	6.82	0.81	6.25 - 7.39
	silver	22	7.21	1.01	5.52 - 9.36
	bronze	3	6.36	0.46	5.84 - 6.73

[a] Standard deviation
[b] Gold and double-gold ratings are combined

Taking into account that glycerol is strongly associated with mouth-feel, the overall opinion of the panel members clearly suggests that the mouth-feel properties of the white wine styles in particular, need to be improved. It follows naturally that the impact of higher glycerol levels on the quality of these wines should be investigated. In two studies where glycerol- overproducing yeast strains were used to produce Chardonnay wines, the quality of the experimental wines was, however, rated less favourably than the control wines, although glycerol levels in excess of 15 g/L were formed in some cases (Prior *et al.* 2000; de Barros Lopes *et al.,* 2000). It should be noted that the attainment of the high glycerol levels suggested by some panel members (10 – 15 g/L) would have major implications on the carbon flux during yeast glycolysis. Reports in the literature show that the formation of high levels of glycerol are also coupled with elevated levels of other metabolites, notably acetic acid. Current genetic studies are focussed on decreasing the acetic acid accumulation of these strains (Eglinton *et al.*, 2002).

On the glycerol levels of SA wines the question was: What is your opinion of the glycerol levels of top quality SA wines?

The general consensus amongst the panel members was that the glycerol levels of top quality SA wines were too low. Some members suggested that the SA wines had lower glycerol levels than their Australian counterparts. Data on a recent large-scale assessment of the glycerol levels of Australian wines are unfortunately not available and data reported in the early 1970's were used for the purposes of comparison. Figure 1 shows a comparison between the average glycerol levels of the SA wines used in this study (190 white and 237 red wines) and those of wines from Australia (37 red and 11 white wines; Rankine & Bridson, 1971), California (15 red and 16 white wines; Ough *et al.,* 1972) and New York State (26 red and 37 red wines; Mattick & Rice, 1970). On average the mean glycerol levels of both SA red and white wines were higher than that of Australian wines and were more similar to the average levels reported for the New York State wines. For all countries represented with this data set, red wines had higher average glycerol levels than white wines. It should be noted that the mean glycerol levels reported for white wines were higher in the earlier studies as opposed to later studies, in both California and SA (Table 3). These differences could be explained partly in terms of the worldwide change towards lower fermentation temperatures for white wines (Ribéreau-Gayon *et al.*, 2000). The decrease in the mean glycerol levels in Californian red wines (10.6 g/L in 1954 versus 6.5 g/L in 1972) was ascribed to better cap management and picking the grapes at lower sugar contents (Ough *et al.*, 1972). In conclusion, although the glycerol levels of wines of different countries are frequently quoted (together with other data) for the purposes of establishing a benchmark for quality, it is clear that such interpretations should be done in the context of the impact of the fermentation conditions used during a particular period and winemaking region and not merely quality *per se*.

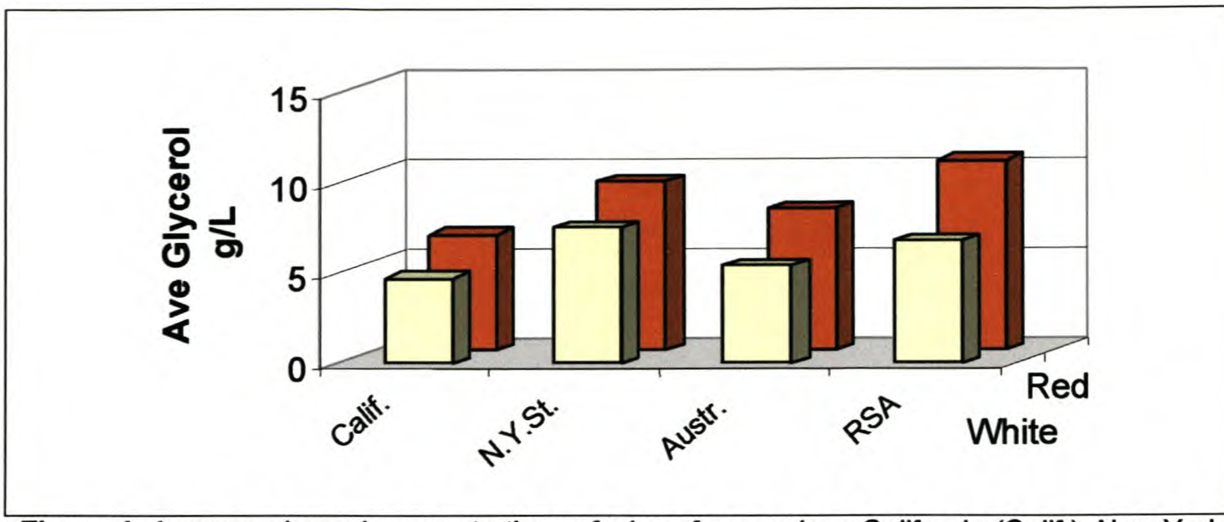

Figure 1. Average glycerol concentrations of wines from various California (Calif.), New York State (N.Y.St.), Australia (Austr.) and South Africa (RSA). See text for further details.

Table 3. Distribution of glycerol levels in commercial table wines from different countries.

Country of origin	Wine style	No. wines	Glycerol g/L		Reference
			Range	Mean	
California	White table	79	6.3 - 16.8	9.6	Amerine, 1954
	Dry red	60	4.6 - 14.3	10.6	Amerine, 1954
	White table	16	1.9 - 7.5	4.8	Ough *et al.*, 1972
	Dry red	15	4.2 - 8.2	6.5	Ough *et al.*, 1972
South Africa	White table	8	6.6 - 9.4	7.8	Venter, 1955
	Red table	10	6.3 - 9.4	7.8	Venter, 1955
	White table	190	4.7 - 9.4	6.8	This study
	Red table	237	6.7 - 14.2	10.5	This study

On factors affecting the glycerol levels and attempts at manipulation of glycerol levels during fermentation the questions were:

1) Which factors do you think contribute to the final level of glycerol in wine?
2) Have you tried practical ways of influencing glycerol levels during fermentation?

Factors considered by the panel members to affect the final glycerol levels of glycerol in wine, included the yeast strain, ripeness of the grapes, fermentation temperature and the degree of Botrytis infection of the grapes. Several panel members have previously tried various practical techniques during fermentation in attempts to increase the final glycerol levels. These included: temperature shocks (high and low), during the dehydration of the active dried yeast; the addition of higher levels of SO_2; the use of spontaneous fermentations; picking grapes at optimal ripeness; and the addition of fresh must during the fermentation process to elicit a stress response from the yeasts. In related research projects with which Martin Meinert (Meinert Wines, Stellenbosch) was associated, a variety of strategies aimed at increasing the glycerol levels were evaluated, but only minor increases could be obtained. Martin is of the opinion that glycerol plays a small role in wine quality at the levels normally found in wine. Similar opinions were recently voiced by Pascal Ribéreau-, who stated that winemakers place too much emphasis on the organoleptic role of glycerol and that the pursuit of winemaking conditions that are more conducive to glycerol production, was in his opinion, of no enological interest (Ribéreau-Gayon *et al.,* 2000).

On the importance of glycerol analyses the questions were:

1) Is glycerol analyses of your wines important to you, and if so, why?
2) Have you had any of your wines analysed for glycerol?

Only a minority (23%) of the winemakers have previously determined the glycerol content of their wines, but more than 75% were of the opinion that glycerol analyses were important to ensure quality. One of the winemakers considered the analyses of the glycerol levels of different blocks of grapes important, particularly in the case of B*otrytis* infected grapes. In a few instances it was stated that no glycerol analyses have previously been done on the wines, due to the lack of or inaccessibility to analytical facilities. These opinions clearly underline the need for the development of a fast and reliable technique for the analyses of glycerol in SA wines.

3. CONCLUDING REMARKS

The assessment of the contribution of glycerol to wine quality is challenging, particularly in view of the complexity and subjective of nature of wine quality. In this study a combined qualitative and quantitative approach was used in an attempt to: a) communicate the perceptions of a specialist panel of leading winemakers and other stakeholders in the SA Wine Industry on this topic; and b) to critically reassess the commonly held perceptions regarding glycerol and wine quality. The results obtained in this study certainly provide new insights regarding several aspects regarding the relationship between glycerol and wine quality, and it should stimulate critical thinking, especially with regards to the advantages and possible risks involved in attempts to manipulate the fermentation conditions to achieve higher glycerol levels.

It should also be noted that there is a renewed interest in the glycerol content of wine, not only from a perspective of wine quality, but also in terms of quality control. In several countries glycerol is used, together with other parameters, to verify the authenticity of the fermentation process itself, but also the source of the glycerol present in wine. The assessment of the origin and amount of glycerol in wine should therefore become a general requirement for SA wines.

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Appendix 2

HEADSPACE SOLID-PHASE MICROEXTRACTION DATA

Table 1a. Absolute peak areas for solid-phase microextraction gas chromatography of a model wine containing different glycerol concentrations. Conditions of analysis are described in Chapter 4, section 4.2.4.

Component	Final glycerol concentation											
	0 g/L						1.0 g/L					
	run 1	run 2	run 3	Average	SD[a]	CV%[b]	run 4	run 5	run 6	Average	SD[a]	CV%[b]
ethanol	12782.0	14458.0	17952.8	15064.3	2638.2	17.5	18060.6	15652.3	14345.0	16019.3	1884.8	11.8
ethyl butyrate	326.6	326.5	306.4	319.8	11.6	3.6	313.3	324.4	333.8	323.8	10.3	3.2
isobutanol	105.1	112.9	109.6	109.2	3.9	3.6	106.8	110.9	114.5	110.7	3.9	3.5
isoamyl acetate	78.9	75.1	72.2	75.4	3.4	4.5	74.5	76.9	81.0	77.5	3.3	4.2
ethyl valerate	117.6	114.7	118.5	116.9	2.0	1.7	112.1	116.0	113.3	113.8	2.0	1.8
isoamyl alcohol	72.0	70.8	68.3	70.4	1.9	2.7	68.0	70.5	73.1	70.5	2.6	3.6
ethyl hexanoate	76.2	70.5	72.5	73.1	2.9	3.9	75.2	77.6	75.1	76.0	1.4	1.9
hexyl acetate	137.3	144.6	148.6	143.5	5.7	4.0	166.2	169.1	164.5	166.6	2.3	1.4
ethyl lactate	38.0	37.5	41.0	38.8	1.9	4.9	38.1	35.2	36.9	36.7	1.5	4.0
acetic acid	12.0	9.3	8.5	9.9	1.8	18.5	8.1	6.6	8.4	7.7	1.0	12.5

Component	5.0 g/L						7.0 g/L					
	run 7	run 8	run 9	Average	SD[a]	CV%[b]	run 10	run 11	run 12	Average	SD[a]	CV%[b]
ethanol	15436.1	9684.8	13236.5	12785.8	2902.0	22.7	14318.7	15573.9	15282.6	15058.4	656.9	4.4
ethyl butyrate	350.4	379.7	348.1	359.4	17.6	4.9	324.9	308.5	295.4	309.6	14.8	4.8
isobutanol	114.9	111.8	104.5	110.4	5.3	4.8	107.6	107.4	102.5	105.8	2.9	2.7
isoamyl acetate	85.5	80.2	79.5	81.7	3.3	4.0	78.3	76.8	80.5	78.5	1.9	2.4
ethyl valerate	131.2	128.6	126.5	128.8	2.4	1.8	121.3	116.6	115.9	117.9	2.9	2.5
isoamyl alcohol	65.9	69.6	67.0	67.5	1.9	2.8	67.7	68.8	65.7	67.4	1.6	2.3
ethyl hexanoate	80.4	81.4	75.5	79.1	3.2	4.0	76.8	74.0	71.8	74.2	2.5	3.4
hexyl acetate	189.4	185.5	175.7	183.5	7.1	3.8	180.7	174.6	171.3	175.5	4.8	2.7
ethyl lactate	38.5	37.0	39.0	38.2	1.0	2.7	36.3	37.2	35.1	36.2	1.1	2.9
acetic acid	8.9	8.9	9.8	9.2	0.5	5.6	6.2	7.8	7.9	7.3	1.0	13.1

Component	10.0 g/L					
	run 13	run 14	run 15	Average	SD[a]	CV%[b]
ethanol	8514.5	10732.2	10372.3	9873.0	1190.2	12.1
ethyl butyrate	317.1	315.3	306.1	312.8	5.9	1.9
isobutanol	108.9	103.3	102.8	105.0	3.4	3.2
isoamyl acetate	78.3	79.1	76.2	77.9	1.5	1.9
ethyl valerate	120.3	124.4	113.5	119.4	5.5	4.6
isoamyl alcohol	68.9	71.8	71.5	70.7	1.6	2.3
ethyl hexanoate	157.6	157.4	156.5	157.2	0.6	0.4
hexyl acetate	252.1	250.3	251.4	251.3	0.9	0.4
ethyl lactate	36.8	38.8	35.8	37.1	1.5	4.1
acetic acid	8.3	9.7	8.7	8.9	0.7	8.1

[a]Standard deviation; [b]Coefficient of variation calculated as SD/average.

Table 1b. Retention times for components in a model wine containing different concentrations of glycerol. microextraction gas chromatography. Conditions of analysis are described in Chapter 4, section 4.2.4.

Final glycerol concentation

	0 g/L						1.0 g/L					
Compound	run 1	run 2	run 3	Average	SD[a]	CV%[b]	run 4	run 5	run 6	Average	SD[a]	CV%[b]
ethanol	5.12	5.18	5.21	5.17	0.04	0.8	5.21	5.19	5.18	5.19	0.02	0.3
ethyl butyrate	7.86	7.88	7.88	7.87	0.01	0.1	7.88	7.87	7.88	7.87	0.00	0.0
isobutanol	10.98	11.00	11.04	11.01	0.03	0.2	11.03	11.02	11.01	11.02	0.01	0.1
isoamyl acetate	11.58	11.59	11.59	11.58	0.01	0.1	11.58	11.58	11.59	11.58	0.00	0.0
ethyl valerate	12.19	12.21	12.16	12.19	0.02	0.2	12.16	12.17	12.20	12.18	0.02	0.2
isoamyl alcohol	17.22	17.24	17.28	17.24	0.03	0.2	17.27	17.27	17.24	17.26	0.01	0.1
ethyl hexanoate	18.01	18.02	17.98	18.00	0.02	0.1	17.98	17.99	18.01	17.99	0.02	0.1
hexyl acetate	20.62	20.63	20.60	20.62	0.02	0.1	20.60	20.61	20.63	20.61	0.01	0.1
ethyl lactate	24.33	24.34	24.35	24.34	0.01	0.0	24.35	24.34	24.34	24.34	0.00	0.0
acetic acid	27.36	27.36	27.37	27.36	0.00	0.0	27.37	27.37	27.36	27.36	0.00	0.0

	5.0 g/L						7.0 g/L					
Compound	run 7	run 8	run 9	Average	SD[a]	CV%[b]	run 10	run 11	run 12	Average	SD[a]	CV%[b]
ethanol	5.20	5.13	5.14	5.16	0.03	0.7	5.17	5.19	5.18	5.18	0.01	0.1
ethyl butyrate	7.88	7.87	7.85	7.87	0.02	0.2	7.87	7.87	7.86	7.86	0.00	0.0
isobutanol	11.02	10.99	10.98	11.00	0.02	0.2	11.00	11.01	11.01	11.01	0.00	0.0
isoamyl acetate	11.59	11.63	11.56	11.59	0.04	0.3	11.57	11.57	11.57	11.57	0.00	0.0
ethyl valerate	12.18	12.26	12.17	12.20	0.05	0.4	12.17	12.15	12.17	12.17	0.01	0.1
isoamyl alcohol	17.26	17.18	17.23	17.22	0.04	0.2	17.23	17.25	17.23	17.24	0.01	0.1
ethyl hexanoate	17.99	18.05	17.98	18.01	0.04	0.2	17.99	17.96	17.91	17.95	0.04	0.2
hexyl acetate	20.62	20.66	20.60	20.62	0.03	0.1	20.60	20.58	20.60	20.59	0.01	0.1
ethyl lactate	24.34	24.32	24.33	24.33	0.01	0.0	24.33	24.33	24.33	24.33	0.00	0.0
acetic acid	27.36	27.35	27.35	27.35	0.01	0.0	27.35	27.36	27.36	27.36	0.00	0.0

	10.0 g/L					
Compound	run 13	run 14	run 15	Average	SD[a]	CV%[b]
ethanol	5.08	5.11	5.09	5.09	0.01	0.3
ethyl butyrate	7.84	7.84	7.83	7.83	0.01	0.1
isobutanol	10.95	10.97	10.95	10.96	0.01	0.1
isoamyl acetate	11.56	11.54	11.51	11.53	0.03	0.2
ethyl valerate	12.18	12.14	12.15	12.16	0.02	0.2
isoamyl alcohol	17.16	17.20	17.17	17.17	0.02	0.1
ethyl hexanoate	17.98	17.94	17.95	17.96	0.02	0.1
hexyl acetate	20.59	20.56	20.56	20.57	0.02	0.1
ethyl lactate	24.30	24.30	24.30	24.30	0.00	0.0
acetic acid	27.33	27.34	27.34	27.34	0.00	0.0

Table 2. Absolute peak areas for solid-phase microextraction gas chromatography of a model wine containing 0 g/L glycerol, as determined on three consecutive days. Analysis conditions are described in Chapter 4, section 4.2.3.

Compound	Final glycerol concentation											
	Day 1						Day 2					
	run 1	run 2	run 3	Average	CV%[b]	CV%[b]	run 4	run 5	run 6	Average	CV%[b]	CV%[b]
ethanol	12782.0	14458.0	17952.8	15064.3	2638.2	17.5	11466.9	14237	12168.8	12624.3	1440.3	11.4%
ethyl butyrate	326.6	326.5	306.4	319.8	11.6	3.6	316.4	321.4	319.2	319.0	2.5	0.8%
isobutanol	105.1	112.9	109.6	109.2	3.9	3.6	107.7	114.9	110.3	111.0	3.6	3.3%
isoamyl acetate	78.9	75.1	72.2	75.4	3.4	4.5	81.9	81.6	84.3	82.6	1.5	1.8%
ethyl valerate	117.6	114.7	118.5	116.9	2.0	1.7	126.4	126.8	120.4	124.5	3.6	2.9%
isoamyl alcohol	72.0	70.8	68.3	70.4	1.9	2.7	67.2	62.9	61.3	63.8	3.1	4.8%
ethyl hexanoate	76.2	70.5	72.5	73.1	2.9	3.9	68.9	66.6	69.2	68.2	1.4	2.1%
hexyl acetate	137.3	144.6	148.6	143.5	5.7	4.0	148.9	140.2	136.6	141.9	6.3	4.5%
ethyl lactate	38.0	37.5	41.0	38.8	1.9	4.9	36.7	35.2	36.4	36.1	0.8	2%
acetic acid	12.0	9.3	8.5	9.9	1.8	18.5	16.8	12	13.2	14.0	2.5	18%
	Day 3											
	run 7	run 8	run 9	Average	CV%[b]	CV%[b]	Day 1	Day 2	Day 3	Average	CV%[b]	CV%[b]
ethanol	16352.5	13835.3	14287.8	14825.2	1341.9	9.05%	15064.3	12624.3	14825.2	14171.3	1345.0	9.49%
ethyl butyrate	305.4	312.9	319.2	312.5	6.9	2.21%	319.8	319.0	312.5	317.1	4.0	1.27%
isobutanol	115.6	107.9	111.6	111.7	3.9	3.45%	109.2	111.0	111.7	110.6	1.3	1.17%
isoamyl acetate	79.3	81.9	79.2	80.1	1.5	1.91%	75.4	82.6	80.1	79.4	3.7	4.61%
ethyl valerate	115.3	112.9	109.8	112.7	2.8	2.45%	116.9	112.7	115.6	115.1	2.2	1.88%
isoamyl alcohol	65.1	67.6	68.4	67.0	1.7	2.57%	70.4	67.0	65.4	67.6	2.5	3.75%
ethyl hexanoate	73.5	70.1	72.5	72.0	1.7	2.43%	73.1	68.2	72.0	71.1	2.6	3.59%
hexyl acetate	148.6	144.5	139.1	144.1	4.8	3.31%	143.5	141.9	144.1	143.2	1.1	0.79%
ethyl lactate	40.2	38.5	37.6	38.8	1.3	3.41%	38.8	36.1	38.8	37.9	1.6	4.14%
acetic acid	12.2	13.4	9.1	11.6	2.2	19.18%	9.9	15.3	11.6	12.3	2.7	22.37%

[a]Standard deviation; [b]Coefficient of variation calculated as SD/average.

Table 3. Absolute peak areas for solid-phase microextraction gas chromatography of a whitel wine containing different glycerol concentrations. Conditions of analysis are described in Chapter 4, section 4.2.4.

	Final glycerol concentation											
	5.4 g/L						**6.4 g/L**					
Compound	**run 1**	**run 2**	**run 3**	**Average**	**SD[a]**	**CV%[b]**	**run 4**	**run 5**	**run 6**	**Average**	**SD[a]**	**CV%[b]**
isoamyl acetate	66.35	65.18	62.67	64.74	1.88	2.9	66.09	67.61	65.46	66.38	1.10	1.7
isoamyl alcohol	78.25	78.95	79.40	78.87	0.58	0.7	80.70	80.60	79.20	80.17	0.84	1.1
ethyl hexanoate	105.57	107.87	108.97	107.47	1.73	1.6	110.40	108.2.	107.60	109.00	1.98	1.8

	10.4 g/L						**12.4 g/L**					
Compound	**run 7**	**run 8**	**run 9**	**Average**	**SD[a]**	**CV%[b]**	**run 10**	**run 11**	**run 12**	**Average**	**SD[a]**	**CV%[b]**
isoamyl acetate	73.72	75.79	75.61	75.04	1.15	1.5	69.15	68.01	70.74	69.30	1.37	2.0
isoamyl alcohol	79.20	81.10	78.05	79.45	1.54	1.9	282.25	296.90	299.80	292.98	9.41	3.2
ethyl hexanoate	107.03	109.53	108.63	108.40	1.26	1.2	108.83	109.13	107.40	110.32	0.92	1%

	15.4 g/L					
Compound	**run 7**	**run 8**	**run 9**	**Average**	**SD[a]**	**CV%[b]**
isoamyl acetate	68.32	65.49	64.90	66.24	1.83	2.8
isoamyl alcohol	81.60	78.61	79.20	79.80	1.58	2.0
ethyl hexanoate	108.45	106.13	109.32	107.97	1.65	1.5

[a]Standard deviation; [b]Coefficient of variation calculated as SD/average.