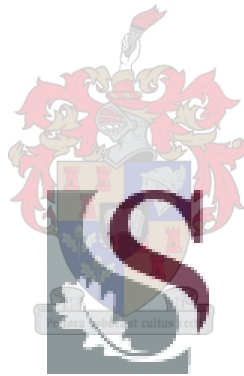


Industry-wide assessment and characterisation of problem fermentations

by

Sulette Malherbe



*Thesis presented in partial fulfilment of the requirements for the degree of
Master of Sciences at Stellenbosch University.*

March 2007

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis 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.



Sulette Malherbe

Date

SUMMARY

In order to remain competitive in the international wine market, wineries need to increase productivity and improve quality with the aid of available technologies. Problematic wine fermentations directly impact both productivity and the quality of wine and therefore, by reducing stuck and sluggish fermentations, less wine will be lost or downgraded. This requires early identification and classification of problem fermentations with the use of high throughput analytical tools and multivariate data analysis in order to take preventative measures.

In this study, a non-directed, holistic approach was used to investigate the occurrence of problem fermentations in the South African wine industry. Data obtained with various analytical techniques was used for the purpose of multivariate data analysis. The use of high throughput analytical techniques such as Fourier transform infrared (FT-IR) spectroscopy proved extremely valuable as a fast screening method to monitor fermentation progress. Principal component analysis of the spectral fingerprints obtained with this technique indicated that all the fermentation problems occurred from the middle of fermentation onwards. In addition, successful discrimination between control and problem fermentations for different red and white cultivars was achieved. This demonstrates the feasibility of this type of methodology to investigate and monitor fermentation.

Solid phase dynamic extraction (SPDE) headspace analysis was used to obtain a volatile fingerprint of the fermenting must samples. Similarly to FT-IR spectra, successful discrimination between problem and control fermentations was achieved with the use of PLS-discriminant analysis. Discrimination between red and white cultivars was achieved with headspace data. This excluded the data from tannin and colour compounds normally used for this discrimination. SPDE coupled to gas chromatography mass spectrometry (GC-MS) proved to be a novel and suitable analytical method for wine analysis, although further method development is required.

A preliminary research and development strategy for evaluating FT-IR spectroscopy for quantification purposes in fermenting must was established. Although these calibration and validation results were preliminary, it was shown that the calibrations for fermenting must under South African conditions need urgent attention.

In conclusion, this study illustrated the potential of using an alternative approach to investigate stuck and sluggish fermentation with definite prediction possibilities for future research endeavours.

OPSOMMING

Indien kelders in die internasionale wynmark kompetend wil bly, moet beskikbare tegnologieë benut word om produktiwiteit te verhoog en kwaliteit te verbeter. Produktiwiteit en wynkwaliteit word egter direk deur probleemfermentasies beïnvloed en daarom is dit dus belangrik dat slepende of steekfermentasies verminder word, sodat minder wyn verlore gaan of afgegradeer word. Ten einde hierdie doel te bereik, word die vroeë identifikasie en klassifikasie van probleemfermentasies met behulp van vinnige-deurvloei analitiese metodes en multiveranderlike data-analises benodig om voorkomende stappe te kan neem.

In hierdie studie is 'n nie-gerigte, holistiese benadering gevolg om die voorkoms van probleemfermentasies in die Suid-Afrikaanse wynbedryf te ondersoek. Multiveranderlike data-analise is op die data van verskeie analitiese tegnieke toegepas. Die gebruik van vinnige-deurvloei analitiese tegnieke, soos Fourier-transformasie-infrarooi (FT-IR) -spektroskopie, is bevestig as 'n uiters waardevolle en vinnige analitiese metode om die vordering van fermentasies mee te monitor. Hoofkomponentanalise van die spektrale vingerafdruk wat met hierdie tegniek verkry is, het aangetoon dat al die probleemfermentasies slegs vanaf die middel van die fermentasieproses en verder voorgekom het. Daar kon ook met sukses 'n duidelike onderskeid getref word tussen probleem- en kontrolefermentasies vir verskillende rooi en wit kultivars. Dit bewys die lewensvatbaarheid van hierdie tipe benadering om fermentasies te monitor en ondersoek.

'n Vlugtige-komponent vingerafdruk van fermenteerende mos is bekom deur die gebruik van soliede-fase-dinamiese-ekstrasie (SPDE) -dampfase-analise. Daar kon ook deur middel van PLS-diskriminantanalise suksesvol tussen probleem- en kontrolefermentasies onderskei word. 'n Soortgelyke onderskeiding tussen rooi en wit kultivars is behaal met dampfasedata, uitsluitende tanniene en kleurkomponente, wat normaalweg vir hierdie onderskeiding gebruik word. Daar is gevind dat, indien SPDE aan gaschromatografie-massaspektrometrie (GC-MS) gekoppel word, dit 'n nuwe en geskikte analitiese metode vir wynanalises is, alhoewel verdere metode-ontwikkeling benodig word.

'n Voorlopige navorsings- en ontwikkelingsstrategie om FT-IR-spektroskopie vir kwantifiseringsdoeleindes in fermenteerende mos te ondersoek, is ontwikkel. Alhoewel hierdie kalibrasie- en validasieresultate slegs voorlopig is, het dit wel aangedui dat daar dringend aandag gegee sal moet word aan die kalibrasies vir fermenteerende mos onder Suid-Afrikaanse toestande.

Hierdie studie demonstreer dus die potensiaal van 'n alternatiewe benadering tot die ondersoek van steek- en slepende fermentasie, met definitiewe voorspellingsmoontlikhede vir toekomstige navorsingsaktiwiteite.

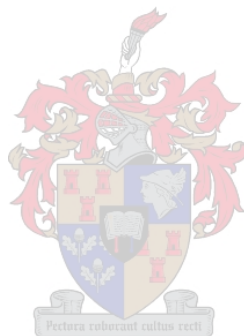
*This thesis is dedicated to my family for their continuous support,
encouragement and motivation*

*Hierdie tesis is opgedra aan my familie vir hul volgehoue
ondersteuning, aanmoediging en motivering*



BIOGRAPHICAL SKETCH

Sulette Malherbe was born on 20 July 1980 and matriculated at Paarl Gymnasium High School in 1998. She obtained her BSc degree at the Stellenbosch University in 2003, majoring in Chemistry. In 2004, Sulette enrolled at the Institute for Wine Biotechnology and obtained her BSc Honours degree in Wine Biotechnology in December of that year. In 2005 she enrolled for a Masters degree in Wine Biotechnology at the same university.



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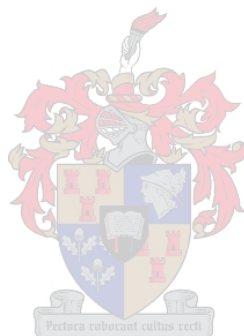
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PREFACE

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

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**

Understanding problem fermentations

Chapter 3 **RESEARCH RESULTS**

The assessment and characterisation of problem fermentations: An industrial case study to investigate the discrimination possibilities of FT-IR spectroscopy

Chapter 4 **RESEARCH RESULTS**

Automated headspace solid-phase dynamic extraction (SPDE) coupled to GC-MS for cultivar discrimination and classification purposes

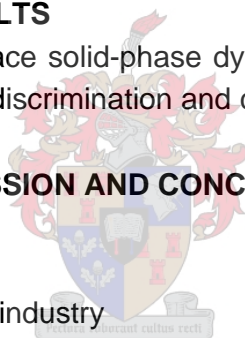
Chapter 5 **GENERAL DISCUSSION AND CONCLUSIONS**

Chapter 6 **ADDENDUMS**

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B: Wineland Article

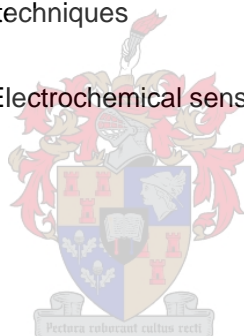
C: Preliminary research and development strategy for evaluating FT-IR spectroscopy for quantification purposes in fermenting must



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1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

One of the most important objectives of the winemaking process for most natural wines is the completion of alcoholic fermentation to dryness (residual fermentable sugar in the wine is less than 4 g/L). Despite improvements in fermentation control, stuck and sluggish fermentations remain chronic problems for the wine industry worldwide. Incomplete or “stuck” fermentations are defined as those having a higher than desired residual sugar content at the end of alcoholic fermentation, while slow or “sluggish” fermentations are characterised by a low rate of sugar utilisation (Bisson, 1999). Fermentation problems arise due to the presence, impact and synergy of various stress factors in the yeast environment, some of which are unavoidable and others which are the result of inappropriate fermentation management decisions (Bisson, 2005).

In industrial cellars the occurrence of stuck and sluggish fermentations has practical, logistical and economical implications to winemakers. Sluggish fermentations require extended fermentation times which could consequently consume tank space for an unknown period of time. During this time, these fermentations need additional management and this is often laborious, especially during harvest when resources are limited. The wine might not be blanketed with sufficient carbon dioxide if the fermentation is slow and protection against oxidative damage needs to be ensured (Bisson, 1999). Furthermore, sluggish fermentations are very susceptible to microbial spoilage from non-*Saccharomyces* yeasts and bacteria which could metabolise residual sugars and consequently lead to increased volatile acidity and the formation of unwanted esters (O'Connor-Cox and Ingledew, 1991). The lack of anaerobic conditions on the surface can encourage the growth of aerobic spoilage organisms, such as acetic acid bacteria. The growth of these organisms can additionally stress the *Saccharomyces* wine yeast, resulting in an arrest of fermentation (Bisson, 2005). The residual sugar concentrations in finished wine are important for two reasons. Firstly, most sugars are sweet and effect flavour by increasing the body of a wine. Secondly, and an equally important consideration, is its involvement in microbial instability. Any residual sugar could potentially be fermented after bottling or metabolised by spoilage microorganisms such as *Brettanomyces*. In addition, winemakers might have to change the style of the wine as a result of the undesired amounts of residual sugar. This could possibly result in a product with decreased quality and consequent financial implications. The economic and logistical/practical consequences of sluggish and stuck wine fermentations therefore demand significant investigation into the causes thereof and determination methods to prevent or possibly predict the occurrence of this problem.

Previous research has identified more than 15 causes of slow and stuck fermentations (Ingledew and Kunkee, 1985; Allen and Auld, 1988; Fugelsang *et al.*, 1991; Kunkee, 1991; Bisson, 1993, 1999, 2005; Henschke and Jiranek, 1993; Henschke, 1997; Varela *et al.*, 2004). Reviewed by several authors (Henschke, 1997; Alexandre and Charpentier, 1998; Bisson, 1999, 2005; Bauer and Pretorius, 2001), the following factors have been identified as potentially responsible: high initial sugar content (Lafon-Lafourcade *et al.*, 1979), nitrogen deficiency (Agenbach, 1977; Ingledew and Kunkee, 1985; Bely *et al.*, 1990), vitamin deficiency, especially thiamine depletion of the must (Peynaud and Lafourcade, 1977; Ough *et al.*, 1989; Salmon, 1989), anaerobic conditions resulting in oxygen deficiency (Thomas *et al.*, 1978; Traverso Rueda and Kunkee, 1982), excessive must clarification (Groat and Ough, 1978; Houtman and Du Plessis, 1986; Alexandre *et al.*, 1994), high ethanol content (Casey and Ingledew, 1986), inhibition of yeast cell activity by fermentation by-products, particularly decanoic acid (Geneix *et al.*, 1983; Lafon-Lafourcade *et al.*, 1984; Viegas *et al.*, 1989; Edwards *et al.*, 1990) and acetic acid (Kreger-Van Rij, 1984; Edwards *et al.*, 1999), pH (Kado *et al.*, 1998), killer toxins (Barre, 1982; Van Vuuren and Jacobs, 1992) and pesticides (Doignon and Rozes, 1992).

Apart from the individual effects of these multiple factors influencing fermentation, the possible synergy between them additionally renders the prediction and diagnosis of the exact cause of problem fermentations even more challenging. As a result, the majority of laboratory studies investigated one or two well-controlled variables, whereas under production conditions these factors interact (Bisson, 2005).

Multiple variables have to be monitored throughout the fermentation process to ensure effective fermentation management and this involves various analytical measurements. As a result of time and cost implications related to the large number of analysis necessary for one wine sample, the focus of analytical methods have shifted towards high throughput techniques providing multiple information in one analysis. Fourier transform infrared (FT-IR) spectroscopy utilises the mid-infrared region of the spectra for accurate and simultaneous determination of chemical components in a short period of time with minimal sample preparation (Patz *et al.*, 2004). The application of this technology has received much attention in recent years (Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003; Patz *et al.*, 2004). Apart from the quantified data obtained with this technology, a complex “fingerprint” spectrum of a sample is also obtained. Chemometrics provides a tool to extract, summarise and visually present additional hidden information in the spectra and/or other variables (Kettanah *et al.*, 2005). In addition to pattern recognition, chemometrics also provide the opportunity to construct discrimination and prediction models (Esbensen, 2002; Kettanah *et al.*, 2005).

1.2 PROJECT AIMS

This project forms part of a larger research program at the Institute for Wine Biotechnology at the University of Stellenbosch to improve the fermentation performance of yeast. The outcomes of this project will be used to establish future goals for projects and to evaluate the direction of the current research.

The principal aim of this work was to establish a database, specifically relating to problem fermentations, from which information could be extracted. A non-directed or holistic approach had to be followed as such a database consisted of a diverse set of information from industrial problem fermentations. To investigate the possibilities of characterising industrial problem fermentations and tendencies amongst these fermentations, analytical techniques were combined with multivariate data analysis techniques. The nature of this approach along with the use of these powerful technologies could possibly discriminate between control and problem fermentations and thereby aid in the construction of predictive models. This was a feasibility study and its outcome would determine future research endeavours. The specific aims of this study were as follows:

- a) to collect problem fermentation samples from commercial cellars during the 2005 and 2006 harvest season;
- b) to establish a data matrix of non-spectral (non FT-IR) variables from the problem fermentations comprising of microbial analysis (populations dynamics), chemical analysis (volatile components) and other basic oenological parameters (free assimilable nitrogen (FAN), sulphur dioxide content, Brix of grapes, fermentation temperature and yeast selection);
- c) to obtain chemical and spectral “fingerprints” of the problem fermentations by using Fourier Transform Infrared (FT-IR) spectroscopy;
- d) to construct a data matrix combining spectral and non-spectral variables in order to investigate tendencies amongst control and problem fermentations with the use of chemometric techniques such as multivariate data analysis;
- e) to investigate the use of dynamic headspace analysis (SPDE-GCMS) for possible application in discrimination between control and problem fermentations and cultivar discrimination;
- f) to evaluate the suitability of the commercially available FOSS Winescan FT120 calibrations for fermenting must in South Africa;
- g) to initiate a South African calibration process for ethanol, glycerol, main organic acids and separate glucose and fructose determinations in fermenting musts, on the FOSS Winescan FT120;

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2. LITERATURE REVIEW

2.1 INTRODUCTION

Alcoholic fermentation, the conversion of the principal grape sugars, glucose and fructose, to ethanol and carbon dioxide is conducted by yeasts of the genus *Saccharomyces*, generally *S. cerevisiae* and *S. bayanus* (Boulton *et al.*, 1996). This complex microbial process probably represents the oldest form of biotechnological applications of a microorganism and has been used by humans for several thousand years (Samuel, 1996).

Despite considerable improvements in our ability to monitor and control fermentation, stuck and sluggish fermentations remain major challenges for the international wine industry, including South Africa. Bisson (1999) defined incomplete or “stuck” fermentations as those having a higher than desired residual sugar content at the end of alcoholic fermentation, while slow or “sluggish” fermentations are characterised by a low rate of sugar consumption by the yeast. The economic and logistical consequences of sluggish and stuck wine fermentations in industrial cellars demand significant investigation into the causes and the determination of methods to avoid this problem.

Extensive research has been conducted since 1977 on elucidating problem fermentations and several causes of sluggish and stuck fermentation have been identified (Ingledew and Kunkee, 1985; Allen and Auld, 1988; Fugelsang *et al.*, 1991; Kunkee, 1991; Bisson, 1993; Henschke and Jiranek, 1993; Henschke, 1997; Alexandre and Charpentier, 1998; Bisson, 1999). Factors such as high initial sugar content (Lafon-Lafourcade *et al.*, 1979), nitrogen deficiency (Agenbach, 1977; Ingledew and Kunkee, 1985; Bely *et al.*, 1990), vitamin deficiency, especially thiamine (Peynaud and Lafourcade, 1977; Ough *et al.*, 1989; Salmon, 1989), oxygen deficiency (Thomas *et al.*, 1978; Traverso Rueda and Kunkee, 1982), excessive must clarification (Groat and Ough, 1978; Houtman and Du Plessis, 1986; Alexandre *et al.*, 1994), high ethanol concentrations (Casey and Ingledew, 1986), inhibition of yeast cell activity by fermentation by-products, particularly the fatty acids (Geneix *et al.*, 1983; Lafon-Lafourcade *et al.*, 1984; Viegas *et al.*, 1989; Edwards *et al.*, 1990) and acetic acid (Kreger-Van Rij, 1984; Edwards *et al.*, 1999), pH (Kado *et al.*, 1998), killer toxins (Barre, 1982; Van Vuuren and Jacobs, 1992), and pesticides (Doignon and Rozes, 1992) have all been identified as potentially responsible for fermentation problems. In addition to the individual effects of each of these factors, possible synergistic effects amongst them add to the complexity of understanding problem fermentations. For this reason the prediction and diagnosis of the exact causes of problem fermentations are often rendered extremely challenging.

In this literature review, the causative factors of problem fermentations and general factors influencing fermentation efficiency will be discussed in more detail. These will include physical (pH and temperature), chemical (nutrients and inhibitory substances) and microbiological factors (microbial competition) and the potential synergistic effects amongst these factors. The issue of must composition, especially the nitrogen content and glucose:fructose ratio, has a definite impact on fermentation efficiency. Since the must composition is also dependent on viticultural practices and harvest considerations, these factors will also be discussed. Along with the development of analytical technology and increased availability of statistical techniques (chemometrics), potentially new and alternative techniques to monitor fermentation evolved. The last section of this review will highlight a selection of these analytical methods and chemometric applications, which could potentially be used to effectively monitor fermentation progress.

2.2 CAUSES OF FERMENTATION ARREST

Previous studies on fermentation problems have identified numerous factors and these can be broadly classified into physical, chemical and microbiological factors influencing fermentation. A spectrum of possible factors, from the vineyard to the cellar, will be discussed.

2.2.1 VINEYARD AND VITICULTURAL FACTORS

Fermentation problems can already originate from the vineyard as the must composition influences the fermentation efficiency. The concentration of nitrogen and yeast-required micronutrients is influenced by a variety of parameters. These include grapevine nutrient deficiencies, fungal degradation and degree of fruit maturity at harvest which is predetermined by cultivar, rootstock, crop load, canopy management, vineyard fertilization and climate (Kliewer, 1970).

Vineyard nitrogen fertilization influences the concentrations of nitrogenous compounds in juice (Spayd *et al.*, 1991, 1994). This affects the formation of higher alcohols and esters by yeast during fermentation (Ough and Bell, 1980; Ough and Lee, 1981; Gallander *et al.*, 1989; Webster *et al.*, 1993) and therefore indirectly wine quality. Spayd *et al.* (1994) found that an increased rate of nitrogen fertilization resulted in increased concentrations of all nitrogen fractions, including individual amino acids, in White Riesling juice. Nitrogen fertilization increased Merlot must arginine concentrations from 279 to 798 mg/L and proline from 1062 to 1639 mg/L in a Bordeaux study (Bertrand *et al.*, 1991). Nitrogen deficiencies in juice can limit yeast growth (Agenbach, 1977; Salmon, 1989; Monteiro and Bisson, 1991; Reed and Nagodawithana, 1991; Spayd *et al.*, 1991) therefore resulting in sluggish or stuck

fermentations (Agenbach, 1977; Vos, 1981; Salmon, 1989; Kunkee, 1991; Spayd *et al.*, 1991) and in the release of H₂S (Vos and Gray, 1979; Henschke and Jiranek, 1991; Jiranek, 1995a).

Agricultural residues (pesticides, fungicides, herbicides) on the exterior surface of grape fruit could also influence fermentation performance. (See section 2.2.3.4.6)

Various cultivars exhibit different glucose and fructose levels in their berries (Kliewer, 1965; Snyman, 2006). The glucose:fructose ratio changes from season to season as a result of climate and ripeness level. Snyman (2006) reported increased fructose levels (lower glucose:fructose ratio) in the grapes of different cultivars during warm and dry seasons. This corresponds to the results obtained by Kliewer (1965). Theories to explain this phenomenon include the conversion of glucose to fructose with sorbitol as an intermediate product. It is not clear whether this reaction is enhanced by increased temperature and/or whether a closer link with other complex biochemical processes exist (Snyman, 2006). Another theory involves the degradation of glucose in the grape berry by the pentose phosphate cycle. If sucrose enters the berry and glucose is respired, the fructose levels would increase and the ratio of glucose:fructose would be reduced. In the case of overripe grapes, increased time on the vine would result in more glucose degradation through respiration leading to a reduced glucose:fructose ratio. The majority of biochemical reactions occur faster during warmer seasons and this could explain the lower levels of glucose in relation to fructose present in the berry (Snyman, 2006). Viticulturally, overripeness can be avoided by monitoring the glucose:fructose ratio during ripening to avoid consequent fermentation problems. The aspects related to glucose:fructose ratios and harvest will be discussed in more detail in the following section on harvest conditions.

2.2.2 HARVEST CONDITIONS

The rate of fermentation by yeast and bacteria is considerably influenced by the amino acid composition of the must. It has been reported that the fruit proline concentrations increase as the °Brix increase during ripening (Ough, 1968; Kliewer, 1970). The increase or decrease of arginine with increased fruit maturity is reported as dependent on the specific cultivar (Kliewer, 1970). Changes in the amino acid profile of grapes during the ripening process have been studied extensively (Kliewer, 1968, 1970; Huang and Ough, 1991; Lehtonen, 1996; Spayd and Andersen-Bagge, 1996; Hernández-Orte *et al.*, 1999; Nicolini *et al.*, 2001) and a wide range of free amino nitrogen concentrations at harvest maturity has been reported (Vos, 1981), depending on the region, cultivar and growing conditions of the grapevine. Peynaud and Lafon-Lafourcade (1961) reported an increase in the less assimilable nitrogen forms (proline and threonine) as grapes ripen. This could explain why musts of overripe grapes sometimes ferment slowly (Kliewer, 1968).

Climatic changes each year often result in various vineyards in a specific viticultural region achieving optimal ripeness simultaneously. This puts enormous pressure on cellars to process these grapes and could result in the pressing of certain vineyards at higher sugar levels and increased grape maturity than desired. In addition to the influence of grape nitrogen content on fermentation, the glucose and fructose concentrations in grapes also exhibit a tremendous effect on fermentation performance. The subject of glucose and fructose concentrations in grapes has been extensively investigated over the years (Amerine, 1954; Amerine and Thoukis, 1958; Kliewer, 1965, 1968; Snyman, 2006). It was found that glucose predominates in unripe grapes, the glucose:fructose ratio at fruit maturity is about 1 and that fructose constitutes the major sugar in overripe grapes. Kliewer (1965) reported a sudden decrease in the glucose:fructose ratio as fruit became overmature and Snyman (2006) reported similar results (**Table 2.1**). These results indicate that overmature grapes become increasingly detrimental for successful fermentation unless the correct yeast strain is used or a different wine style is desired.

Table 2.1 The levels of glucose and fructose in different cultivars pressed at early and late stages of fruit maturity (Snyman, 2006).

| Cultivar | Sugar (°Brix) | | Glucose (g/L) | | Fructose (g/L) | | Glucose:Fructose | |
|--------------------|---------------|------|---------------|-------|----------------|-------|------------------|------|
| | Early | Late | Early | Late | Early | Late | Early | Late |
| Chenin Blanc | 19.5 | 25.1 | 9.12 | 12.12 | 9.73 | 13.15 | 0.94 | 0.92 |
| Chardonnay | 18.8 | 25.2 | 8.75 | 11.75 | 10.0 | 14.29 | 0.88 | 0.82 |
| Sauvignon Blanc | 22.7 | 24.9 | 11.01 | 12.75 | 12.13 | 14.33 | 0.91 | 0.88 |
| Cabernet Sauvignon | 21.1 | 25.5 | 9.77 | 11.46 | 10.88 | 14.58 | 0.90 | 0.78 |
| Shiraz | 19.2 | 27.0 | 9.34 | 13.16 | 9.76 | 15.66 | 0.96 | 0.84 |

Vineyard mechanization includes mechanical leaf removal, pruning, fruit thinning and harvesting and is a reality of modern viticultural technology (Morris, 2000). The major quality problem with machine harvested grapes is the fruit damage and the handling after harvest (Moyer *et al.*, 1961; Shepardson and Miller, 1962; Bourne *et al.*, 1963; Marshall *et al.*, 1971; Christensen *et al.*, 1973). A considerable interval between machine harvesting and processing of the grapes can result in increased enzymatic activity and browning, oxidation (loss of color) and development of off-flavours and microbial growth (Bourne *et al.*, 1963; Marshall *et al.*, 1971; Marshall *et al.*, 1972; Christensen *et al.*, 1973; Splittstoesser *et al.* 1974; Peterson, 1979). Temperature during this time interval influences the quality of machine harvested grapes tremendously (Marshall *et al.*, 1971; 1972; Morris *et al.*, 1972, 1973, 1979; Peterson, 1979). The transport of machine harvested grapes from the vineyard to wineries

could enhance the onset of alcoholic fermentation (of the released juice) by wild yeasts. The resulting high initial wild yeast populations could produce high concentrations of acetic acid and ethanol resulting in inhibition of the desired yeast starter culture or fermentation difficulties (Morris *et al.*, 1973; Alexandre and Charpentier, 1998). Sulphur dioxide addition to machine harvested grapes has been shown to discourage bacterial spoilage and can serve as an antioxidant to prevent juice browning (Bourne *et al.*, 1963; Morris *et al.*, 1972, 1973, 1979; Nelson and Ahmedullah, 1972; Benedict *et al.*, 1973; Christensen, 1973; O'Brien and Studer, 1977). The above-mentioned considerations and precautions are also applicable for hand harvested grapes, however, due to the increased fruit damage observed for machine harvested grapes, the effect might be more detrimental to yeast fermentation.

2.2.3 CELLAR MANAGEMENT: ALCOHOLIC FERMENTATION

2.2.3.1 Yeast strain

Yeast performance is determined partly by its genetic makeup (genotype), which is species and strain dependent. Strain differences are more pronounced in stress conditions, suggesting differences in adaptation to the environment, a hypothesis that is supported by transcriptome data (Gasch, 2003). Wine yeast strains differ largely in nitrogen requirements and ability to utilise sugars, especially during the later stages of fermentation (McClellan *et al.*, 1989; Schütz and Gafner, 1995). Selection of yeast strains which efficiently utilise available nitrogen in low nitrogen musts and juices, in addition to nitrogen supplementation appears to be one approach to resolve fermentation difficulties due to nitrogen deficiencies (Jiranek *et al.*, 1991, 1995b). Strains also differ in their ability to utilise glucose (glucophilic yeast) and fructose (fructophilic yeast). The selection of appropriate fructophilic yeasts for fermentations of grapes suspected to have low glucose: fructose ratios could avoid fermentation problems.

Challenging fermentation conditions such as high level of juice clarification, high protection from air (low oxygen content), low assimilable nitrogen and high sugar content requires yeast strains to have a high sugar and ethanol tolerance to complete fermentation successfully without producing any off-flavours (Henschke, 1997). Degré (1993) described various characteristics for the selection of good wine yeast strains to conduct fermentation successfully. Tolerance to both ethanol and temperature is also very strain dependent (Bisson, 1999).

2.2.3.2 Yeast preparation

Apart from the importance of yeast strain selection, the preparation of the inoculum is equally critical. In order to achieve maximum viability, commercial active dried yeast should not be directly inoculated into the must. Rehydration, according to the manufacturer's instructions, at

the recommended temperature without exceeding the recommended rehydration period is required to re-establish functional membranes and metabolic activity (Boulton *et al.*, 1996). The suspension should be mixed properly, although excessive mixing could result in loss of cell viability (Bisson, 2005). Deviations from the rehydration instructions such as extended rehydration in water and cold or hot rehydration will reduce the yeast viability (Bisson, 2005). During rehydration and inoculation the yeast is exposed to respectively hypo-osmotic and hyper-osmotic shock (Bauer and Pretorius, 2000). Additional temperature shock (5 to 7°C difference between culture and must temperature) when rehydrated yeast is introduced into the must greatly reduces the cell concentration of the inoculum (Zoecklein, 2005). Ingledew and Kunkel (1985) showed high cell numbers promoted faster rates of fermentation. The use of old or expired active dried yeasts might also cause fermentation problems.

Initial yeast populations should be large enough (2×10^6 to 5×10^6 yeast cells/mL) (Zoecklein, 2005) to dominate indigenous microflora and ensure rapid, complete fermentation (Bauer and Pretorius, 2000). Unsuccessful inoculation could result in incomplete fermentation due to the growth of less alcohol tolerant indigenous yeast (Henschke, 1997).

2.2.3.3 Yeast nutrition

2.2.3.3.1 Nitrogen – essential macronutrients

Nitrogenous compounds are important components of grape juice and impact on the production of yeast biomass, fermentation rate and time to complete fermentation (Bisson, 1991). The formation of fermentation flavours, such as hydrogen sulfide, organic acids, higher alcohols and esters are also influenced by nitrogen (Bell *et al.*, 1979; Simpson, 1979; Vos and Gray, 1979; Ough and Bell, 1980; Vos, 1981; Juhasz and Torley, 1985; Dukes *et al.*, 1991; Henschke and Jiranek, 1991; Rapp and Versini, 1991; Jiranek *et al.*, 1995a; Webster *et al.*, 1993). This spectrum of yeast metabolism end products directly influences wine quality.

Saccharomyces species of yeast are capable of synthesizing all required nitrogen-containing compounds from ammonium (NH_4^+), carbon and energy sources. Ammonia and free alpha amino acids (collectively referred to as FAN) are therefore readily assimilated, while peptides and proteins are assimilated for the production of amino acids via hydrolysis (Reed and Nagodawithana, 1991). Nitrogenous compounds are used by yeast to produce structural and functional proteins that result in increased yeast biomass and the production of enzymes that facilitate many biochemical changes occurring during yeast fermentation (Spayd and Andersen-Bagge, 1996). The importance of nitrogenous compounds in fermentation of grape juice and beer worts were reviewed by Bisson (1991) and O'Connor-Cox and Ingledew (1989), respectively.

Nitrogen deficiency (less than 150 mg/L FAN) slows down yeast growth and the fermentation or may even result in a stuck fermentation (Agenbach, 1977; Vos *et al.*, 1978;

Monk, 1982; Jiranek *et al.*, 1991; Kunkee, 1991; Monteiro and Bisson, 1991; Butzke and Dukes, 1996), possibly due to the inhibition of the synthesis of proteins transporting sugar through the cell membrane to the interior of the cells (Busturia and Lagunas, 1986; Salmon, 1989; Huang and Ough, 1991). It has been shown that an adequate supply of nitrogen increases yeast growth provided the other essential yeast nutrients are not lacking (Aries and Kirsop, 1977; Strydom *et al.*, 1982; Ingledew and Kunkee, 1985; Henschke, 1990; Dukes *et al.*, 1991). However, additions of ammonia after the early yeast growth phase may be ineffective in that the inhibited sugar transport into yeast cells may be irreversible in low nitrogen juices (Salmon, 1989). Yeast may use amino acids not only as nitrogen sources but also as redox agents to balance the oxidation-reduction potential under conditions of restricted oxygen (Albers *et al.*, 1996; Mauricio *et al.*, 2001).

2.2.3.3.2 Phosphate

Phosphate limitation has been shown to affect cell growth and biomass formation as well as directly affecting fermentation rate (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Gancedo and Serrano, 1989; Boulton *et al.*, 1996).

2.2.3.3.3 Oxygen and other survival factors

Oxygen and/or the presence of certain lipids, referred to as oxygen substitutes, are critical for yeast growth (Munoz and Ingledew, 1989a, 1989b, 1990). These 'survival factors' are compounds required to minimize the inhibitory effects of ethanol (Lafon-Lafourcade *et al.*, 1979; Lafon-Lafourcade and Ribéreau-Gayon, 1984).

During the early stages of growth, the oxygen consumed by yeast appears to have an energy role (Henschke, 1997). Oxygen is essential for the biosynthesis of sterols and unsaturated fatty acids which are both essential to membrane structure and function (Casey and Ingledew, 1986) and cell viability.

The production of toxic fatty acids, octanoic and decanoic acid, are affected by oxygen deprivation (Bardi *et al.*, 1999) and the toxicity of these medium chain fatty acids increases as the ethanol concentration increases (Henschke, 1997). This effect elevates the risk of problem fermentations to occur.

Oxygen deficiency could be responsible for sluggish fermentation as a consequence of inhibition of lipid biosynthesis which results in decreased ergosterol and unsaturated fatty acid content ('survival factors'), decreased biomass production and yeast viability.

Must aeration could therefore stimulate lipid biosynthesis, increase ethanol tolerance as a result of increased lipid composition in the cell membrane, decrease the release of MCFA and reduce the potential toxicity and the risk of fermentation problems (Henschke, 1997; Alexandre and Charpentier, 1998).

The addition of yeast hulls, the cell wall material remaining after yeast extract preparation, has been suggested as supplements to juice to prevent stuck fermentations (Ribereau-Gayon, 1985). Studies showed the ability of yeast hulls to remove certain toxic fermentation side-products (Lafon-Lafourcade, 1984). In addition, Munoz and Ingledew (1989b) reported that yeast hulls could also supply beneficial unsaturated fatty acids and the importance of yeast hulls in the stimulation of fermentation and prevention of stuck and sluggish fermentations was verified (Munoz and Ingledew, 1989a).

2.2.3.3.4 Vitamins

Insufficient availability of vitamins (essentially thiamine) may be associated with some sluggish fermentations (Peypaud and Lafon-Lafourcade, 1977; Ough *et al.*, 1989). *Saccharomyces cerevisiae* is capable of synthesising all essential vitamins except biotin, however, research has shown the presence of extracellular vitamins is highly stimulatory to growth and fermentation (Monk, 1982; Lafon-Lafourcade and Ribereau-Gayon, 1984; Ough *et al.*, 1989; Fleet and Heard, 1993). It was shown that wild yeasts, such as *Kloeckera apiculata*, decrease thiamin levels to a deficient situation for *Saccharomyces* (Bataillon *et al.*, 1996). Acetic acid has been reported to reduce the ability of *Saccharomyces* to transport and retain thiamine (Iwashima *et al.*, 1973). Thiamine is cleaved and its biological activity destroyed by sulphur dioxide, further reducing the concentration of this vitamin (Alexandre and Charpentier, 1998; Bisson, 1999).

2.2.3.3.5 Minerals

Deficiencies and imbalances in minerals and cations, serving as co-factors for glycolytic and other enzymatic reactions, can result in fermentation arrest (Dombeck and Ingram, 1986; Blackwell *et al.*, 1997; Walker and Maynard, 1997). Magnesium plays a key role in metabolic control, growth and cell proliferation, glycolytic pathway and subsequently ethanol production (reviewed by Walker, 1994). Limitation of zinc and magnesium directly affects sugar catabolism and consequently fermentative activity (Jones *et al.*, 1981; Jones and Greenfield, 1984; Dombeck and Ingram, 1986; D'Amore *et al.*, 1987; Monk, 1994). Calcium limitation increases ethanol sensitivity (Nabais *et al.*, 1988). High manganese depresses uptake of magnesium and vice versa (Blackwell *et al.*, 1997) which may lead to a deficiency situation. Additionally, an imbalance of pH and potassium ions present in grapes from vines with poor potassium uptake ability from the soil could result in stuck fermentations (Kudo *et al.*, 1998).

2.2.3.4 Inhibitory substances

2.2.3.4.1 Ethanol

Ethanol inhibits different transport systems utilised by *S. cerevisiae* (Leao and Van Uden, 1982; Cartwright *et al.*, 1987a; Pascual *et al.*, 1988; Mauricio and Salmon, 1992; Salmon *et al.*, 1993), influences proton fluxes (Leao and Van Uden, 1984; Cartwright *et al.*, 1986, Cartwright *et al.*, 1987b; Killian *et al.*, 1989) and affects yeast plasma membrane composition (Jones and Greenfield, 1987; Jones, 1989, 1990) resulting in subsequent growth inhibition (Thomas and Rose, 1979; Ingram and Butke, 1984) and decrease in fermentation rate as a result of inhibiting sugar transport activity (Salmon *et al.*, 1993). Fermentation temperature influences ethanol tolerance. At lower temperatures, greater tolerance to ethanol occurs (Henschke, 1997). An important property of ethanol is it increases the toxicity of other compounds. The availability of sterols and fatty acids has a definite impact on ethanol sensitivity (Lafon-Lafourcade and Ribéreau-Gayon, 1984).

2.2.3.4.2 Acetic acid

High levels of acetic acid are often associated with stuck or sluggish fermentations. The heterofermentative lactic acid bacteria, including strains of *Lactobacillus* and *Oenococcus*, certain non-*Saccharomyces* yeasts such as *Brettanomyces*, *Hansenula anomala*, *Kloeckera apiculata* and *Candida krusei* (Fleet and Heard, 1993), commercial wine yeasts (Hanneman, 1985) and acetic acid bacteria (Drysdale and Fleet, 1985, 1988, 1989) all have the ability to produce high levels of acetic acid that directly increases volatile acidity (Lambrechts, 2000). Elevated acetic acid concentrations can inhibit yeast growth, enhance ethanol toxicity and prevent the completion of fermentation. Controversially, the arrest of fermentation could allow the growth of spoilage organisms which could lead to high levels of volatile acidity.

2.2.3.4.3 Medium chain fatty acids

Medium chain fatty acids which are intermediates in the biosynthesis of long chain fatty acids can inhibit alcoholic fermentation (Lafon-Lafourcade *et al.*, 1984). Fatty acid toxicity increase as pH decreases with decanoic acid being more inhibitory than octanoic acid (Viegas *et al.*, 1989). Both inhibit hexose transporter systems resulting in reduced fermentation rate (Zamora *et al.*, 1996).

2.2.3.4.4 Toxins and killer toxins

Killer yeast strains (phenotype K^+R^+) secrete a proteinaceous extracellular toxin that kills other sensitive yeast strains (phenotype K^-R^-) of *S. cerevisiae*. Neutral yeasts (phenotype K^-R^+) are resistant to killer toxins but do not produce it (Bevan and Makower, 1963; Woods

and Bevan, 1968; Medina *et al.*, 1997). The killer toxin can change the nitrogen metabolism by decreasing the ion gradient across the membrane of the sensitive yeasts and consequently interrupting the coupled transport of protons and amino acids (De la Peña *et al.*, 1981). The toxin also causes the cellular loss of small metabolites such as ATP, glucose and amino acids (Bussey, 1974). Killer toxins can inhibit wine fermentation by sensitive yeasts (Van Vuuren and Wingfield, 1986; Radler and Schmitt, 1987; Carrau *et al.*, 1988, 1993). The interactions between killer and sensitive yeasts and the effect on nitrogen metabolism in winemaking conditions have been studied extensively (Shimizu, 1993; Medina *et al.*, 1997; Torrea-Goñi and Ancín-Azpilicueta, 2002).

Moulds present on the fruit may produce mycotoxins to which *Saccharomyces* is susceptible (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Bisson, 1999). In addition, plant produced compounds (the phytoalexins) and enzymes (the pathogenesis-related proteins) may impact yeast growth (Bisson, 1999) since these compounds are produced in response to fungal infection.

2.2.3.4.5 Sulphites

Sulphites are highly toxic to microorganisms. Molecular SO₂ is more active at low pH. Thus molecular SO₂ is extremely active against yeasts in low pH (3-3.5) must. Excessive use of SO₂ is toxic to yeast cells (Alexandre and Charpentier, 1998).

2.2.3.4.6 Agricultural residues

Fungicides and pesticides used in the vineyard may negatively affect yeast viability if present at high enough residual concentrations at the time of harvest (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Bisson, 1999). These residues can act directly or indirectly to inhibit yeast growth during fermentation (Specht, 2003).

2.2.3.5 Physical factors

2.2.3.5.1 Excessive must clarification

Excessive must clarification can often cause sluggish fermentation due to the loss in fatty acid content, sterol content and macromolecules (Alexandre and Charpentier, 1998). The level of solids also affect alcohol tolerance, therefore the choice of an alcohol tolerant strain is more important in a clarified juice than a high solid must (Henschke, 1997). Must clarification affects the assimilation of nitrogen compounds. Must clarification reduces nutrients and eliminates fatty acids, especially many unsaturated fats. As a result the amino acid transport system is affected (Ayestarán *et al.*, 1995; Ancín *et al.*, 1998; Ayestarán *et al.*, 1998).

2.2.3.5.2 pH

Saccharomyces is tolerant to low pH fermentations and can grow in juice pH range of 2.8 to 4.2 (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Heard and Fleet, 1988; Bisson, 1999). Sulphite toxicity to yeast is largely dependent on the level of SO₂ accumulation in the cell. Once inside the cell, the sulphites cause a rapid decrease in the intracellular ATP level, resulting in cell death (Hinze and Holzer, 1986).

2.2.3.5.3 Temperature extremes

Temperature extremes during fermentation could severely affect yeast growth and metabolism (Specht, 2003). Ethanol resistance is also influenced by temperature (Heard and Fleet, 1988; Bisson, 1999; Bisson and Butzke, 2000). At higher temperatures, the cell membrane fluidity increases and ethanol can enter the cell more readily, adversely affecting metabolism and cell viability. Cooler temperatures may enhance ethanol resistance by increasing sterol levels in yeast cell membranes (Suutari *et al.*, 1990; Torija *et al.*, 2003) resulting in lower accumulation of intracellular ethanol (Lucero *et al.*, 2000).

2.2.3.6 Microbial incompatibility

Initial high populations of non-*Saccharomyces* yeast and bacteria increase the risk of stuck and sluggish fermentations to occur (Drysdale and Fleet, 1989; Bisson, 1999; Edwards *et al.*, 1990, 1998). This is due to competition for nutrients and production of toxic substances. Using unsanitized equipment (cellar hygiene) increases the possibility for microbiological factors such as wild killer yeasts and bacteria (spoilage) influencing the fermentation process. The interactions between *O. oeni* and *S. cerevisiae* are also described by Alexandre *et al.* (2004). Malolactic bacteria have elaborate nutritional requirements (Buckenhüskes, 1993) and competition for these may inhibit or delay yeast activity during the alcoholic fermentation (Huang *et al.*, 1996; Edwards *et al.*, 1998). Lonvaud-Funel (1995) suggests that inoculation of must with starter cultures should take place only after the conclusion of the alcoholic fermentation to avoid the increase of wine volatile acidity due to sugar metabolism by *O. oeni*. Incompatible pairings of wine yeast and malolactic bacteria is also a possibility. Edwards *et al.* (1998) reported on *Lactobacillus kunkeei* frequently causing stuck fermentations, regardless of the yeast strain present.

2.2.3.7 Metabolic basis of stuck and sluggish fermentation

The metabolic basis of stuck and sluggish fermentation has been fairly well established. The decrease in rate of sugar consumption is correlated with a decrease in sugar uptake capacity. Glucose and fructose consumption are reduced in response to various environmental or cellular stress conditions. Nutrient limitation (macronutrient and micronutrient), low pH, lack of

oxygen, lack of adequate agitation, temperature extremes, presence of toxic substances, presence of other microorganisms, imbalance of cations, and poor strain tolerances (particularly to ethanol or acetaldehyde). All of these have been associated with stuck and sluggish fermentations and have an impact on glucose and fructose transporter expression and activity (Alexandre and Charpentier, 1998).

According to literature, fructose levels in stuck wine are found to be 10 times higher than the glucose concentration. Stuck fermentation can therefore be expected for wines with glucose/fructose ratio smaller than 0.1 (Gafner and Schütz, 1996).

Apart from the influence of nutrients, physical and microbial factors on the metabolism of the yeast which could result in decreased rate of fermentation or even complete fermentations arrest, apoptosis have been suggested as an additional mechanism influencing fermentation (Büttner *et al.*, 2006; Ludovico *et al.*, 2001). Apoptosis refers to the programmed cell death of the yeast cell which is also a regulated suicide program crucial for metazoan development (Madeo *et al.*, 2004; Büttner *et al.*, 2006).

2.2.4 CELLAR MANAGEMENT: MALOLACTIC FERMENTATION

Despite considerable research (Wibowo *et al.*, 1985; Britz and Tracey, 1990), the malolactic fermentation (MLF) process remains to be an imperfectly controlled process and at times MLF can be difficult to get started. The occurrence of MLF problems and the possible causes thereof has been studied (Nel *et al.*, 2001) less extensively than in the case of alcoholic fermentation problems. Various factors influencing the start and successful completion of MLF will be highlighted.

2.2.4.1 Inoculation considerations

Malolactic fermentation is a biological process of wine deacidification in which the dicarboxylic L-malic acid (malate) is converted to the monocarboxylic L-lactic acid (lactate) and carbon dioxide (Davis *et al.*, 1985). This process is normally conducted by lactic acid bacteria (LAB) isolated from wine, including *Oenococcus oeni* (previously *Leuconostoc oenos*, Dicks *et al.*, 1995), *Lactobacillus* spp. and *Pediococcus* spp. (Wibowo *et al.*, 1985). *O. oeni* is the preferred starter culture to conduct MLF due to its tolerance to low pH, high ethanol and SO₂ levels and flavor profile produced (Kunkee, 1967; Wibowo *et al.*, 1985; Tracey and Britz, 1987; Van Vuuren and Dicks, 1993). It has been shown that the ability to perform MLF in harsh conditions is closely related to the physiological properties of the *O. oeni* strain inoculated (Nannelli *et al.*, 2004).

Although MLF may occur spontaneously, the fermentation management could be simplified with the introduction of *O. oeni* cultures (Krieger, 1993; Nielsen *et al.*, 1996). The lag phase associated with spontaneous MLF (wild/uncultured strains) increase the risk of

spoilage organisms and production of volatile acidity (as a result of malolactic bacterial sugar metabolism) due to the low SO₂ levels. Inoculation with a LAB culture avoids these problems by immediately providing the population (more than 2x10⁶ cells/mL) necessary to conduct MLF. Semon *et al.* (2001) suggests that pre-fermentation inoculation results in increased volatile acidity concentrations. However, the success of MLF is not always guaranteed due to changes in fermentation conditions, grape must composition and microbial competition (Krieger, 1993). Compatibility of yeast and LAB should be considered when time of inoculation is considered. Very often, starter culture failures are due to improper preparation and inoculation procedures. In some cases, starter culture failure may be due to antagonistic interactions between yeast and bacteria.

2.2.4.2 Nutritional requirements

Malolactic fermentation difficulty could be the result of insufficient nutrients important for the development of lactic acid bacteria (Nygaard and Prahl, 1996). Yeast can reduce the nutrients available to malolactic bacteria (MLB) considerably and therefore time of inoculation is critically important to avoid competition for nutrients. For this reason, winemakers often add a MLB nutrient when inoculating with MLB to assist their development. This addition is especially important if the must and wine initially has low levels of nutrients or if yeast strains with inherently high nutritional requirements were used. The addition of bacterial nutrients ensures a quick onset and completion of MLF and could also prevent delayed and/or stuck MLF.

MLB have elaborate nutritional requirements (Buckenhüskes, 1993) with limited means of synthesizing growth requiring compounds (Fourcassier *et al.*, 1992; Fugelsang, 1996). *Oenococcus oeni* has very specific and at times very fastidious nutritional requirements to support sufficient growth and development of the bacteria. Studies suggest that wine carbohydrates (Melamed, 1962; Ribéreau-Gayon *et al.*, 1975; Dittrich *et al.*, 1980) and amino acids (Mayer *et al.* 1973; Temperli and Kuensch, 1976; Beelman and Gallander, 1979) may be utilised by these bacteria during malolactic fermentation and this metabolism as well as that of organic acids (Pilone *et al.* 1966; Kunkee, 1974; Beelman and Gallander, 1979; Lafon-Lafourcade and Ribéreau-Gayon, 1984; Ribéreau-Gayon *et al.*, 1975) can lead to changes in the concentration of constituents which affect sensory quality of wines (Davis *et al.*, 1986). Inorganic nitrogen [supplied in the form of diammonium phosphate (DAP)] cannot be used by these bacteria (Loubser, 2005). Vitamins, especially from the B-group, as well as pantothenic acid, are required. In addition, certain trace elements (including magnesium and manganese) also form part of the very specific nutritional requirements of *O. oeni* (Loubser, 2005). Liu (2002) reviewed the current knowledge on the metabolism of LAB (predominantly oenococci)

comprehensively. However, the biochemical mechanisms by which LAB grow in wines are still not clearly understood.

2.2.4.3 Inhibitory factors

The physico-chemical properties that influence LAB growth are well known, mainly: pH, acidity, ethanol and SO₂ concentrations and temperature (Bousbouras and Kunkee, 1971; Ingram and Butke, 1984; Wibowo *et al.*, 1985; Davis *et al.*, 1988; Wibowo *et al.* 1988; Henschke, 1993). A study by Vaillant *et al.* (1995) investigating the effects of 11 physico-chemical parameters, identified ethanol, pH and SO₂ as having the greatest inhibitory effect on the growth of malolactic bacteria in wine. Another argument is that inhibitory substances are accumulated in wine and all these factors could have possible synergistic effects on each other, enhancing the inhibitory effect of a specific factor.

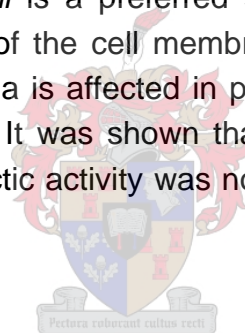
2.2.4.3.1 Ethanol content

Malolactic bacteria are sensitive to ethanol and usually struggle above 13.5% exhibiting very slow or non-existent growth. *O. oeni* is a preferred starter culture due to its tolerance to ethanol. The fatty acid composition of the cell membrane of wine LAB can be modified by ethanol. The viability of these bacteria is affected in particular by the saturated/unsaturated fatty acid ratio (Henick-Kling, 1995). It was shown that ethanol (12% v/v) had an inhibitory effect only on cell growth but malolactic activity was not affected (Capucho and San Romão, 1994).

2.2.4.3.2 pH

The effect of pH on the growth rate of LAB in wines is well demonstrated in the literature (Bousbouras and Kunkee, 1971; Castino *et al.*, 1975; Liu and Gallander, 1983). Davis *et al.* (1986) showed the rate of bacterial growth and malolactic fermentation increased as wine pH was increased from 3.0 to 4.0. The pH of wine has a selective effect upon the species that grow in wine. Usually, *O. oeni* is the only species isolated from wines with a pH below 3.5 (Davis *et al.*, 1986). Generally, malolactic bacteria favour higher pH's and for most strains, minimal growth occurs at pH 3.0. Under winemaking conditions, pH's above 3.2 are advised.

The lag phase before MLF, in the case of spontaneous MLF, can be prolonged the lower the pH. The species of LAB dominant in the must or wine is determined by the pH (Bousbouras and Kunkee, 1971). At a low pH (3.2 to 3.4) *O. oeni* is the primary LAB species, different strains of which will dominate throughout MLF. At a higher pH (3.5 to 4.0), *Lactobacillus* and *Pediococcus* dominate over *Oenococcus* (Costello *et al.*, 1983).



2.2.4.3.3 Temperature

The influence of temperature on the growth of LAB and the occurrence of MLF has been thoroughly researched (Van der Westhuizen & Loos, 1981; Wibowo *et al.*, 1985). Research results confirm that MLF occurs much more rapidly at temperatures of 20°C and above than 15°C and below (Loubser, 1999; Du Plessis, 2005). In the absence of SO₂ the optimum temperature range for MLF is 23 to 25°C. Maximum malic acid degradation will occur at 20 to 25°C. However, these temperatures decrease with an increase in SO₂ concentrations resulting in 20°C being more acceptable. Most strains of *O. oeni* grow very slowly or cease to grow below 15°C. Cells may however remain viable at low temperatures.

2.2.4.3.4 Sulphur dioxide

Yeast produce SO₂ during alcoholic fermentation and this may inhibit the growth of malolactic bacteria (Lonvaud-Funel *et al.*, 1988; Henick-Kling and Park, 1994). The levels of SO₂ produced by yeast depend on the yeast strain, availability of nutrients and the presence of compounds in the must (e.g. acetaldehyde) which binds SO₂ (Nygaard and Prahl, 1996). Already in 1994 Henick-Kling *et al.* demonstrated the inhibition of malolactic starter cultures by active growing yeasts due to the production of high levels of SO₂ during the early stage of alcoholic fermentation.

Apart from the selective effect of pH on the growth of LAB, the long-term survival of *O. oeni* under practical wine conditions is determined by the addition of SO₂ (Lafon-Lafourcade *et al.*, 1983). According to other studies (Somers and Wescombe, 1982; Lafon-Lafourcade, 1983), a total SO₂ concentration of more than 50 mg/L generally restricts the growth of lactic acid bacteria in wines, especially at the lower pH values when a greater proportion of the SO₂ is in the undissociated, antimicrobial form. It is therefore not recommended to add SO₂ to must after alcoholic fermentation (Henick-Kling, 1994).

2.2.4.4 Microbial interactions

Yeast (*S. cerevisiae*) may deplete complex nutrients and growth factors required by malolactic bacteria and may release bioactive metabolites (SO₂, fatty acids and macromolecules) that can stimulate, inhibit or have negligible effect on the metabolism of MLB (Lonvaud-Funel *et al.*, 1988; Edwards *et al.*, 1990; Capucho and San Romao, 1994; Henick-Kling and Park, 1994; Rosi *et al.*, 1999; Alexandre *et al.*, 2004). Interactions between co-existing yeast (*S. cerevisiae*) and *O. oeni* can cause problems with MLF. Fermentations of must with low levels of nutrients may cause the yeast used during alcoholic fermentation to produce increased levels of SO₂ which may inhibit MLF. In the case of inoculation before the completion of alcoholic fermentation, bacterial inhibition decreases towards the end of fermentation. This could be explained by the death phase of yeast which reduces the SO₂

produced and the availability of nutrients as a result of yeast autolysis (Nygaard and Prah, 1996).

The presence of bacteriophages (bacterial viruses) can also cause sluggish or stuck MLF (Henick-Kling, 1994) and can be problematic if wooden barrels used for maturation are contaminated (Berthelot, 2000).

The growth of *Pediococcus* spp. are favoured in high pH wines, resulting in volatile acidity or the production of bacteriocins (antimicrobial proteins or peptides) which may inhibit the growth of *O. oeni* (Green *et al.*, 1997; Van Reenen *et al.*, 1998).

King and Beelman (1986) suggested that the growth of *O. oeni* during alcoholic fermentation might be retarded by the production of toxic compounds by yeasts other than ethanol and sulfur dioxide. Alcohol, temperature and pH can modify the fatty acid composition of the cell membrane of wine LAB. In particular the saturated/unsaturated fatty acids ratio affects the viability of these bacteria (Henick-Kling, 1995).

2.3 TECHNOLOGY TO MONITOR FERMENTATION

2.3.1 MICROBIOLOGICAL

2.3.1.1 Enumeration by traditional plating

The identification and enumeration of microorganisms throughout the fermentation process by plating on selective growth media is a widely applied technique (Gueimonde and Salminen, 2004). However, this method of enumeration is often time consuming, laborious and could be inaccurate as a result of the possible viable but nonculturable (VBNC) state of microorganisms. Cells in VBNC state are defined by Olivier (1993) as cells which are metabolically active but unable to undergo the cellular division for growth in liquid or agar. The evolution to a VBNC state is related to the intensity of the stress (Olivier *et al.*, 1995).

2.3.1.2 Polymerase Chain Reaction (PCR) related technologies

Many molecular techniques have been developed for yeast identification and characterization (Querol and Ramón, 1996; Guillamón *et al.*, 1998; Esteve-Zarzoso *et al.*, 1999; Loureiro and Querol, 1999; Querol *et al.*, 2000), most of them based on colonies obtained on plates.

Real-time or quantitative PCR (QPCR) methods have been developed to enumerate several species of LAB, including those found in wine (Delaherche *et al.*, 2004; Furet *et al.*, 2004; Pinzani *et al.*, 2004; Neeley *et al.*, 2005). González *et al.* (2006) reported the use of nested PCR and real-time PCR for the detection (qualitative) and enumeration (quantitative) of acetic acid bacteria in wine conditions.

López *et al.* (2003) demonstrated the use of a new PCR-based method to monitor inoculated wine fermentations and ensure the fermentation is conducted by the inoculated yeast. The method is based on the variation in the number and position of introns in the mitochondrial gene *COX1* (López *et al.*, 2003).

One of the most commonly used culture-independent fingerprinting techniques is denaturing gradient gel electrophoresis (DGGE). It is based on the separation of PCR amplicons of the same size but different sequences (Ercolini, 2004). The theoretical aspects of this separation were first described by Fisher and Lerman (1983). Many applications of PCR-DGGE in microbial ecology have been previously described and reviewed (Muyzer *et al.*, 1997; Muyzer and Smalla, 1998; Muyzer, 1999). Applications of PCR-DGGE include the identification of microorganisms, the evaluation of microbial diversity and microbiological quality assessment (Ercolini, 2004). A study by Cocolin *et al.* (2000) demonstrated PCR-DGGE is a viable alternative to standard plating methods for qualitative assessment of the microbial constituents in model wine fermentations. The quantitation of DGGE profiles however, is problematic due to the complex nature of multitemplate PCR (Wagner *et al.*, 1994; Suzuki and Giovannoni, 1996; Hansen *et al.*, 1998; Polz and Cavanaugh, 1998).

2.3.1.3 Flow cytometry

In recent years, flow cytometry (Bruetschy *et al.*, 1994; Bouix and Leveau, 2001; Malacrino *et al.*, 2001, 2005; Thornton *et al.*, 2002; Boyd *et al.*, 2003) has been used to monitor live and dead yeast cell concentrations during fermentation (Chaney *et al.*, 2006). Validation of this method has been performed by comparison with other viability analysis techniques such as haemocytometry and plating (Fiala *et al.*, 1999; Thornton *et al.*, 2002). Thiazol orange, a permeant DNA-reactive stain that enters live and dead cells, fluoresces at 530 nm and is used to differentiate cells from debris. Additional staining with propidium iodide, an impermeant DNA-reactive stain which cannot penetrate cells with intact membranes, fluoresces at 625 nm and differentiates live and dead cells.

Since cell viability stains often rely on membrane integrity, they do not necessarily report on the metabolic activity of cells. Varela *et al.* (2004) suggested that fermentation rate is a combination of intracellular (metabolic) activity and the mass of cells actively fermenting. It may therefore be more informative to monitor the metabolic activity or yeast 'vitality' than the cell viability alone. This information could possibly serve as a better predictor of stuck fermentations by determining the physiological state of the yeast populations using flow cytometry and fluorescent viability staining (Bouchez *et al.*, 2004) combined with fluorescent vitality staining.

2.3.1.4 FT-IR spectroscopy for the identification of microorganisms

Naumann and coworkers suggested Fourier transform infrared (FT-IR) spectroscopy as a rapid and inexpensive method to identify microorganisms (Naumann, 1985; Naumann *et al.*, 1988, 1990, 1991; Helm *et al.*, 1991). Absorption of infrared light by cellular compounds results in a fingerprint-like spectrum that can be identified by comparison to reference spectra. The success of the method is therefore directly dependent on the complexity of the reference spectrum library. The application of FT-IR spectroscopy for the identification of microorganisms was firstly reported in the food industry for some species of the genera *Lactobacillus* (Curk *et al.*, 1994), *Actinomyces* (Haag *et al.*, 1996), *Listeria* (Holt *et al.*, 1995), *Streptococcus* (Goodacre *et al.*, 1996) and *Clostridium* (Franz, 1994). Additional research reported the identification of yeasts by FT-IR (Serfas *et al.*, 1991; Henderson *et al.*, 1996; Kümmerle *et al.*, 1998).

FT-IR microspectroscopy is a novel tool to characterize microorganisms (Ngo Thi *et al.*, 2000) in which the spectra of single colonies are recorded by a mid-IR spectrometer coupled to a microscope. Isolation and purification of the organisms to be measured are not necessary. Wenning *et al.* (2002) compared identification by FT-IR macrospectroscopy and FT-IR microspectroscopy and found similar results. Therefore, the time-consuming isolation of organisms prior to identification, as is the case with FT-IR macrospectroscopy, is not necessary.

2.3.2 CHEMICAL ANALYSIS

An accurate measurement of various chemical components throughout the winemaking process is a necessity. As a result of the development and improvement of technology, the focus of wine analysis shifted towards evaluating and establishing high-throughput analytical methods.

2.3.2.1 Chromatographic techniques

High Performance Liquid Chromatography (HPLC) is an analytical technique for the separation and determination of organic and inorganic solutes in a variety of samples. Analysis of the major organic acids, carbohydrates, glycerol and ethanol in wine and grape must using HPLC systems with refractive index (RI) and ultraviolet (UV) detection have been reported in numerous studies (McCord *et al.*, 1984; Frayne, 1986; Falque Lopez and Gomeze, 1996; López-Tamames *et al.*, 1996; Michnick *et al.*, 1997; Aragon *et al.*, 1998; Castellari *et al.*, 2000; Reynolds *et al.*, 2001; Palacios *et al.*, 2002). The coupling of HPLC and FT-IR for the determination of carbohydrates, alcohols and organic acids was presented by Vonach *et al.* (1998). Since most compounds absorb in the infrared region, FT-IR can provide

qualitative information about the compounds and can be regarded a general detector for liquid chromatography (reviewed by Somsen and Visser, 2000). Edelman *et al.* (2003) reported on another HPLC application with diamond attenuated total reflectance (ATR)-FT-IR detection for the determination of carbohydrates, alcohols and organic acids in red wine.

Gas chromatography (GC) is a technique almost routinely used to determine the volatile composition of wine and fermenting must. Several studies using GC in combination with mass spectrometry (GC-MS) and/or additional sorptive extraction techniques such as solid phase micro extraction (SPME), solid phase dynamic extraction (SPDE) and solid phase extraction (SPE) have been reported (Ferreira *et al.*, 1996; Vianna and Ebeler, 2001; Alves *et al.*, 2005; Howard *et al.*, 2005; Liu *et al.*, 2005; Câmara *et al.*, 2006; Esti and Tamborra, 2006). Mallouchos *et al.* (2002) and Hernández-Orte *et al.* (2002) used SPME GC-MS and GC-FID (flame ionization detection) respectively to investigate the relationship between the amino acid profile and aroma profile of wines.

2.3.2.2 Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has become a popular technique for wine and grape juice analysis (Clark *et al.*, 2006). Many studies have used NMR (^{13}C and ^1H) to determine amino acids, organic acids, sugars, alcohols, glycerol, polyphenols, catechin, epicatechin and gallic acid in wine or phenolic extracts (reviewed by Clark *et al.*, 2006) and in grape juice or must (reviewed by Clark *et al.*, 2006). Clark *et al.* (2006) reported on monitoring a commercial fermentation using ^1H NMR analysis and the aid of chemometrics for the simultaneous determination of a spectrum organic analytes. The potential of this technique as a tool to monitor commercial wine fermentations is however limited due to the availability and access to high field NMR instrumentation.

Infrared spectroscopy (IR) has been used successfully to monitor large scale fermentations, since various compounds are measured simultaneously from a single sample without prior treatment (Urtubia *et al.*, 2004). Infrared spectroscopy is a technique based on the interaction of infrared radiation with the vibrations and rotations of atoms of a molecule. Infrared radiation is passed through a sample and the fraction of incident radiation absorbed at a particular energy results in the absorption spectrum. The vibration frequency of a chemical bond in a molecule is related to the energy at which any peak in an absorption spectrum appears. Since all molecules absorb infrared radiation at different wavelengths, an infrared spectrum therefore contains both qualitative and quantitative information of the sample material (Griffiths and de Haseth, 1986; Andersen *et al.*, 2002).

Initially, routine wine analysis used vibrational spectroscopy in the near-infrared (NIR) region (Baumgarten, 1987). The use of FT-IR technology in the MIR region for wine analysis is due to a more accurate determination of more constituents and properties than the NIR

method (Nieuwoudt, 2004; Patz *et al.*, 2004). Application of FT-MIR spectroscopy is of special interest due to the presence of sharp and specific absorption bands (Schindler *et al.*, 1998). Multivariate prediction models are constructed through a calibration process (Eriksson *et al.*, 1999; Esbensen, 2002) for predicting wine constituents from the FT-IR absorbance spectrum (Andersen *et al.*, 2002). Recently, this technique has been extensively evaluated for quantification purposes in industrial applications (Patz *et al.*, 1999; Dubernet and Dubernet, 2000; Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003; Nieuwoudt *et al.*, 2004).

2.3.2.3 New technology: Electrochemical sensors

One of the most promising directions for the development of innovative analytical methods is the use of electrochemical techniques. These devices consist of chemical sensor arrays coupled with an appropriate pattern recognition system capable of extracting information from the complex signals. The electronic nose consists of an array of gas sensors with different selectivity, a signal collecting unit and pattern recognition software. It is useful for the analysis of headspace of liquid or solid food samples (Schaller *et al.*, 1998) and numerous attempts using the electronic nose for wine analysis have also been reported (Di Natale *et al.*, 1995, 1996). Similar in principle to the electronic nose, the electronic tongue consists of an array of sensors designed for liquids for the evaluation and classification of complex liquids. Various techniques such as conductimetric, potentiometric and voltammetric techniques can be used for the electronic tongue (Winquist *et al.*, 2000). Studies using potentiometric electronic tongue for beverage analysis and wine discrimination (Legin *et al.*, 1999, 2003) have been reported. Buratti *et al.* (2004) used an electronic nose and an amperometric electronic tongue to differentiate and classify Italian wines according to region by applying multi-dimensional chemometric techniques. The application of various types of electrochemical sensors (electronic nose and tongue) in combination with other analytical techniques for discrimination and classification in different media has been reported (Toko, 2000; Legin *et al.*, 2004; Ciosek *et al.*, 2005; Cozzolino *et al.*, 2005; Gallardo *et al.*, 2005; Lozano *et al.*, 2005; Ciosek and Wróblewski, 2006; García *et al.*, 2006; Lvova *et al.*, 2006; Parra *et al.*, 2006). The development of electronic tongues and their analytical applications in the food and beverage industry is reviewed by Vlasov *et al.* (2002).

2.3.3 CHEMOMETRICS

The application of sophisticated statistical techniques, the so-called “chemometrics”, in wine production and laboratory is widely referenced in the literature from several points of view. Multivariate data, such as spectra, are often rich in information. The methods used in chemometrics for the analysis of such large data sets are principal component analysis (PCA Wold *et al.*, 1987; Jackson, 1991) and projections to latent structures (PLS Wold, 1982;

Tenenhaus, 1998; Wold and Josefson, 2000; Wold *et al.*, 2001). These methods provide a strategy for utilising this richness in information for summarizing data (Wold, 1982; Wold *et al.*, 1987), classification and discriminant analysis (Wold, 1976; Stahle and Wold, 1987) and modelling relationships between variables (Wold, 1982; Martens and Naes, 1989; Tenenhaus, 1998; Wold and Josefson, 2000; Wold *et al.*, 2001).

Supervised and non-supervised pattern recognition techniques have been used to distinguish different varieties, geographical areas, elaboration processes etc. Câmara *et al.* (2006) used multivariate analysis for the classification and differentiation of Madeira wines according to the main grape varieties using headspace analysis. Multivariate data analysis have been extensively used to differentiate wines with different geographic origins on the basis of volatile wine compounds (Noble *et al.*, 1980; García-Jares *et al.*, 1995; García-Martin *et al.*, 1995), specific compounds (hexan-1-ol and cyclo-hexane) (Kwan and Kowalski, 1980), sugars, organic acids and amino acids (Arminda Alves, 1992; Guedes de Pinho, 1994) and trace elements (Day *et al.*, 1995). The value of chemometrics has also been proven a versatile and valuable tool for assessing wine authenticity (Arvanitoyannis *et al.*, 1999).

Multivariate regression, such as principal component regression (PCR) and partial least squares regression (PLSR) are standard procedures in chemometrics, which has been used for developing equations for the determination of quantitative parameters in wine and other food industries using the data provided by the spectroscopic techniques.

FT-IR spectroscopy in combination with chemometric data evaluation provides valuable quantitative information even for highly complex problems such as wine analysis.

2.4 CONCLUSIONS

Fermentation predictability and wine quality are principally dependent on wine yeast attributes even if a wide range of factors affect the fermentation performances of yeasts. In particular, the ability to adapt to nutritional deficiency and to cope with the presence of inhibitory substances is of vital importance to fermentation performance.

Difficulties arise from a combination of factors and a variety of sources. It is the impact of two or more conditions together that may cause a problem of much greater difficulty than what would have been predicted by a single parameter acting alone. Therefore, each step of the winemaking process needs to be approached with as complete an understanding as possible. The cause of a stuck or sluggish fermentation is rarely the result of one factor in isolation. Generally, various factors would have a synergistic effect on each other, enhancing the effect of a specific factor.

A better understanding of the aspects of wine microorganism physiology will allow us to better match combinations of yeast and bacteria starter cultures with grape varieties, and select the timing of yeast and bacteria inoculations.

Fermentation problems usually arise due to the presence and impact of various stress factors in the yeast and bacteria environment. Some of these stress factors are however unavoidable and others are the result of inappropriate fermentation management decisions. New analytical technologies in combination with chemometrics such as multivariate data analysis could provide powerful tools to monitor industrial fermentations and prevent fermentation problems in the future.

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3. RESEARCH RESULTS

The assessment and characterisation of problem fermentations: An industrial case study to investigate the discrimination possibilities of FT-IR spectroscopy

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ABSTRACT

Sluggish and stuck fermentations are major problems in the international wine industry, including South Africa. Several empirical studies to identify the causes of sluggish and stuck fermentations have been conducted in the past, and data suggest that numerous factors can have a significant influence on the successful completion of fermentations. Due to the multiple factors which can lead to slow or incomplete fermentations, previous studies on problem fermentations were mostly limited to investigating one or two well-controlled factors at a time. In this study, we attempt to overcome this limitation by applying chemometrics and multivariate data analysis to investigate the factors affecting the likelihood of stuck fermentations in industrial wine fermentations. For this purpose collection of problem fermentation samples from commercial cellars took place in the 2005 and 2006 vintages and a multivariate approach was implemented. Fourier transform infrared (FT-IR) spectroscopy and additional measurements including microbial analysis, SO₂ determinations, free assimilable nitrogen (FAN) determinations and volatile flavour components determined by gas chromatography were incorporated in this multi-instrument and multivariate data analysis approach. Principle component analysis (PCA) was used to identify the main sources of variation in the spectra of the 379 must/wine samples. Reducing sugar, alcohol content, cultivar and stage of fermentation represent the main sources of variation amongst the samples. Multivariate partial least squares (PLS) regression models were established to discriminate between problem and control fermentations. The data suggest that FT-IR spectroscopy represents a high throughput and cost-effective technique to monitor fermentation. It was also shown that multivariate data analysis could be used to effectively discriminate problem fermentations from control fermentations and this feature shows potential for prediction purposes in order to identify potential problem fermentations and to take preventative measures.

3.1 INTRODUCTION

The completion of fermentation (to dryness) is one of the important objectives during the winemaking process. Incomplete or “stuck” fermentations are defined as those leaving a higher than desired residual sugar content at the end of alcoholic fermentation, while slow or sluggish fermentations are characterized by a low rate of sugar utilisation (Bisson, 1999). Although these terms are often associated with alcoholic fermentation, similar problems might also occur in the case of malolactic fermentation. Fermentation problems can be encountered at various stages during the winemaking process. Indirectly starting as early as in the vineyard with inappropriate viticultural practices leading to nutrient deficiencies in the must (Kliewer, 1970), harvest considerations (Snyman, 2006), inappropriate fermentation management (Bisson, 2005) and possible problems with refermentation during the final bottling stages as a result of microbial instability.

Despite advances in winemaking technology and improvements in fermentation control, stuck and sluggish fermentations remain a major concern for winemakers due to their negative economic impact. The determination of the causes of fermentation arrest in specific industrial cases is difficult despite the fact that many causes of incomplete fermentation are known. This is because the majority of studies investigate one or two well-controlled variables, whereas under production conditions these factors interact (Bisson, 2005).

Current literature related to stuck and sluggish fermentation is substantial. A significant amount of information on the metabolism, physiology, cell biology, and stress adaptation in *Saccharomyces cerevisiae* under laboratory conditions has been generated. Although extrapolation from such studies to industrial winemaking conditions has to be done with caution, results obtained point to numerous factors that may affect fermentation rate and cause sluggish or stuck fermentations. These include high initial must sugar content, nutrient- and in particular nitrogen limitation, ethanol toxicity, organic- and fatty acid toxicity, presence of killer factors or other microbial-produced toxins, must cation imbalances, temperature extremes, pesticide and fungicide residues, microbial competition and poor oenological practices (reviewed by Ingledew and Kunkee, 1985; Henschke, 1997; Bisson, 1999). These factors may slow down the metabolism of the yeast and consequently result in decreases in biomass production, cell viability and fermentation rate. Fermentation problems are complex, because they are rarely due to one factor in isolation, and rather the result of a combination of several factors. Furthermore, possible synergistic effects between these factors render problem fermentations a rather challenging subject to investigate (Alexandre and Charpentier, 1998).

This work aimed at overcoming previously encountered inadequacies (such as investigating only one or two variables simultaneously) by implementing a more holistic

approach. For this purpose a database was established to capture chemical composition and Fourier transform infrared (FT-IR) spectra of control and problem fermentations. Additional measurements included microbial enumeration, sulphur dioxide determination, free assimilable nitrogen (FAN) determination and volatile component determination by means of gas chromatography (GC). Samples were collected from participating commercial wineries during the 2005 and 2006 harvest seasons. A multivariate data matrix described by the above-mentioned variables was constructed for the purpose of multivariate data analysis. Multivariate chemometric techniques, such as principal component analysis (PCA) and partial least squares (PLS) regression, were implemented in this study. PCA was used to identify the main sources of variability amongst the samples and to identify potential relationships or similarities between the samples. FT-IR spectroscopy was evaluated as a rapid, high throughput analytical method to monitor fermentations effectively. Consequently, the possibility of discrimination between control and problem fermentations, using FT-IR spectroscopy, was investigated. Discriminant partial least squares (PLS) regression models were constructed for this purpose. Several subsets of samples which include different stages of fermentation and different cultivar subsets were used for data modelling purposes. The data show that multi-instrument and multivariate data analysis provides an alternative approach to investigate and characterise possible causes of problem fermentations under industrial conditions.

3.2 MATERIALS AND METHODS

3.2.1 ESTABLISHMENT OF CONTACT WITH INDUSTRY

Questionnaires (see addendum A) were distributed throughout the South African wine industry by means of email, the South African Society for Enology and Viticulture (SASEV pre-harvest workshop and website www.sasev.co.za) and personal contact. A promotional article was published (see addendum B) in the January 2006 edition of the Wineland magazine in an attempt to encourage participation of commercial wine cellars in this project. This study relied completely on the involvement of winemakers from industry for the collection of control and problem fermentation samples. A total of 400 questionnaires were distributed and of the original contact group, 55 winemakers agreed to participate in this project. This represents a 14% return of questionnaires. Samples were however only collected from 36 cellars during the two harvest seasons and this represent a 9% return. This is in agreement with a previous study (Sheridan, 2003) which required involvement from industry. During the 2005 season problem fermentation samples were collected from 22 wineries and during the 2006 season 14 cellars provided control and problem fermentation samples for the study.

Seasonal differences, in terms of grape quality, had an effect on the occurrence of problem fermentations. As a result, participation from commercial wineries varied between the 2005 and 2006 harvest seasons. Although cellar and fermentation management is crucial and time-consuming during harvest, it remains a concerning fact that participation and interest from the winemakers appears limited, even with an applied project investigating a relevant problem to the wine industry.

3.2.2 SAMPLE COLLECTION AND STORAGE

Upon notification by winemakers, samples (500 mL quantities) were collected from large scale fermentation tanks in various South African commercial wineries. The samples were collected from the specific problem fermentation tank after rinsing the plastic sampling bottle with the wine. The sample tap of these commercial fermentation tanks (12 000L, 25 000L, 33 000L) were situated either a third from the bottom on the side of the tank or at the top of the tank in the case of 100 000 L tanks. These sampling conditions are not optimal in terms of the Theory of Sampling (Pitard, 1993; Gy, 1998; Smith, 2001; Petersen *et al.*, 2005). Ideally, samples should be taken incrementally in a one dimensional space with an upward flow of material. Although such sampling possibilities presents itself with pump-over of fermenting must from one fermentation vessel to another, these pipes do not always possess the necessary sampling valve. Secondly, sampling was not always performed during these pump-over sessions. Therefore, since this was an industrial study, sampling was performed in the traditional manner (at the sampling tap) with the awareness of the limitation of this sampling method.

A total of 64 problem fermentation samples were collected during the 2005 vintage. During the 2006 vintage a total of 315 samples were collected of which 286 were control and 29 were problem fermentation samples. The cultivar distribution of all the samples (379) collected was as follows: Cabernet Franc (4), Cabernet Sauvignon (24), Chardonnay (38), Chenin blanc (38), Colombard (6), Malbec (7), Merlot (56), Muscat varieties (6), Nouvelle (3), Petit Verdot (4), Pinot blanc (2), Pinot noir (9), Pinotage (25), Rosè (10), Sauvignon blanc (61), Semillon (7), Shiraz (51), Viognier (12), Weisser Riesling (2), Gewürztraminer (8), Gamay noir (1), Sangiovese (1), Barbara (1), Mouverdre (1), Cabernet Sauvignon/Merlot (1) and one unknown white cultivar.

Samples were transported to the laboratory in temperature controlled conditions and analysis performed immediately after the appropriate sample preparation. Following the initial microbial, free assimilable nitrogen (FAN), FT-IR spectroscopy and sulphur dioxide (SO₂) analysis, 45 mL of the remaining must sample was stored at -20°C for GC analysis.

3.2.3 SAMPLE PREPARATION FOR FOURIER TRANSFORM INFRARED SPECTROSCOPY

Effective degassing of actively fermenting must samples prior to the acquisition of spectra obtained with the Winescan FT120 instrument was evaluated. The calibrations of this apparatus, associated with the quantification of various important wine parameters, are extremely sensitive to high levels of carbon dioxide (CO₂) and therefore sample preparation by means of filtration, sonication, centrifugation and vacuum was investigated. The effect of pimaricin (Vin-O-Cid, Wintrust, Stellenbosch, S.A.) addition was also investigated. This substance does not eliminate CO₂ present in the must, but it inhibits yeast growth which prevents additional CO₂ formation in the fermenting must samples. The CO₂ levels were quantified (in mg/L) by using the Winescan FT120 instrument equipped with global calibrations. The target concentration of CO₂ prior to scanning was below 300 mg/L.

Must samples were filtered using a filtration unit (type 79500, FOSS Electric, Denmark) connected to a vacuum pump and filter paper circles graded at 20 – 25 µm with a diameter of 185 mm (Schleicher & Schnell, reference number 10312714). The effects of successive filtration (one filtration, two filtrations and three filtrations) on the CO₂ levels were investigated.

Sonication in an ultrasonic bath for 5 min, 15 min and 25 min respectively was tested to evaluate the effect on the CO₂ levels. Samples were filtered once prior to sonication in order to remove solid particles from the must.

Must samples were supplemented with pimaricin (a spatula tip equal to 0,1 g), mixed and 15 min reaction time was allowed. The effect of the pimaricin combined with different treatments was investigated. The following combinations were tested: pimaricin addition followed by one filtration, pimaricin addition followed by one filtration and 10 min sonication and pimaricin addition followed by centrifugation at 8000 rpm for 10 min. These results were compared with the above mentioned treatments without the pimaricin addition to evaluate the effect of pimaricin on the CO₂ levels of the must.

Replacing filtration with centrifugation of must samples was also investigated as a means to remove the solid particles from must and to evaluate the effect of centrifuging on CO₂ levels. Centrifugation for 10 min at 5000 rpm was tested. This was followed by an increase in rotation speed (10 min at 8000 rpm) and finally an increase in centrifugation time (15 min at 8000 rpm).

The effect of vacuum on the CO₂ levels was tested in combination with filtration and centrifugation respectively. Samples were centrifuged at 8000 rpm for 10 min to remove the solid particles, followed by vacuum for 1 min and 2 min respectively. In order to evaluate the effect of filtration combined with vacuum and to evaluate the use of two different vacuum systems, samples were filtered once, followed by vacuum until no CO₂ was visible. The

vacuum systems tested were an Erlenmeyer with a side arm and a modified Falcon tube, both connected separately to a vacuum pump.

3.2.4 FOURIER TRANSFORM INFRARED SPECTRAL MEASUREMENTS

FT-IR spectra were generated by using a Winescan FT120 instrument (software version 2.2.1) equipped with a purpose built Michelson interferometer (FOSS Electric A/S, Hillerød, Denmark). Most samples were centrifuged and degassed by vacuum prior to analysis. Duplicate spectra were acquired in the spectral range 4992.25 to 929.778 cm^{-1} for each sample and the spectra averaged for data processing. Samples are pumped through the heat exchanger and the CaF_2 -lined cuvette. Each spectrum is based on an average of 20 repeat scans at 4 cm^{-1} intervals within the wavenumber range 4992.25 to 929.778 cm^{-1} under fixed instrument settings as described (**Table 3.1**). (Winescan FT120 Type 77110 and 77310 Reference manual, FOSS Electric, Denmark, 2001).

Table 3.1 Winescan FT120 instrument settings.

| Instrument settings | |
|---------------------|------------------|
| cell path length | 37 μm |
| sample temperature | 40°C |
| sample volume | 7-8 mL |

The Winescan instrument generates an interferogram (calculated from a total of 20 scans and generated by the software of the instrument) based on the recorded frequencies of infrared radiation transmitted by the sample. Fourier transformation converts the interferogram into a single-beam transmittance spectrum. In order to correct for the background absorbance of water, a zero setting using FOSS Zero liquid S-6060 is done prior to the sample and a zero-beam spectrum is thus obtained. The sample single-beam spectrum is divided by the zero-beam spectrum to give a transmittance spectrum which is converted into a linearized absorbance spectrum by a series of mathematical procedures (Winescan FT120 Type 77110 and 77310 Reference manual, FOSS Electric, Denmark, 2001).

3.2.5 QUANTIFICATION OF CHEMICAL COMPONENTS BY FOURIER TRANSFORM INFRARED SPECTROSCOPY

Quantified chemical data were obtained from the infrared spectra to indicate the distribution of important wine components (**Table 3.2**). These components are important to monitor fermentation progress and the possible formation of undesired compounds (acetic acid

resulting in volatile acidity). The conversion of the linearized absorbance spectra into quantifiable results was achieved with the commercially available calibrations referred to as global calibrations (provided with the instrument). Winescan FT120 2001 version 2.2.1 software was used. These global calibrations are constructed on the basis of a partial least squares (PLS) regression. Absorptions at selected frequencies (cm^{-1}) or groups of frequencies are used to generate a regression algorithm which best fit the reference values in the data set (Patz *et al.*, 2004). A selection of the global calibrations was validated and calibrations established for additional wine parameters (discussed in Chapter 6).

Table 3.2 Chemical components obtained from FT-IR spectra.

| Chemical analysis | |
|---------------------|-----------------------|
| pH | ethanol |
| total acidity (TA) | acetic acid |
| malic acid | volatile acidity (VA) |
| glycerol | |
| residual sugar (RS) | |

3.2.6 MICROBIAL ENUMERATION

Microbial populations for yeast, lactic acid bacteria, acetic acid bacteria and *Brettanomyces* were determined by plating 100 μL of a dilution series (made in sterile water) of must on selective media. Samples were microscopically evaluated prior to plating. Plates were incubated at 30°C for 4 to 14 days (depending on the microorganism) and colonies were counted and colony forming units per mL (cfu/mL) were determined.

3.2.6.1 Yeast

Yeast counts were determined using YPD agar (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) (Biolab, Merck, Wadeville, Gauteng). Lactic acid bacteria (LAB) and acetic acid bacteria (AAB) growth was inhibited by the addition of respectively streptomycin sulfate (Sigma, USA) and kanamycin sulfate (Roche, Germany) at 25 mg/L (25 mg/L dissolved in water and 1 mL added to 1 L medium). YPD plates were incubated for 3 days at 30°C.

3.2.6.2 Lactic acid bacteria

MRS agar (Biolab, Merck, Wadeville, Gauteng) was used for the enumeration of lactic acid bacteria (*Lactobacillus*, *Pediococcus* and *Leuconostoc*). Plates were incubated at 30°C for 5 to 7 days. Actistab (Gist-Brocades, France) (100 mg/L dissolved in methanol and 1 mL

added to 1 L medium) was added for the elimination of yeast growth and kanamycin sulfate (25 mg/L) was added to the medium as an inhibitor for acetic acid bacteria.

3.2.6.3 Acetic acid bacteria

Acetic acid bacteria were isolated and enumerated on GYC agar (5% w/v glucose, 1% w/v yeast extract, 3% CaCO₃ and 2% w/v agar) (Biolab, Merck, Wadeville, Gauteng) and on MRS agar (Biolab, Merck, Wadeville, Gauteng) supplemented with 2% ethanol. To both media Actistab (Gist-Brocades, France) (100 mg/L) and streptomycin (25 mg/L) was added to eliminate yeast and lactic acid bacteria growth. Plates were incubated at 30°C for 5 to 7 days.

3.2.6.4 *Brettanomyces*

DBDM agar (0.67% w/v yeast nitrogen base (YNB), 6% v/v ethanol, 0.01% w/v *p*-coumaric acid, 0.0022% w/v bromocresol green, 0.005% w/v cycloheximide, 2.5% agar, pH adjusted to 5.4) was used for the isolation of *Brettanomyces* spp. Plates were incubated for 11 to 14 days at 30°C. Lactic acid bacteria growth was eliminated by the addition of streptomycin (25 mg/L) to the medium and kanamycin (25 mg/L) was added as an inhibitor for acetic acid bacteria.

3.2.7 SULPHUR DIOXIDE DETERMINATION

The free and total sulphur dioxide (SO₂) levels of the samples were determined using Ripper's method (Methods of analysis for wine laboratories, compiled by the South African Wine Laboratories Association). A Metrohm Titrino apparatus (702 SM Titrino, Swiss lab) equipped with a 722 stirrer (Swiss lab) was used for this analysis.

3.2.8 FREE ASSIMILABLE NITROGEN DETERMINATION

The formol titration method was used to estimate the assimilable nitrogen concentration in the must samples. The sample is first neutralized with 1N NaOH to pH 8.5. An excess of neutralised formaldehyde (pH 8.5) is added followed by the re-titration of the solution to the endpoint. The formaldehyde reacts with the free amino groups of the alpha-amino acids (FAN). The ammonia is also titrated. Both the NH₄⁺ and FAN concentrations were determined with this method (Zoecklein *et al.*, 1995).

3.2.9 ANALYSIS OF VOLATILE FLAVOUR COMPOUNDS BY GAS CHROMATOGRAPHY

Analysis of volatile higher alcohols, esters and carbonyl compounds was performed in triplicate with a Hewlett Packard 6890 Plus gas chromatograph (Little Falls, USA) equipped with a split/splitless injector and an FID detector. A J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length × 0.32 mm inside

diameter \times 0.5 μm film thickness, was used for separation. All samples were centrifuged for 3 minutes at 4000 rpm to remove solid particles in the must. The extracts for injection into the GC were then prepared by extracting 5 mL of wine with 1 mL of diethyl ether (99.5%, Merck) after the addition of 10 mg/L internal standard (4-methyl-2-pentanol) (Fluka, $\geq 97\%$). This was followed by sonication for 5 minutes to facilitate mixing of the diethyl ether layer and the wine and centrifugation for 3 minutes at 4000 rpm to separate the diethyl ether layer from the wine. The diethyl ether layer was removed from the wine and dried on anhydrous sodium sulphate (Na_2SO_4) (Merck, 99%). The dried diethyl ether extract was transferred to a vial insert and capped. The instrument parameters used for analysis are found in **Table 3.3**. The carrier gas was hydrogen at a flow rate of 30 mL/min. Subsequently, the flavour compounds (listed in **Table 3.4**) were calculated by comparing their retention times and areas with those from calibration standard curves on a data handling system (HP GC Chemstation, Revision A.07.01 [682]). The large standard deviations indicate the amount of variation present in this sample set. Additional gas chromatography-mass spectroscopy (GC-MS) analysis was performed on a selection of the samples and these were not included in the data analysis of this chapter. The GC-MS data will be discussed in Chapter 4.

Table 3.3 GC instrumental parameters

| Oven Program | |
|-----------------------|-----------------------------------|
| initial temperature | 33°C for 17 min |
| ramp | 12°C/min to 240°C, hold for 5 min |
| Front inlet | |
| injection volume | 3 μL |
| mode | Split |
| split ratio | 15:1 |
| split flow | 49.5 mL/min |
| injection temperature | 200°C |
| initial pressure | 84.5 kPa |
| flow mode | Constant flow |
| column flow | 3.3 mL/min |
| Detector | |
| temperature | 250°C |
| H ₂ flow | 30 mL/min |
| air flow | 350 mL/min |
| make up flow | N ₂ 30 mL/min |

Table 3.4 Range of volatile components determined by GC-FID.

| Volatile components | Number | Range (mg/L) | mean | SD |
|-------------------------------------|---------------|---------------------|-------------|-----------|
| Esters | | | | |
| ethyl decanoate | 299 | 0.10 – 6.2 | 0.75 | 0.78 |
| ethyl hexanoate | 350 | 0.11 – 3.14 | 1.09 | 0.59 |
| ethyl butyrate | 191 | 0.1008 – 0.88 | 0.409 | 0.139 |
| ethyl octanoate | 352 | 0.13 – 6.44 | 1.56 | 1.28 |
| ethyl lactate | 105 | 0.12 - 222 | 22.6 | 38.5 |
| Alcohols and higher alcohols | | | | |
| n-hexanol | 375 | 0.22 – 3.46 | 1.0 | 0.46 |
| n-butanol | 273 | 0.23 – 4.24 | 1.41 | 0.66 |
| methanol | 318 | 10.74 – 470 | 128.0 | 101.81 |
| 2-phenylethanol | 373 | 0.18 - 199 | 29.1 | 32.5 |
| n-propanol | 358 | 2.25 – 281.03 | 40.86 | 33.16 |
| isobutanol | 361 | 0.13 – 133.4 | 11.18 | 20.87 |
| isoamyl alcohol | 373 | 0.56 – 752.0 | 200.6 | 133.8 |
| Acetates | | | | |
| ethyl acetate | 366 | 0.12 - 243.20 | 67.91 | 39.68 |
| isoamyl acetate | 353 | 0.11 – 18.32 | 3.85 | 3.49 |
| hexyl acetate | 197 | 0.10 – 1.38 | 0.56 | 0.21 |
| 2-phenylethyl acetate | 305 | 0.10 – 2.3 | 0.41 | 0.27 |
| diethyl succinate | 115 | 0.10 – 11.9 | 1.5 | 2.3 |
| Acids and fatty acids | | | | |
| acetic acid | 374 | 21.3 -1209 | 336.7 | 214.3 |
| propionic acid | 293 | 1.32 – 75.7 | 15.5 | 11.8 |
| isobutyric acid | 356 | 0.2 – 6.1 | 1.6 | 0.88 |
| butyric acid | 341 | 0.17 – 2.6 | 1.1 | 0.40 |
| isovaleric acid | 359 | 0.16 – 5.2 | 1.3 | 0.9 |
| valeric acid | 29 | 0.10 – 0.44 | 0.20 | 0.10 |
| hexanoic acid | 372 | 0.22 – 7.27 | 2.6 | 1.5 |
| octanoic acid | 366 | 0.27 – 7.9 | 2.6 | 1.7 |
| decanoic acid | 369 | 0.11 – 3.7 | 1.2 | 0.76 |

3.2.10 CHEMOMETRICS AND DATA ANALYSIS

3.2.10.1 Univariate analysis

Specific compositional elements of the samples were characterized. The concentration range of chemical components present in the wine matrix, the mean and standard deviation were determined.

3.2.10.2 Multivariate analysis

3.2.10.2.1 Data processing

Data obtained (FT-MIR, GC, microbial counts and chemical composition) during the 2005 and 2006 harvest seasons were imported into *The Unscrambler* software (version 9.2, CAMO ASA, Norway) for the purpose of chemometric analysis. A matrix was constructed with rows representing wine samples (objects) and variables in the columns corresponding to spectra and chemical compounds. Data were pretreated by autoscaling in order to avoid the differences in measurement units. Autoscaling is a widely used scaling technique and the result is a variable with zero mean and a unit standard deviation (Kowalski and Bender, 1972). The whole data matrix comprised 379 objects, 1054 spectral variables (mid-infrared wavenumbers), 26 GC chemical components, 17 non-spectral chemical variables and 6 category variables.

3.2.10.2.2 Principal component analysis (PCA)

Principal component analysis (PCA) was used to extract information from multivariate data (Kettanah *et al.*, 2005) and summarize the data (Wold *et al.*, 1987; Wold, 1982). PCA is a multivariate technique that reduces the dimensionality of the original data matrix containing numerous variables to a more visually understandable model while retaining the maximum amount of variability. This allows for the main sources of variation in the data set to be identified. This mathematical procedure resolves sets of data into orthogonal components of which linear combinations approximate the original data to any desired degree of accuracy (Adams, 1995; Otto, 1999; Martens and Martens, 2001; Naes *et al.*, 2002). Complex relationships between samples were investigated and expressed with the use of these models (Kettanah *et al.*, 2005). These models therefore allow for the identification of possible groupings of samples (Mardia *et al.*, 1979) with similarities and relating them to specific variables or groups of variables (Wold *et al.*, 1982, 2001; Martens and Naes, 1989; Tenenhaus, 1998; Wold and Josefson, 2000). The data structure was explored with this technique and possible outliers detected.

3.2.10.2.3 Discriminant partial least squares regression (DPLS or PLS-Discrim)

Discriminant analysis was performed by developing DPLS (PLS-Discrim) models using a no metric dummy Y-variable as a reference value. The dummy variable is an arbitrary number for a sample which belongs to a particular group. Each sample is assigned a dummy variable (signified by -1 for stuck/sluggish samples and +1 for control fermentation samples) to test the ability of the method to discriminate between control and problem fermentation samples. The DPLS model is then developed by regression of the spectral and other additional data (X-variables/matrix) against the assigned reference value (dummy variable). This approach is referred to as a discriminant PLS (DPLS or PLS-Discrim) (Osborne *et al.*, 1993; Ding *et al.*, 1999; Naes *et al.*, 2002) and has wide application (Cozzolino *et al.*, 2002, 2003).

3.2.10.2.4 General strategy for data analysis

As a result of the complexity of the data matrix, a multivariate strategy was devised for data exploration purposes (K.H. Esbensen, Aalborg University, Esbjerg, Denmark, personal communication, 2006). Samples were classified into three stages of fermentation in order to make the analysis more meaningful:

Stage 1: early fermentation (<4% v/v alcohol);

Stage 2: middle fermentation (>4% v/v alcohol, >20 g/L residual sugar);

Stage 3: late fermentation (<20 g/L residual sugar).

Delta (Δ) depicts the difference between fermentation stages of the samples (**Table 3.5**).

Table 3.5 Explanation of delta (Δ) values.

| Delta (Δ) | Description |
|--------------------|--|
| $\Delta = 0$ | samples are in the same stage of fermentation |
| $\Delta = 1$ | samples differ from each other with one fermentation stage |
| $\Delta = 2$ | samples differ from each other with more than one fermentation stage |

The following strategy was implemented for the exploration of the whole data matrix (data matrix shown in **Fig. 3.1**). Firstly, PCA modelling was performed on matrix A (**Fig. 3.2**). This was done in order to gain insight into the dominant structure of the data and to examine the possible grouping and relationships amongst the problem fermentation samples with respect to different chemical compounds (obtained from infrared spectroscopy and gas chromatographic analysis), microbial data as well as FT-IR spectra. GC-MS data was also obtained for a subset of samples and will therefore be discussed separately in Chapter 4.

| 6 ID | MIR FOSS | Chem FOSS | GC-FID | Microbial | GC-MS | SO ₂ |
|---|----------|-----------|--------|-----------|-------|-----------------|
| 2005 Problem fermentation samples | | | | | | A |
| 2006 Problem fermentation samples | | | | | | |
| 2006 Control fermentation samples $\Delta = 0$ | | | | | | |
| 2006 Control fermentation samples $\Delta = 1$ | | | | | | |
| 2006 Control fermentation samples $\Delta = 2$ | | | | | | |

Figure 3.1 A visual display of the data matrix. Matrix A consist of problem fermentations collected during 2005 and the analysis performed. Matrix B consist of problem and control fermentations collected during the 2005 and 2006 seasons. Delta (Δ) describes the difference in stage between the control samples and the problem samples collected. ($\Delta = 0$: control samples and problem samples in the same stage of fermentation; $\Delta = 1$: control samples and problem samples differ with 1 stage; $\Delta = 2$: control samples and problem samples differ with 2 stages)

| 6 ID | MIR FOSS | Chem FOSS | GC-FID | Microbial | GC-MS | SO ₂ |
|-----------------------------------|----------|-----------|--------|-----------|-------|-----------------|
| 2005 Problem fermentation samples | | | | | | A |
| 2006 Problem fermentation samples | | | | | | |
| 2006 Control fermentation samples | | | | | | |

Figure 3.2 PCA was performed on Matrix A to investigate and visualise the dominant structure of the problem fermentations in term of chemical composition and FT-IR spectroscopy.

Secondly, the same approach of PCA modelling was implemented to visualise the structure of the data in terms of differences or comparisons, possible patterns and groupings amongst problem and control fermentation samples in matrix B (**Fig. 3.3**).

| 6 ID | MIR FOSS | Chem FOSS | GC-FID | Microbial | GC-MS | SO ₂ |
|---|----------|-----------|--------|-----------|-------|-----------------|
| 2005 Problem fermentation samples | | | | | | |
| 2006 Problem fermentation samples | | | | | | |
| 2006 Control fermentation samples $\Delta = 0$ | | | | | | |
| 2006 Control fermentation samples $\Delta = 1$ | | | | | | |
| 2006 Control fermentation samples $\Delta = 2$ | | | | | | |

B

Figure 3.3 PCA was performed on Matrix B to evaluate the structure of the data with regards to comparisons and differences between problem and control fermentations. PLS-discriminant regression was also performed on Matrix B including all samples from all stages of fermentation ($\Delta = 0,1, 2$).

| 6 ID | MIR FOSS | Chem FOSS | GC-FID | Microbial | GC-MS | SO ₂ |
|---|----------|-----------|--------|-----------|-------|-----------------|
| 2005 Problem fermentation samples | | | | | | |
| 2006 Problem fermentation samples | | | | | | |
| 2006 Control fermentation samples $\Delta = 0$ | | | | | | |
| 2006 Control fermentation samples $\Delta = 1$ | | | | | | |
| | | | | | | |

B

Figure 3.4 PLS discriminant regression applied to Matrix B ($\Delta = 0,1$) to evaluate the discrimination possibilities between control and problem fermentations excluding samples differing with two fermentation stages.

Thirdly, PLS discriminant regression was applied on matrix B (**Fig. 3.3**) for all three cases of delta ($\Delta = 0,1,2$). All samples in all stages of fermentation were used for this initial analysis. The information in the Y-variable (dummy variable inserted to represent stuck/control) is used

to find the Y-relevant structure in the X-data (chemical and spectral data) used for discrimination between stuck and sluggish.

The PLS discriminant procedure was repeated for matrix B ($\Delta = 0,1$) excluding samples which differed with two fermentation stages ($\Delta = 2$) (**Fig. 3.4**). Two possible combinations of samples with this criterion were investigated: samples in early and middle fermentation and samples in middle and late fermentation.

Additional PLS discriminant models were constructed using matrix B ($\Delta = 0$) with samples in the same stage of fermentations (**Fig. 3.5**). Three different models were constructed for samples in early, middle and late stages of fermentation in an attempt to discriminate between control and problem fermentations. Further classification modelling by PLS discriminant regression was attempted for smaller subsets of data, with regard to different cultivars, in order to find a PLS model which could discriminate between control and problem fermentations (**Fig. 3.6**). Red and white cultivars were modelled separately, followed by additional modelling of the most abundant individual red (Merlot, Shiraz, Pinotage, Cabernet Sauvignon, Pinot noir) and white (Sauvignon blanc, Chardonnay, Viognier) cultivars collected.

| 6 ID | MIR FOSS | Chem FOSS | GC-FID | Microbial | GC-MS | SO ₂ |
|---|----------|-----------|--------|-----------|-------|-----------------|
| 2005 Problem fermentation samples | | | | | | |
| 2006 Problem fermentation samples | | | | | | |
| 2006 Control fermentation samples $\Delta = 0$ | | | | | | |
| | | | | | | |
| | | | | | | |

Figure 3.5 PLS discriminant regression applied to Matrix B ($\Delta = 0$) to evaluate the discrimination possibilities between control and problem fermentations using samples in the same stages of fermentation.

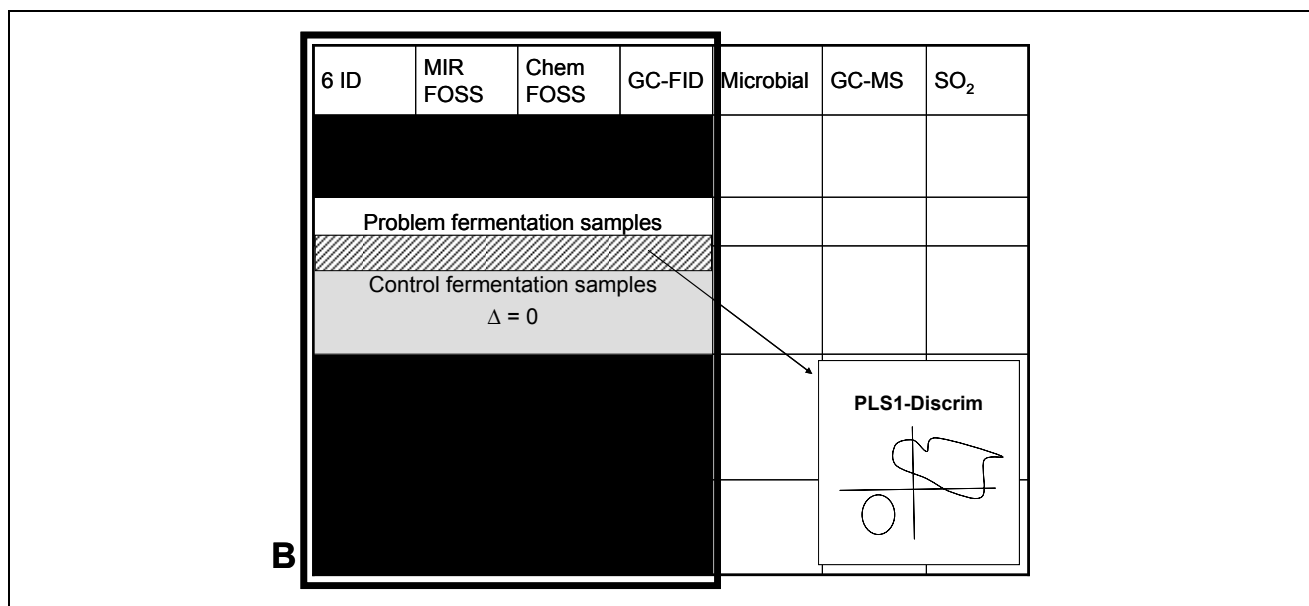


Figure 3.6 PLS discriminant regression models constructed on subsets of Matrix B ($\Delta = 0$) to find a suitable model to discriminate between control and problem fermentations within a specific cultivar.

3.3 RESULTS AND DISCUSSION

3.3.1 EFFECTIVE DEGASSING PRIOR TO FT-IR ANALYSIS

A fast and effective method for sample preparation prior to spectral analysis was needed due to the sensitivity of the Winescan FT120 instrument's calibrations to carbon dioxide (CO₂) levels and several methods were investigated. These were: filtration, centrifugation, sonication, pimaricin addition and vacuum filtration. **Table 3.6** explains the acronyms for the sample treatments used in this experiment.

Filtration of actively fermenting must demonstrated potential for reducing the CO₂ levels in the must. Initial filtration was necessary to eliminate possible particles in the must from entering the spectroscopic instrument. After one filtration 1102 mg/L CO₂ was detected. Two filtrations of the same must decreased the CO₂ level to 596 mg/L and three successive filtrations reduced CO₂ levels even further to 435 mg/L (**Fig. 3.7A**). Although this method of degassing showed potential, it is rather time consuming and repeated filtration could influence the chemical composition of the must samples.

Increased length of sonication (5 min, 15 min, 25 min) resulted in CO₂ levels being elevated from 976 mg/L to 1218 mg/L (**Fig. 3.7B**).

The addition of pimaricin decreased the CO₂ levels compared to the control treatments without pimaricin addition (**Fig. 3.7C**). An undesired 1 g/L increase in sugar concentration was observed with the addition of pimaricin since this substance has 50% sugar content.

Both the increase in speed of centrifugation, from 5000 rpm to 8000 rpm, and the increase in length of centrifugation from 10 min to 15 min resulted in an increase in the CO₂ levels of the must (**Fig. 3.7D**).

The combination of centrifugation and vacuum decreased the CO₂ levels from the original 1102 mg/L (with only one filtration) to 256 mg/L after 1 min of vacuum and an additional reduction to 230 mg/L after 2 min (**Fig. 3.7E**). The centrifugation step was replaced with filtration to remove solid particles from the must (procedure referred to as vacuum filtration) and tested in combination with two vacuum systems. The Falcon tube vacuum system decreased CO₂ levels from 1102 mg/L (control, filtered once) to 218 mg/L in 5 min. The Erlenmeyer system reduced CO₂ levels from 1102 mg/L (control, filtered once) to 170 mg/L in less than one minute (**Fig. 3.7F**). Vacuum filtration was the fastest and most effective sample preparation tested, therefore this was the sample preparation method adopted in the rest of the study.

Table 3.6 Acronyms used in the sample preparation experiment.

| Treatment | Description |
|-----------|---|
| F1 | one filtration |
| F2 | two successive filtrations |
| F3 | three successive filtrations |
| S5F1 | one filtration, 5 min sonication |
| S15F1 | one filtration, 15 min sonication |
| S25F1 | one filtration, 25 min sonication |
| P15F1 | pimaricin reaction time 15 min, one filtration |
| P15F1S10 | pimaricin reaction time 15 min, one filtration, 10 min sonication |
| P15C108 | pimaricin reaction time 15 min, 10 min centrifugation, 8000 rpm |
| C105 | 10 min centrifugation, 5000 rpm |
| C108 | 10 min centrifugation, 8000 rpm |
| C158 | 15 min centrifugation, 8000 rpm |
| C108V1 | 10 min centrifugation, 8000 rpm, 1 min vacuum |
| C108V2 | 10 min centrifugation, 8000 rpm, 2 min vacuum |
| F1V5F | one filtration, 5 min vacuum with Falcon tube system |
| F1V1E | one filtration, <1 min vacuum with Erlenmeyer system |

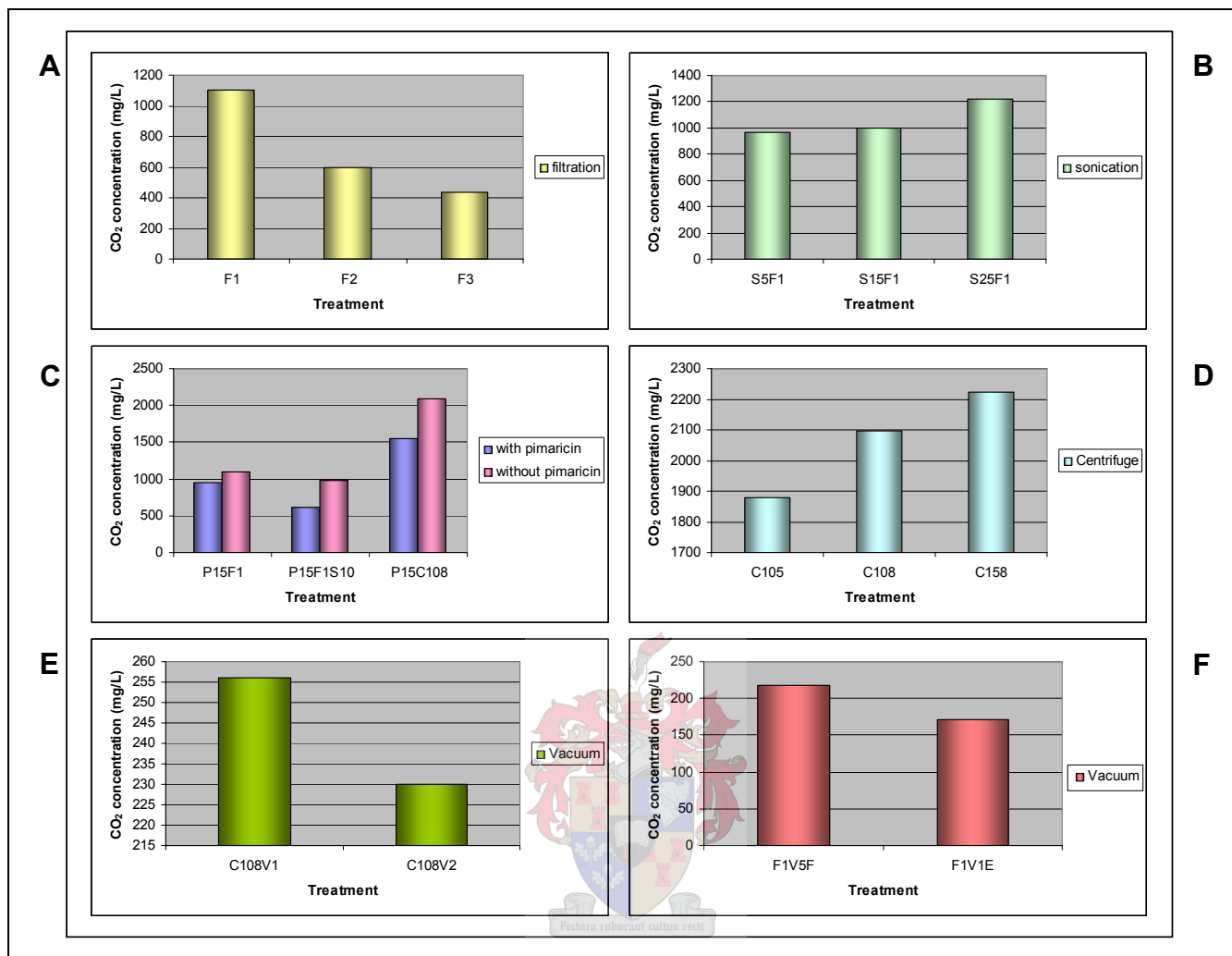


Figure 3.7 Sample preparation by means of (A) filtration, (B) sonication, (C) pimaricin addition, (D) centrifuging and (E & F) vacuum. Successive filtration reduced CO₂ levels significantly while centrifugation and sonication increased CO₂ levels. Pimaricin addition reduced CO₂ levels but a 1 g/L increase in sugar concentration was observed. Vacuum in combination with centrifugation (E) reduced CO₂ levels. Vacuum in combination with filtration (F) was the most effective and fastest method for sample preparation prior to FT-IR analysis.

3.3.2 FT-IR SPECTRAL FEATURES

Absorption bands in the mid infrared range can be detected due to molecular vibrations. FT-IR spectra provide the combined absorbance of all infrared active components present in the sample. This results in a unique fingerprint in the mid infrared spectral region (Smith, 1999). Wine consists of hundreds of chemical components. This complexity necessitates the use of an indirect measurement such as FT-IR spectroscopy to obtain a chemical fingerprint of a specific wine without quantifying each individual component. The spectra however, reflect the chemical composition of the wine. The most prominent features of the spectra are due to

the absorbance of water in the wavenumber regions, $1543 - 1716 \text{ cm}^{-1}$ and $2970 - 3626 \text{ cm}^{-1}$ (Winescan FT120 Type 77110 and 77310 Reference manual, FOSS Analytical, Denmark, 2001; Nieuwoudt *et al.*, 2004) (See **Fig. 3.8**). The “fingerprint region” ($929 - 1600 \text{ cm}^{-1}$) captures a significant amount of information related to the chemical composition of the samples (Smith, 1999).

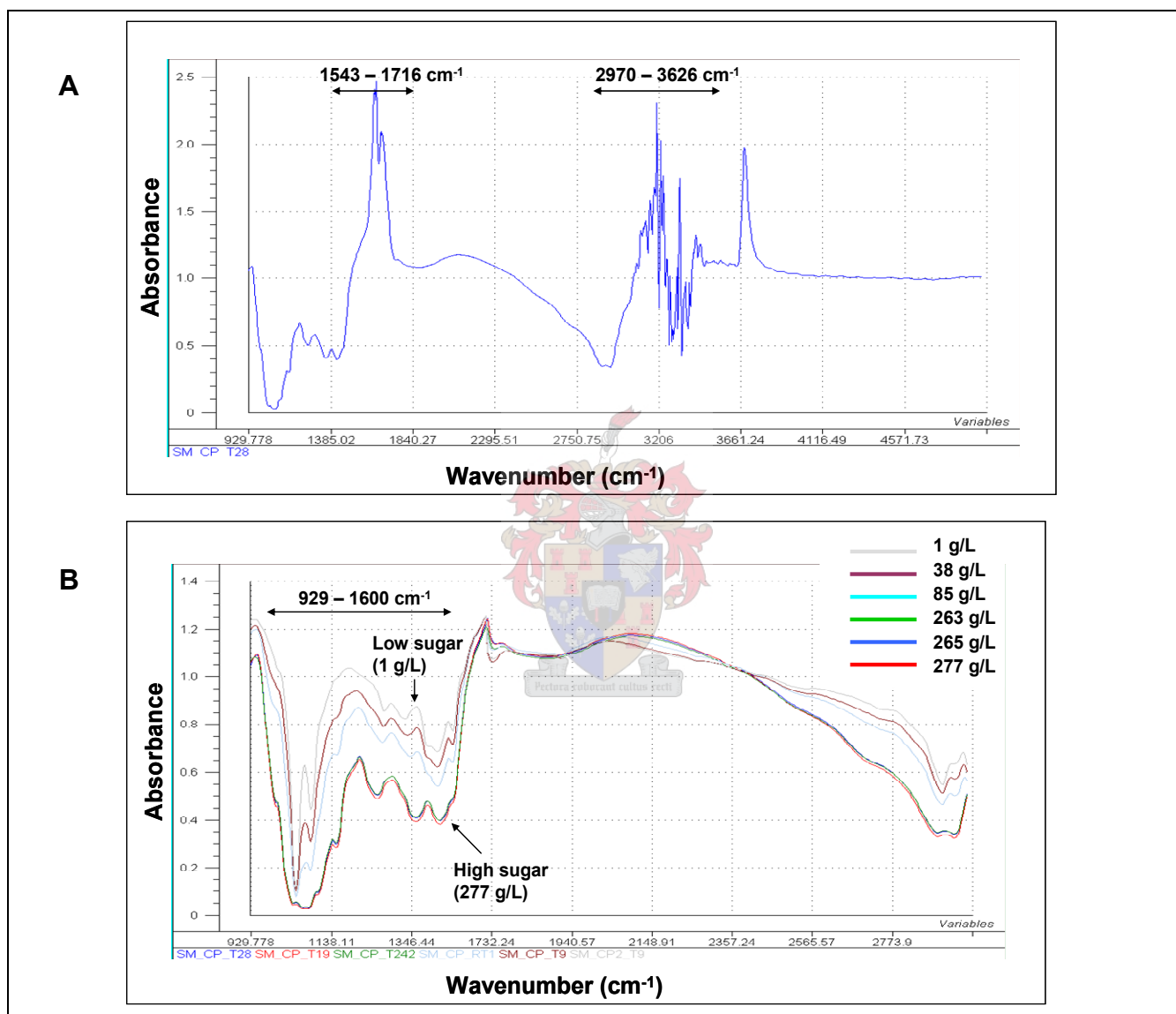


Figure 3.8 (A) FT-IR absorbance spectra of a Pinotage must in the region $929.778 - 4992.25 \text{ cm}^{-1}$. The two water absorbance regions, $1543 - 1716 \text{ cm}^{-1}$ and $2970 - 3626 \text{ cm}^{-1}$, are indicated. (B) Spectral variation between high sugar and low sugar must of Pinotage samples in the “fingerprint” region $929 - 1600 \text{ cm}^{-1}$. Samples are from different stages of fermentation. (red = 277 g/L, blue = 265 g/L, green = 263 g/L, light blue = 85 g/L, purple = 38 g/L, grey = 1 g/L residual sugar).

The FT-IR absorbance spectra of Pinotage must samples at different stages of fermentation progression were obtained. The spectra show distinct differences in absorption patterns especially in the “fingerprint” region of the spectrum (**Fig. 3.8B**). PCA analysis of these samples indicate that the variation in sugar concentration between the samples correlate with 84% of variance explained by PC1. This illustrates the potential of the “fingerprint” region for the extraction of compositional information and the possibility to use FT-IR technology to monitor fermentation progress. This is of special interest since a fast and reliable method to monitor the progress of fermentation is needed.

3.3.3 PCA MODELLING AS A DATA EXPLORATORY TOOL

Principal component analysis was used to summarize the information contained in the multivariate data set. The proposed stepwise multivariate strategy is discussed in section **3.2.10.2.4**. Firstly, PCA was performed on matrix A which included 64 problem fermentation samples from the 2005 season, all wavenumbers (excluding the water absorbance regions $1543 - 1716 \text{ cm}^{-1}$ and $2970 - 3625 \text{ cm}^{-1}$), microbial data, SO_2 determinations and volatile components (**Fig. 3.9**).

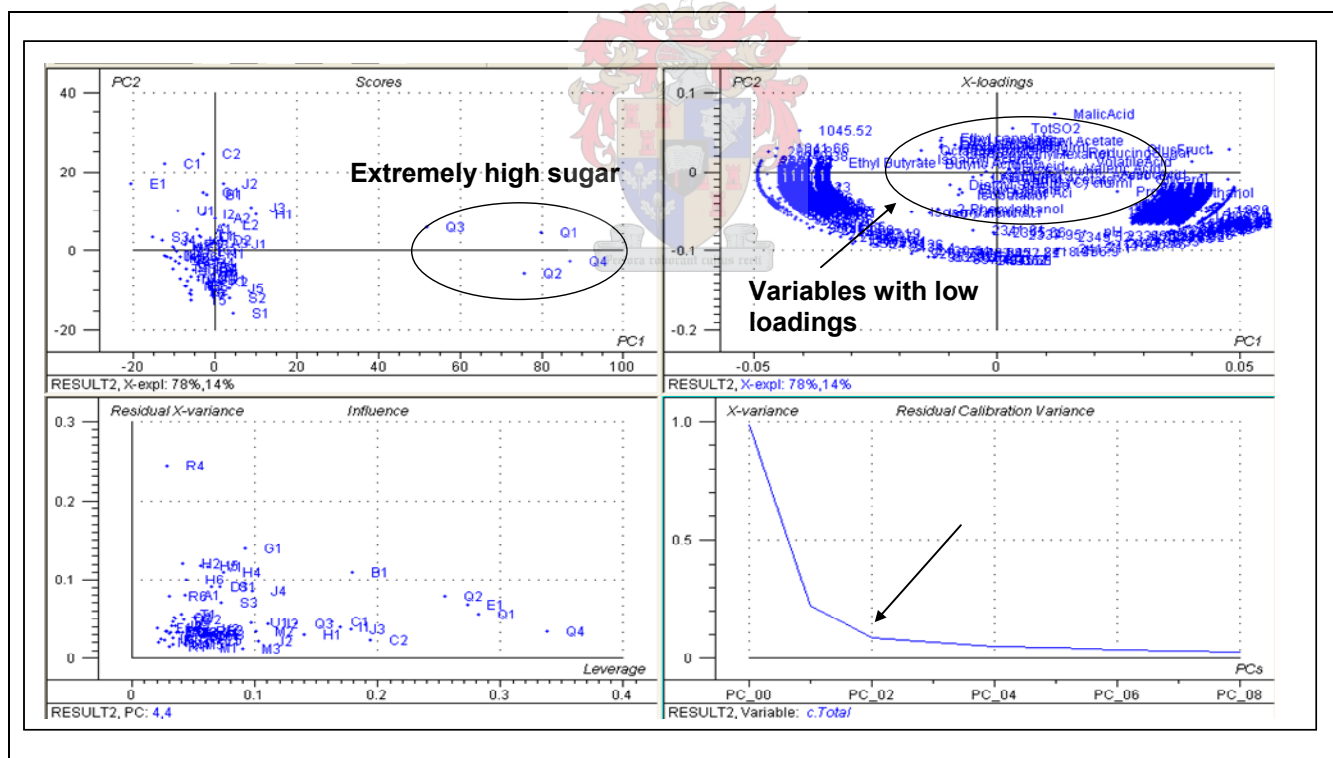


Figure 3.9 PCA score plot (PC1 vs PC2) of all the 2005 problem must samples (matrix A). PC1 explains 78% of variance between the samples. Samples with extremely high sugar concentrations were detected.

The four samples to the right of the score plot are extremely high in sugar and represent a different style of wine. Although the first two PCs explained 92% of the variance between the samples, these four samples dominated PC1 (78%) and for further analysis of this particular dataset they were excluded. On the loadings plot of PC1 versus PC2 it was clear that in comparison with the spectral data, the other measurements which included microbial data, SO₂ determinations and volatile components had insignificant loadings. This is not surprising since the FT-IR spectrum serves as a chemical “fingerprint” and could contain additional hidden information contributing to the separation of the samples along the principal component. Variables with higher loadings (positive or negative) generally indicate the important variables explaining the separation or distribution along a specific principal component.

PCA was performed on the remaining samples after the exclusion of the four “outliers” (**Fig. 3.10**). PC1 now explained 50% of the variance between the samples. On the loadings plot of PC1 it was once again clear that the spectral variables contained comprehensive information about the samples. From some of the quantified data it was evident that PC1 described the relationship between the sugar and ethanol content of the samples, which was expected. These two variables are negatively correlated with each other. Additional category variable information in the matrix made it possible to observe that alcoholic fermentation could be distinguished from malolactic fermentation. However, this might be the effect of different sugar levels present at these different stages of production. Red and white wine cultivars could also be distinguished from each other.

Supplementary PCA was performed (**Fig. 3.11**) on these samples using only the GC-FID data since several features concerning the data structure might have been lost due to the domination of the spectral variables. The four outlier samples mentioned above were excluded from the analysis. On the score plot separation along PC1 was associated with discrimination between red and white cultivars. High loadings for n-propanol, acetic acid, n-butanol, ethyl acetate, n-hexanol, diethyl succinate, isobutanol, isobutyric acid, isovaleric acid, 2-phenylethanol and isoamyl alcohol were correlated with the red cultivars. Separation along the first principal component also demonstrated high loadings for butyric acid, 2-phenylethyl acetate, isoamyl acetate, decanoic acid, hexanoic acid, ethyl octanoate, ethyl hexanoate, ethyl decanoate and octanoic acid which were highly correlated with the white cultivars. The variables with high loadings for the red cultivars were strongly negatively correlated with the variables which had high loadings for the white cultivars.

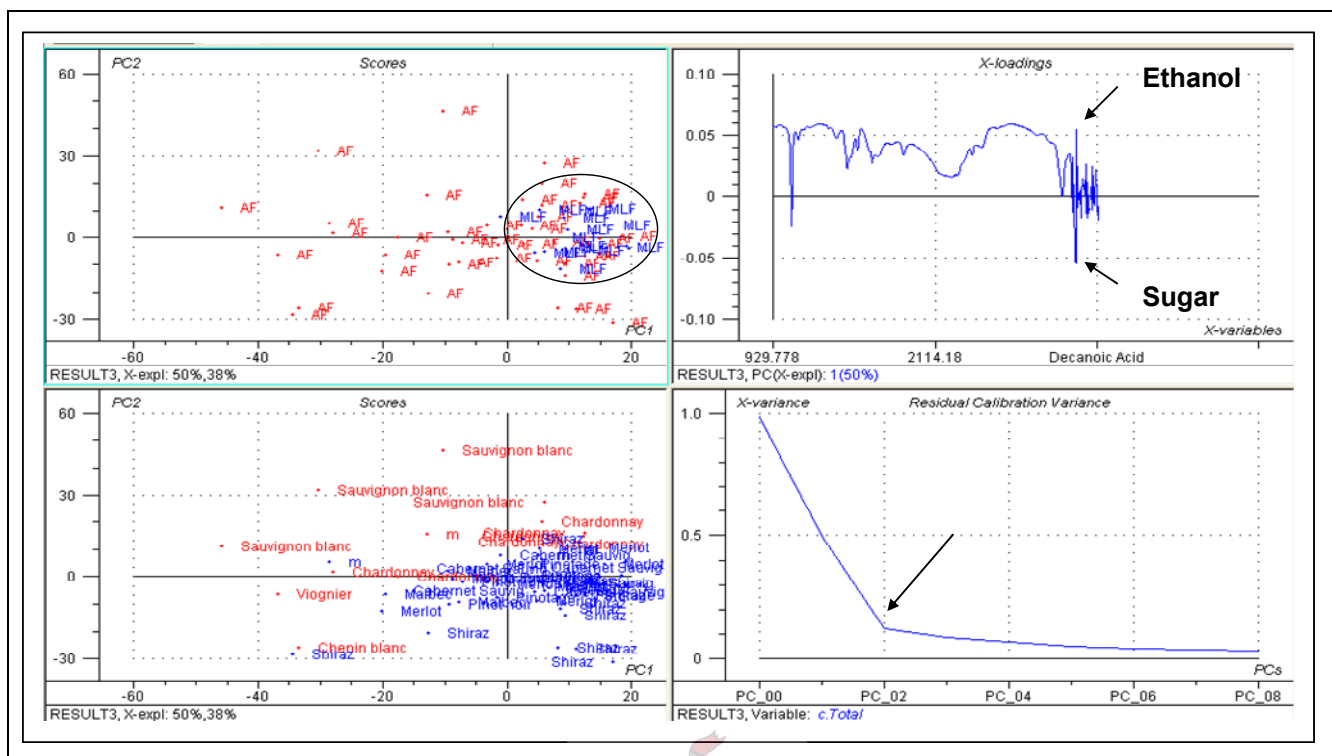


Figure 3.10 Score plot PC1 vs PC2 of all the 2005 problem must samples (matrix A) with the exclusion of the four “outliers”. PC1 explains 50% of variance between the samples. The PC1 loadings plot has high loadings for ethanol and sugar as well as a large portion of the wavenumbers. Discrimination between alcoholic (red AF) and malolactic (blue MLF) fermentation and red (indicated by blue cultivar names) and white (indicated by red cultivar names) cultivars was also observed.

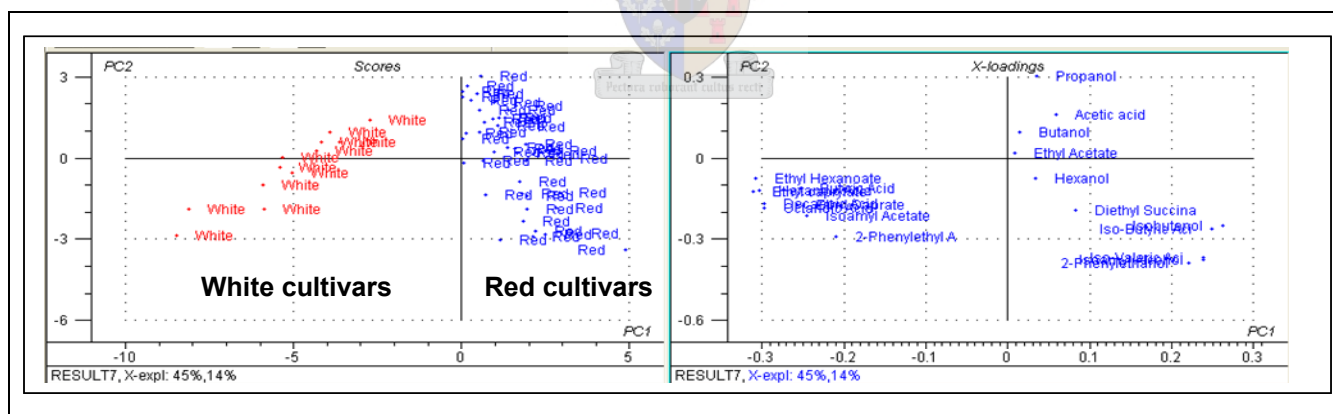


Figure 3.11 Score plot of PC1 vs PC2. PCA was performed on the volatile components only and discrimination between red and white cultivars was observed. PC1 explained 45% of the variance in the data structure.

PCA was done on the complete data matrix (matrix B) which included 379 must samples, volatile components and all wavenumbers excluding the water absorbance regions ($1543 - 1716 \text{ cm}^{-1}$ and $2970 - 3625 \text{ cm}^{-1}$) (Fig. 3.12). PC1 explains 61% of the variation between the samples. Category variables included in the data matrix demonstrated that this data set

contained samples from early fermentation (<4% alcohol) till late in fermentation (<20 g/L sugar). On the score plot of PC1 versus PC2 it is also clear that all the problem fermentations occurred from mid-fermentation onwards. This is in accordance with literature (Lambrechts, 2000; Bisson, 2005) which remarks that problem fermentations usually occur above 10% v/v ethanol.

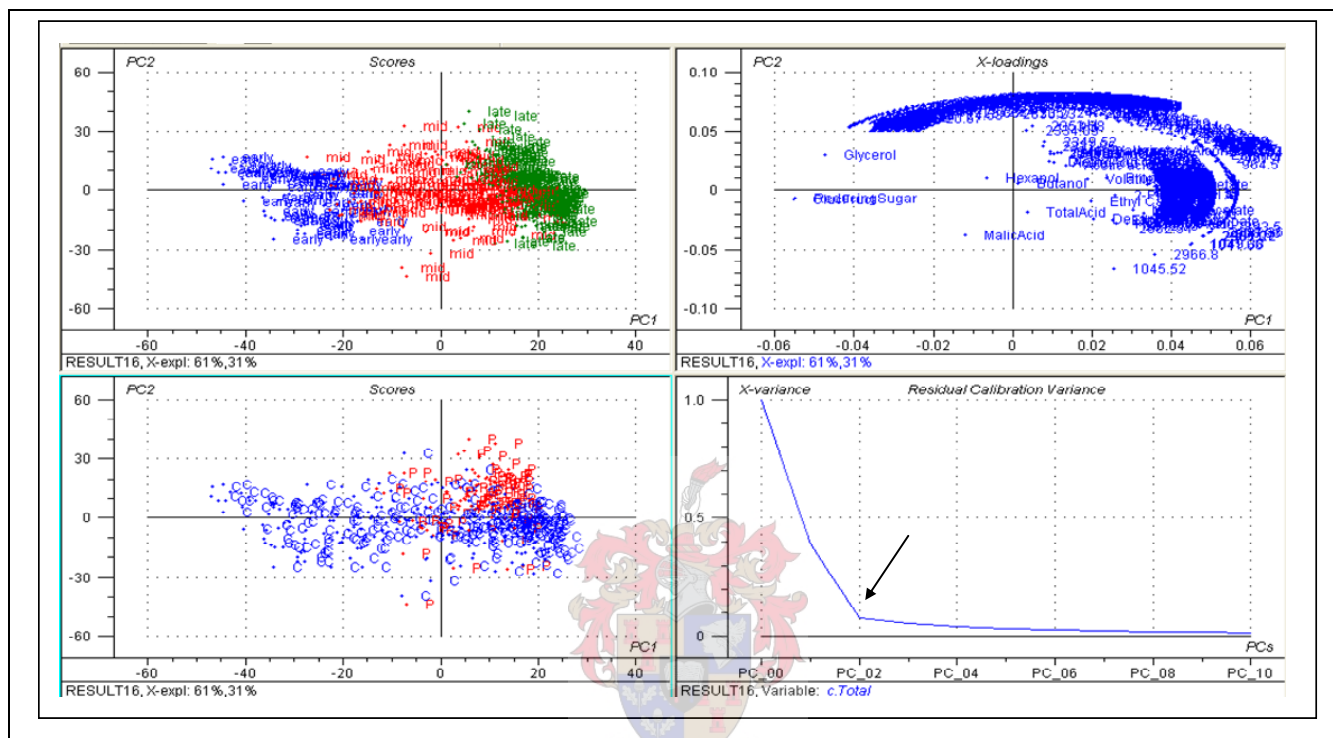


Figure 3.12 PCA score plot of all the must samples (matrix B), PC1 versus PC2. PC1 explains 61% of variance between the samples. Samples were collected throughout the fermentation process (blue=early, red=mid, green=late) resulting in huge variance in the data set. It is demonstrated that problem fermentations (red letter P) only occurred above 10% ethanol (from mid fermentation onwards).

Additional PCA modelling on the 2006 control and problem fermentation samples also indicated that the spectral variables contained ample information regarding the structure of the data and the FAN determinations appeared insignificant in comparison with these spectral data in explaining the variance observed in the data and the discrimination between control and problem fermentations.

3.3.4 PLS-DISCRIM REGRESSION USED FOR DISCRIMINATION PURPOSES

Fig. 3.13 shows the discriminant PLS regression plot for the discrimination of problem and control fermentation samples in matrix B for all the stages of fermentation ($\Delta = 0, 1, 2$). Generally, a tendency of separation between problem and control samples was observed, but some samples did overlap. This could be as a result of the extreme variation in terms of

cultivar and stage of fermentation. A correlation between some of the variables and the desired property (ability to discriminate between problem and control samples) was observed. Volatile components acetic acid, volatile acidity and diethyl succinate were strongly negatively correlated while 2-phenylethyl acetate was positively correlated with the ability to discriminate between control and problem fermentations. It should be noted that 45% of the X-variance modelled only 24% of the variance in the Y-variable. In effect, almost 50% of information in the X space had no relation with the desired property, the Y-variable.

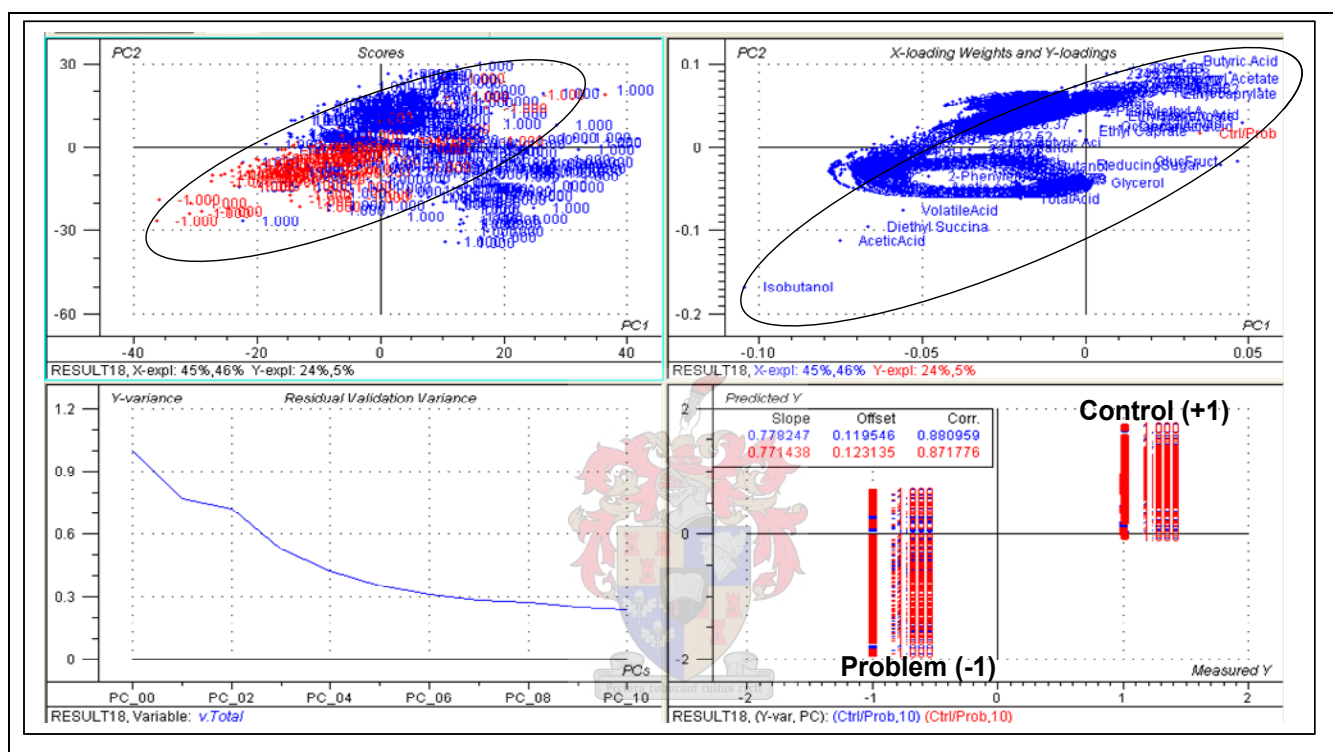


Figure 3.13 PLS discriminant plot of problem (red) and control (blue) fermentation samples (matrix B: $\Delta = 0,1,2$). All fermentation stages were included in this first discriminant analysis. No clear discrimination was observed although there were tendencies for separation between problem and control fermentations.

Samples from the late stage of fermentation were excluded and PLS discriminant analysis on matrix B ($\Delta = 0, 1$) was performed. Results indicate better separation between problem and control fermentation samples (**Fig. 3.14**). Samples still overlapped due to variation in sugar concentration and cultivar, but 29% of the X-variance of PC1 explained 23% of the Y-variance. Isobutanol had a high loading on PC1.

Another combination of matrix B ($\Delta = 0,1$) with the exclusion of early fermentation samples was utilised to evaluate the potential of a DPLS model to discriminate between control and problem fermentations (**Fig. 3.15**). PC1 and PC2 explained 62% of the X-variance and 40% of the Y-variance and partial discrimination between problem and control fermentations were observed. The corresponding loading weights plot established the relative

importance of each variable. The variables with the highest contribution to the first component explaining 36% of the total X-variance and 26% of the Y-variance are isobutanol, butyric acid, acetic acid and diethyl succinate.

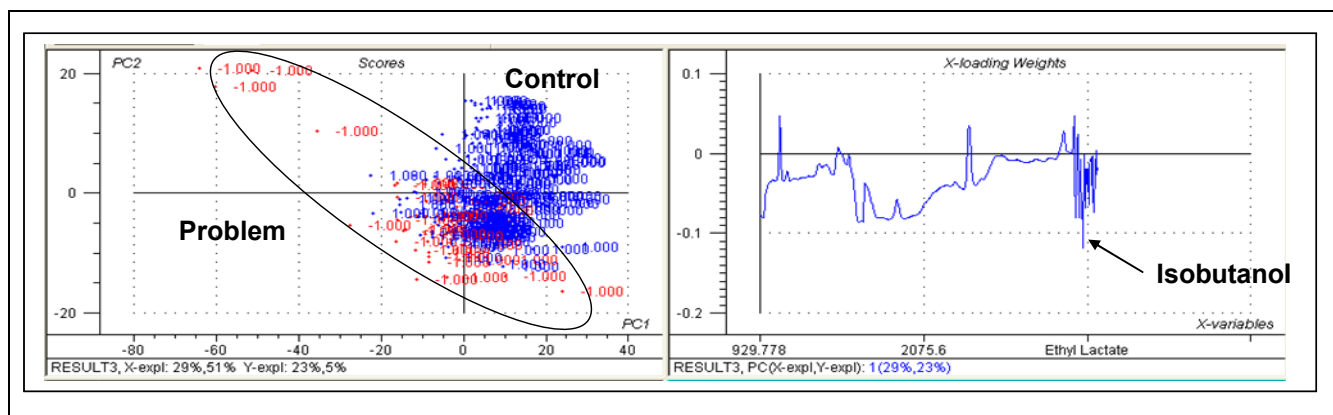


Figure 3.14 Score plot of PC1 vs PC2 for the discriminant PLS model. Better discrimination between problem (red) and control (blue) fermentations was achieved with the exclusion of late fermentation samples.

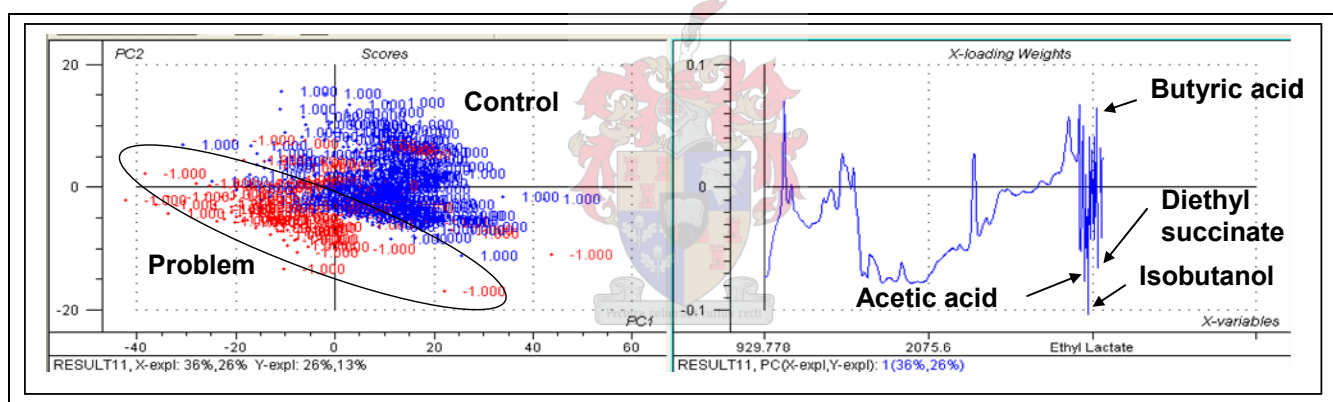


Figure 3.15 Score plot of PC1 vs PC2 for the PLS discrimination model. Samples from the early fermentation stage were excluded. 36% of the X-variance in PC1 explained 26% of the Y-variance.

The final step in this multivariate approach was to investigate samples within similar stages of fermentation in matrix B ($\Delta = 0$) and find a DPLS model with the best discrimination ability and evaluate whether the development of prediction models for the specific problem in question is indeed feasible for future work.

Fermentation samples from only the middle stage of fermentation (matrix B: $\Delta = 0$) were used to test the discrimination ability of the DPLS model. The resulting model was not effective in achieving this goal because of overlaps between problem and control fermentations (**Fig. 3.16**).

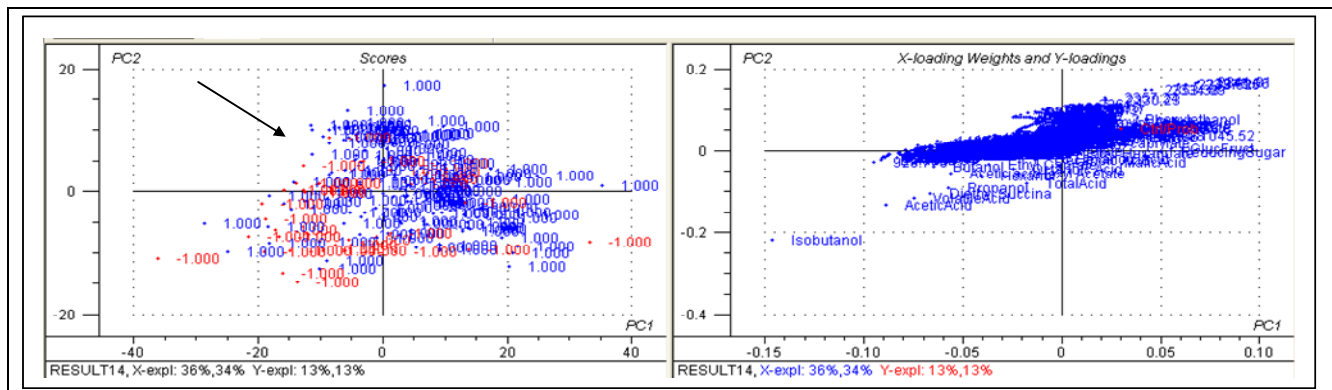


Figure 3.16 Score plot of PC1 vs PC2 for the DPLS model. Only samples from the middle fermentation stage were included in this model. No clear discrimination was observed.

Modelling only samples from the late fermentation stage resulted in 47% of the Y-variance being explained by 64% of the X-variance in PC1 (**Fig. 3.17**). Interestingly enough, when PCs 2 to 6 were inspected, the respective PCs modelled less variation in the X-variance and more of the Y-variance. The Y-relevant information usually appears in early components, but in situations where the variation related to Y is very subtle it could only be explained in later PCs (Esbensen, 2002). Isobutanol and butyric acid had high loadings on the loading weights plot.

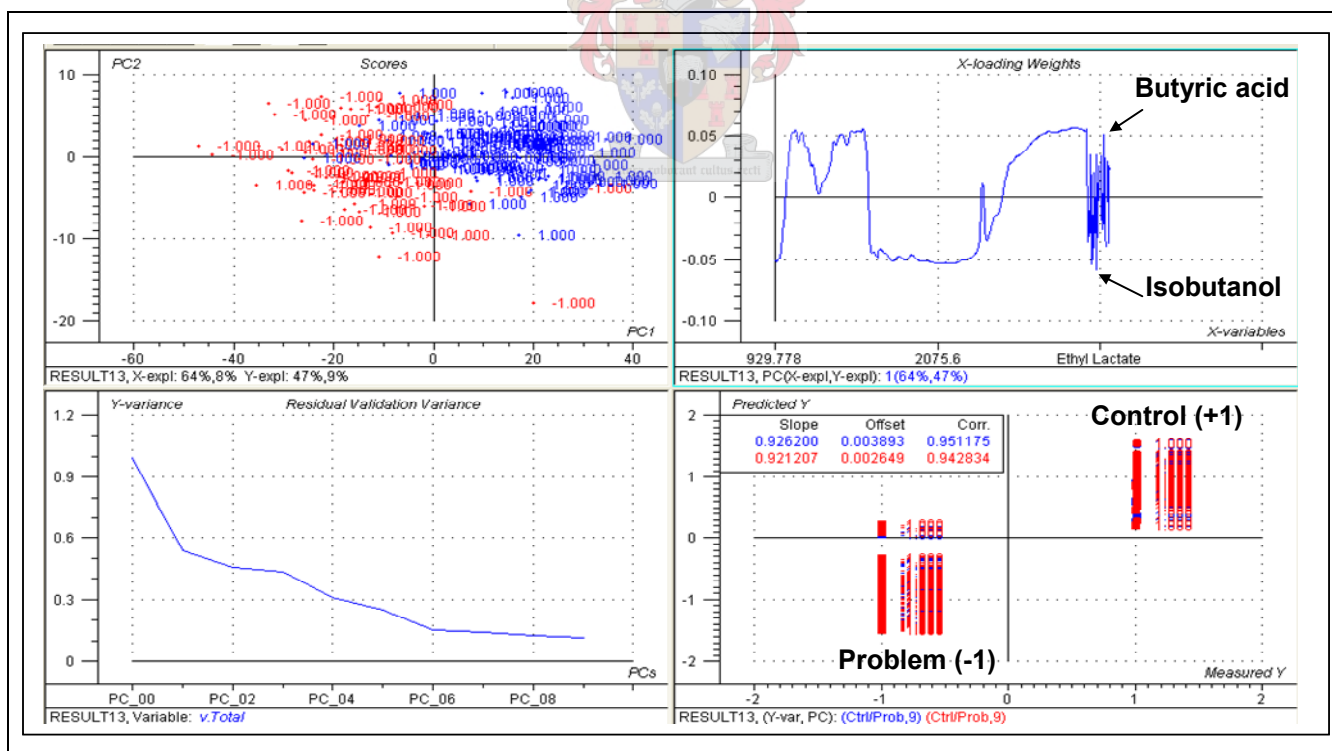


Figure 3.17 Score plot of PC1 vs PC2 for the DPLS model. Samples from the late fermentation stage (matrix B: $\Delta = 0$) resulted in better discrimination between problem (red) and control (blue) fermentations.

As a result of the large variation built into this dataset due to chemical composition, cultivar and origin, the dataset was divided into smaller subsets. Firstly, samples composed of only white cultivars in the middle stage of fermentation were modelled (**Fig. 3.18**). There was no clear discrimination between control and problem fermentations, although there was a tendency for separation of the different classes in the score plot. On the loading weights plot of PC1, isobutanol had a high loading which indicates the significance of this component in explaining the variability along PC1. As mentioned before, with inspection of relevant PCs explaining the Y-variance the later principal components (PCs 3, 4 and 5) explained a large portion of the Y-variance. PC1 explains 35% of the X-variance and 15% of the Y-variance. PC2 explains less of the Y-variance (12%). The third PC explained 24% of the X-variance and 15% of the Y-variance and on the loading weights plot, glycerol and isobutanol had high loadings.

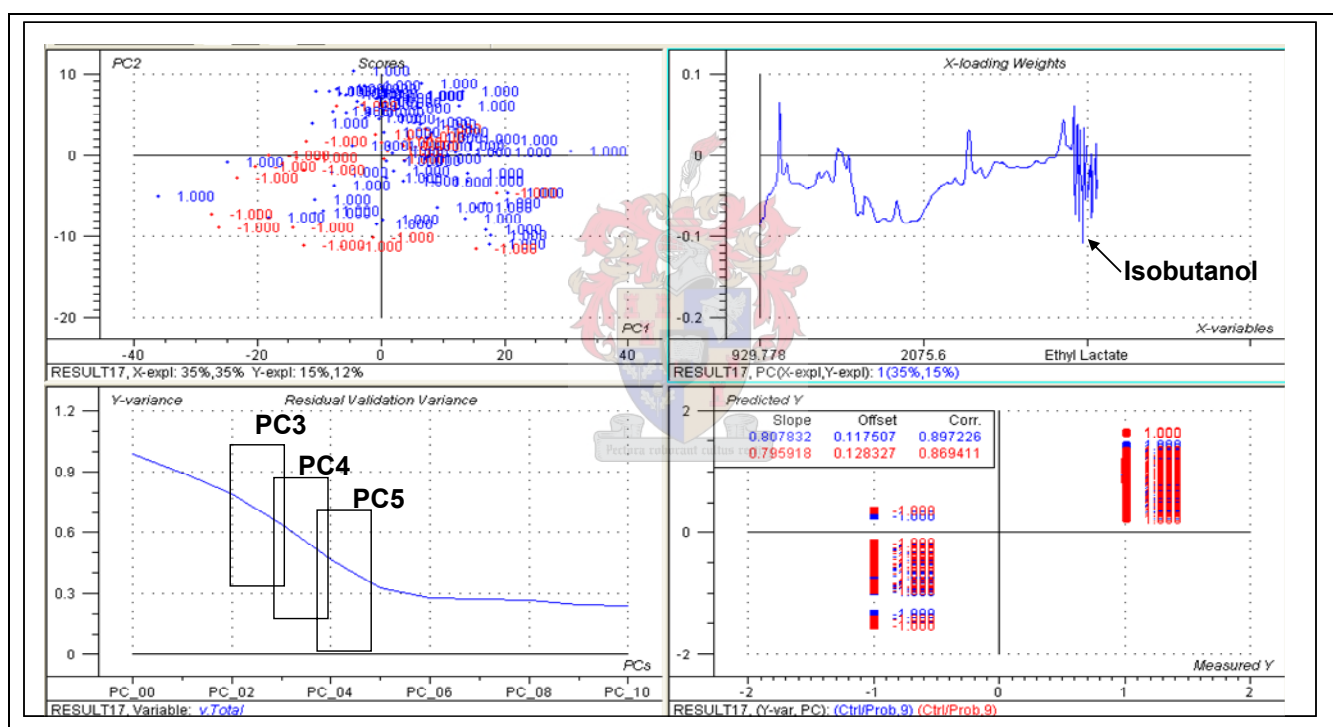


Figure 3.18 Score plot of PC1 vs PC2 shows no clear discrimination between problem (red) and control (blue) fermentation samples. Only white cultivars (middle stage of fermentation) were modelled.

PC4 and PC5 plotted against PC1 showed better discrimination between problem and control fermentations (**Fig. 3.19**). This could be explained by the fact that these two PCs combined explained 2% of the X-variance and a substantial fraction of the total Y-variance (29%). Total acid, isoamyl alcohol, 2-phenylethanol, diethyl succinate and isobutanol had high loadings on PC4 and the variables most significant in explaining the variance in PC5 were isobutanol, isoamyl acetate and pH.

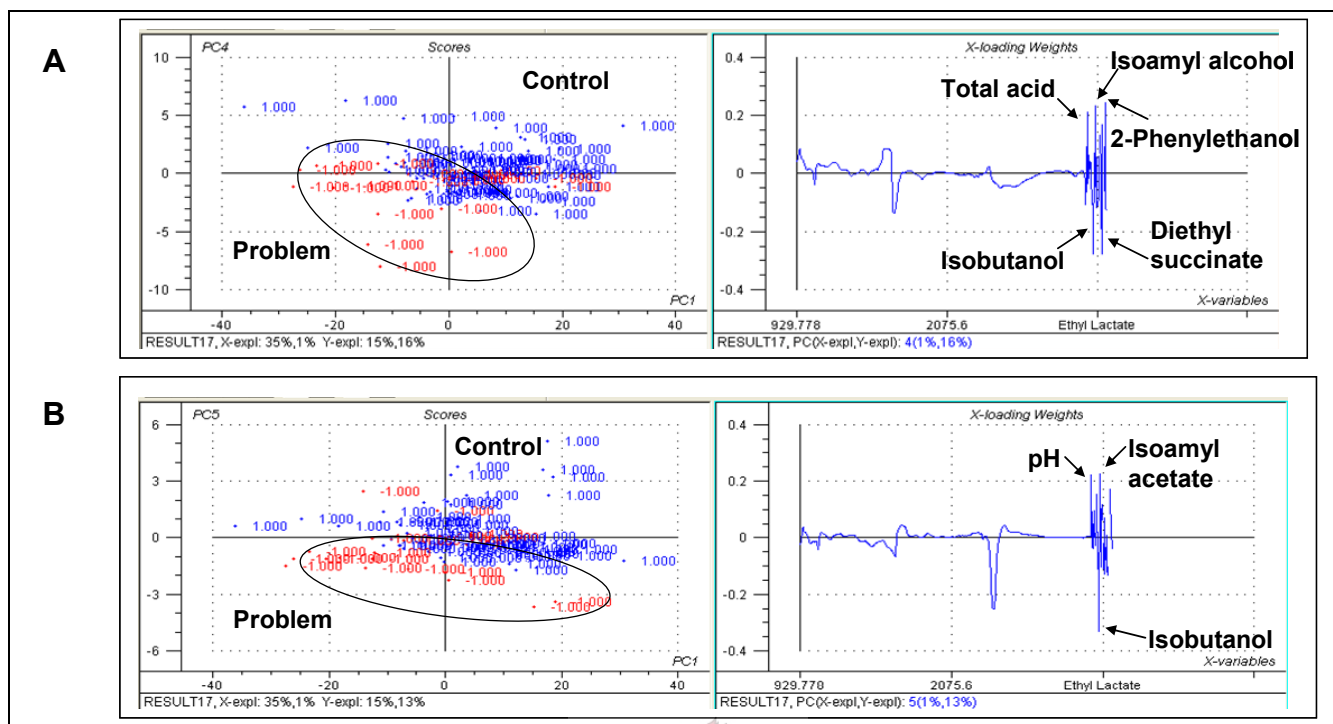


Figure 3.19 (A) Score plot of PC1 vs PC4 shows partial discrimination between problem (red) and control (blue) fermentation samples (matrix B: $\Delta = 0$). (B) Score plot of PC1 vs PC5. Only white cultivars from the middle stage of fermentation were included in this model. 2% of the X-variance modelled 29% Y-variance.

A discriminant PLS model consisting of red cultivars in the middle stage of fermentation (matrix B: $\Delta = 0$) was also constructed to investigate the discrimination possibilities (**Fig. 3.20**). Subtle discrimination between control and problem fermentations was observed. The score plot reveals that PC1 accounted for 43% of the X-variance and 18% of the Y-variance with high loading weights for butyric acid, acetic acid and isobutanol.

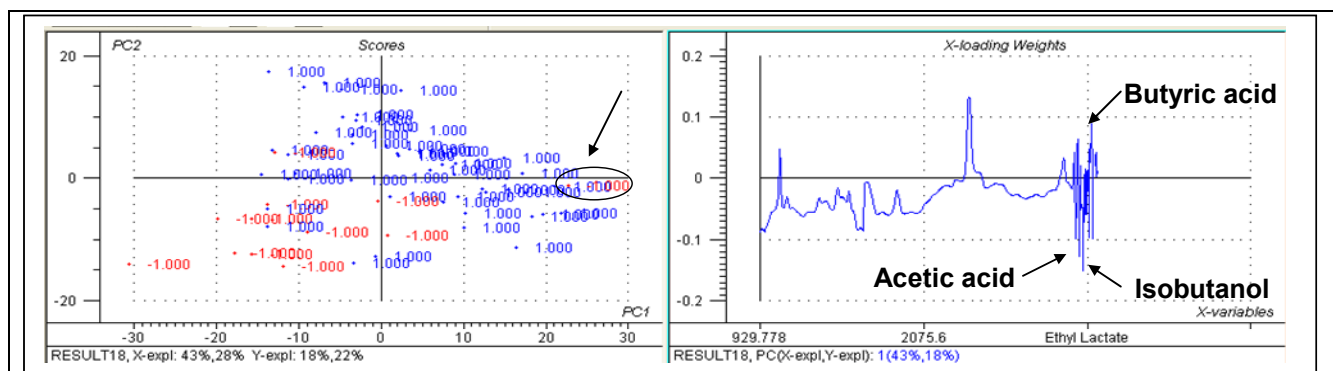


Figure 3.20 Score plot of PC1 vs PC2 indicates partial discrimination between problem (red) and control (blue) fermentation samples (matrix B: $\Delta = 0$). Red cultivars from the middle stage of fermentation were included in this model. PC1 modelled 43% of the X-variance and 19% Y-variance.

After removing an outlier that was detected in the score plot and remodelling, the Y-variance modelled by PC1 increased (**Fig. 3.21**). The outlier removed was a Pinot noir sample. The influence of this cultivar on the entire model was evident since PC1 modelled 43% of the X-variance and 18% of the Y-variance but after removing the outlier, PC1 modelled less of the X-variance (37%) and more Y-variance (30%). The second model (**Fig. 3.22**) however, with the exclusion of the outlier, was better for discrimination purposes of the model since it then explained more of the Y-related information.

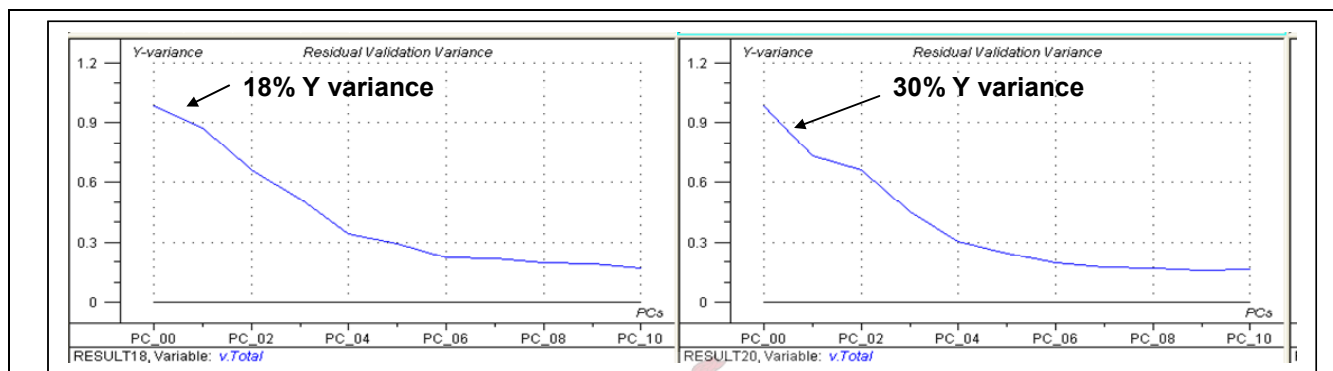


Figure 3.21 The Y-variance plot indicates the Y-variance explained by principal components. An increase from 18% Y-variance explained by PC1 to 30% was observed after the exclusion of one outlier in the DPLS model.

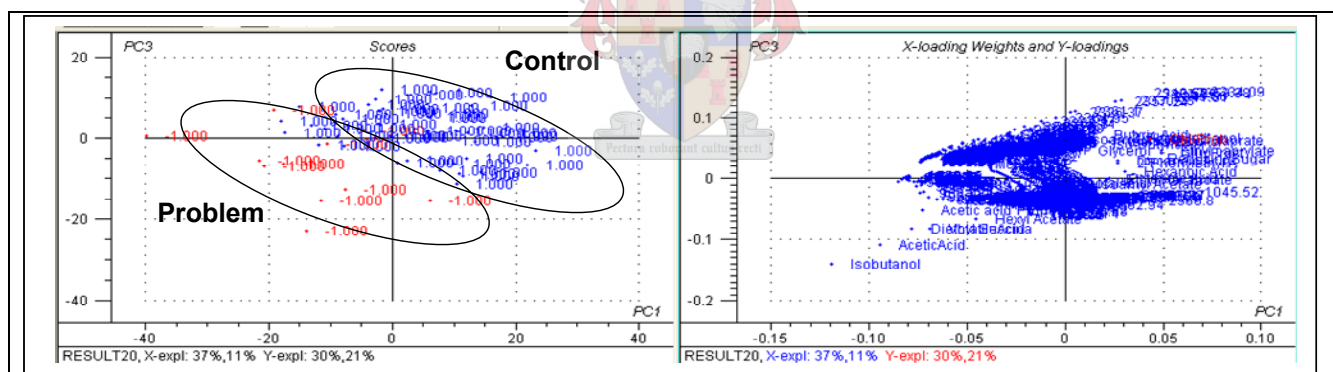


Figure 3.22 Score plot of PC1 vs PC3 for the DPLS model after removing the outlier. Increased discrimination was observed, with PC3 modelling an additional 11% of the X-variance and 21% of the Y-variance.

White cultivars in the late stage of fermentation were subsequently modelled (**Fig. 3.23**). Discrimination between problem and control fermentation samples was achieved with the exception of some samples. PC1 explained 40% of the X-variance and 26% of the Y-variance. Isobutanol and octanoic acid had high loadings on the loading weights plot.

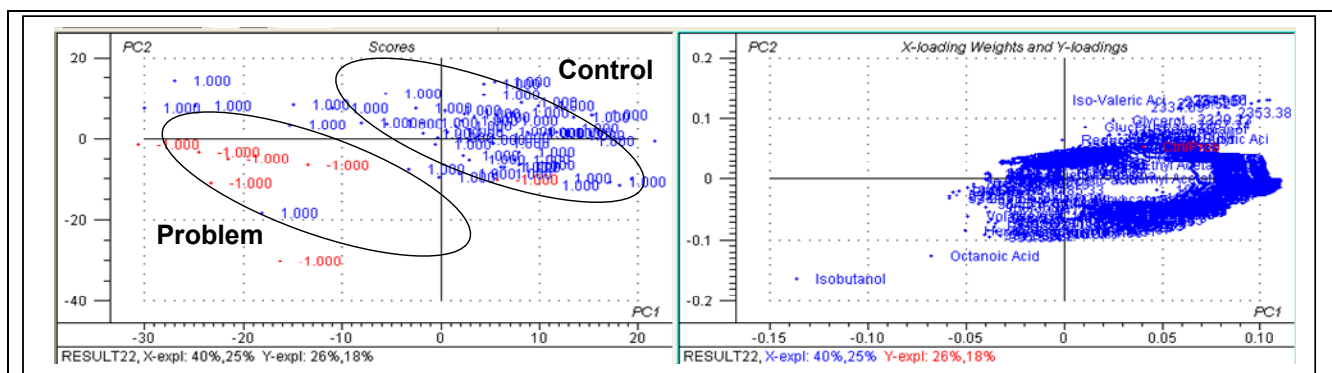


Figure 3.23 Score plot of PC1 vs PC2 for the DPLS model consisting of white cultivars in the late stage of fermentation. Discrimination between problem (red) and control (blue) fermentations was achieved with minor overlapping. 66% X-variance and 44% Y-variance were modelled by PC1 and PC2.

Red cultivars in the late stage of fermentation was also used to construct a discriminant PLS model (**Fig. 3.24**). Discrimination between problem and control fermentation samples was achieved. PC1 explained 57% of the X-variance and 33% of the Y-variance. It is clear from the residual validation variance plot that PC1, 3 and 4 modelled the largest portion of Y-variance. PC2 modelled 28% X-variance but only 7% Y-variance. PC3 modelled only 7% of the X-variance but 14% of the Y-variance.

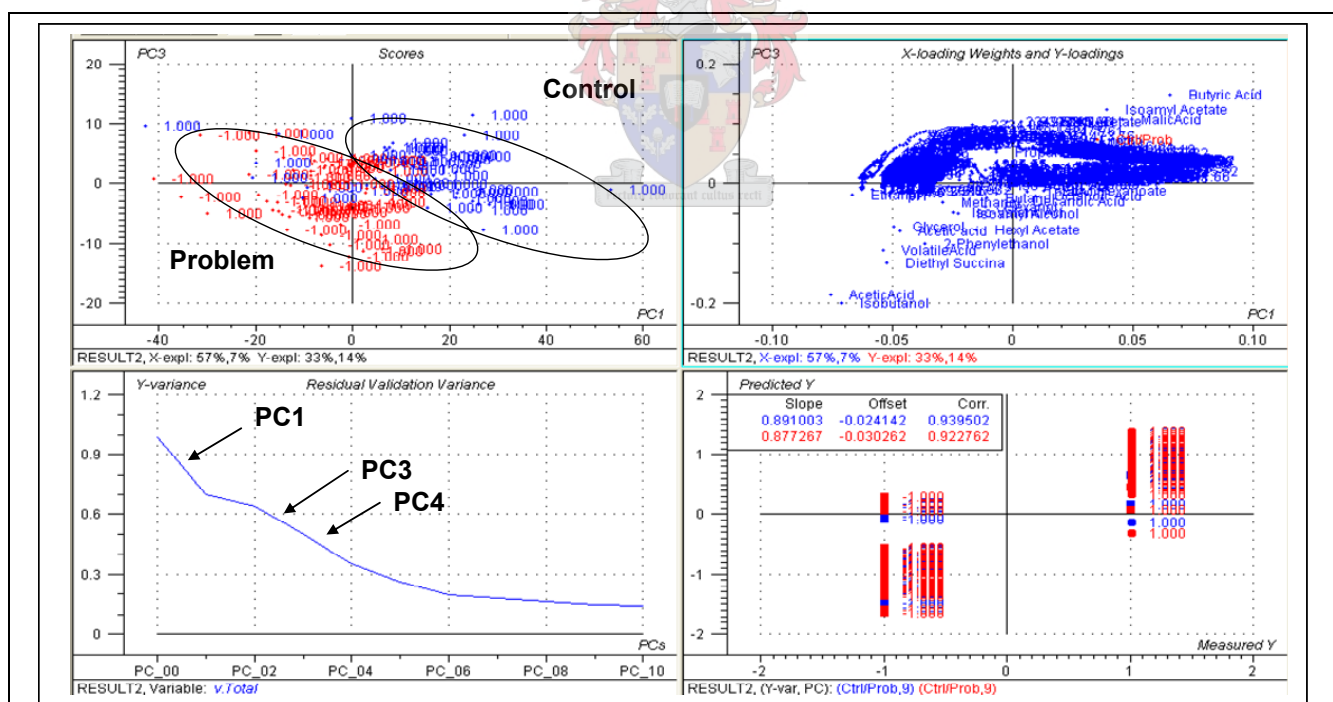


Figure 3.24 Score plot of PC1 vs PC3 indicates discrimination between problem (red) and control (blue) fermentation samples (matrix B: $\Delta = 0$). Only red cultivars from the late stage of fermentation were included in this model.

The loading weights plot indicate the variables which contribute most to the explanation of the total variance and which variables are correlated with the Y-variable. Acetic acid, isobutanol, volatile acidity, diethyl succinate had high loadings. These variables were negatively correlated with butyric acid and isoamyl acetate.

3.3.4.1 PLS-discrimination modelling for specific cultivars

The most abundant varieties within the original dataset (matrix B) were divided into various smaller subsets. These cultivar subsets were utilised to obtain a suitable discrimination model which had the ability of discriminating between control and problem fermentation samples. The following cultivars from the middle and late stages of fermentation were used for modelling: a) Sauvignon blanc; b) Chardonnay; c) Viognier; d) Merlot; e) Shiraz; f) Pinotage; g) Cabernet Sauvignon; h) Pinot noir.

- a) **Sauvignon blanc:** Problem fermentation samples could be distinguished from control fermentation samples (**Fig. 3.25**). PC1 modelled 29% X-variance and 23% Y-variance.

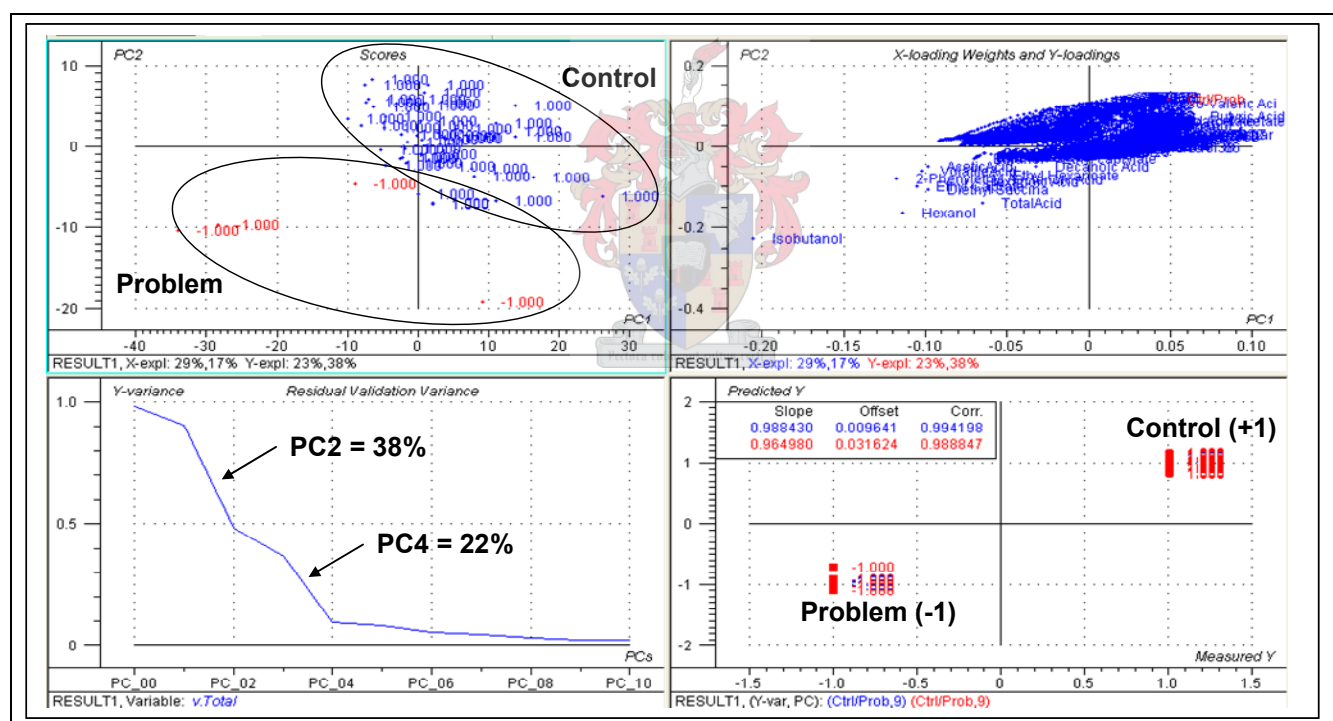


Figure 3.25 Score plot of PC1 vs PC2 for the DPLS model containing Sauvignon blanc samples from the middle and late fermentation stages. Discrimination between problem (red) and control (blue) fermentation samples was achieved. 4PCs explains 96% X-variance and 92% Y-variance.

The second principal component modelled 17% of the X-variance and 38% of the Y-variance. PC3 modelled 49% of the X-variance, but only 9% of the Y-variance is captured by this component. Remarkably, PC4 models only 1% of the X-variance and 22% of the

Y-variance. The first 4 PCs therefore models 96% of the X-variance and 92% of the Y-variance. The score plot of PC2 vs PC4 also provides clear differentiation between control and problem fermentations (data not shown) with 18% of the X-variance in these PCs modelling 60% of Y-variance. On the loading weights plot isobutanol, 2-phenylethyl acetate, n-hexanol, ethyl decanoate, diethyl succinate, volatile acidity and acetic acid had high loadings correlating with problem fermentations. Butyric acid and isovaleric acid had high loadings, to a much lesser extent, on the loading weights plot which were positively correlated with control fermentations.

b) Chardonnay: Clear discrimination between control and problem fermentations was obtained with the exception of a few samples (**Fig. 3.26**). PC1 explained 37% of the X-variance and 36% of the Y-variance. Observation of the loading weights suggests that the variables which contributed most significantly to the variance explained by the first two principal components are isobutanol, diethyl succinate, ethyl decanoate and octanoic acid.

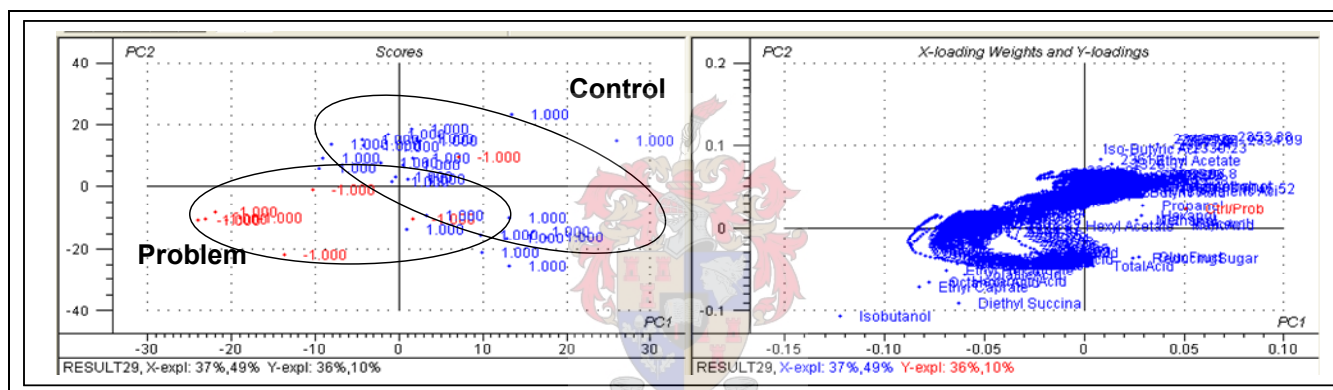


Figure 3.26 Score plot of PC1 vs PC2 for the DPLS model containing Chardonnay samples from middle and late stages of fermentation. PC1 explained 37% of the X-variance and 36% of the Y-variance.

c) Viognier: The score plot suggests clear discrimination between problem and control fermentations (**Fig. 3.27**). PC1 explained 70% of the X-variance and 77% of the Y-variance. The first principal component is strongly correlated with decanoic acid and the second principal component explaining 19% X-variance and 7% Y-variance is strongly correlated with isoamyl alcohol, 2-phenylethyl acetate and 2-phenylethanol. The third principal component modelled 6% X-variance and 11% Y-variance and had high loadings for the above mentioned compounds as well as acetic acid.

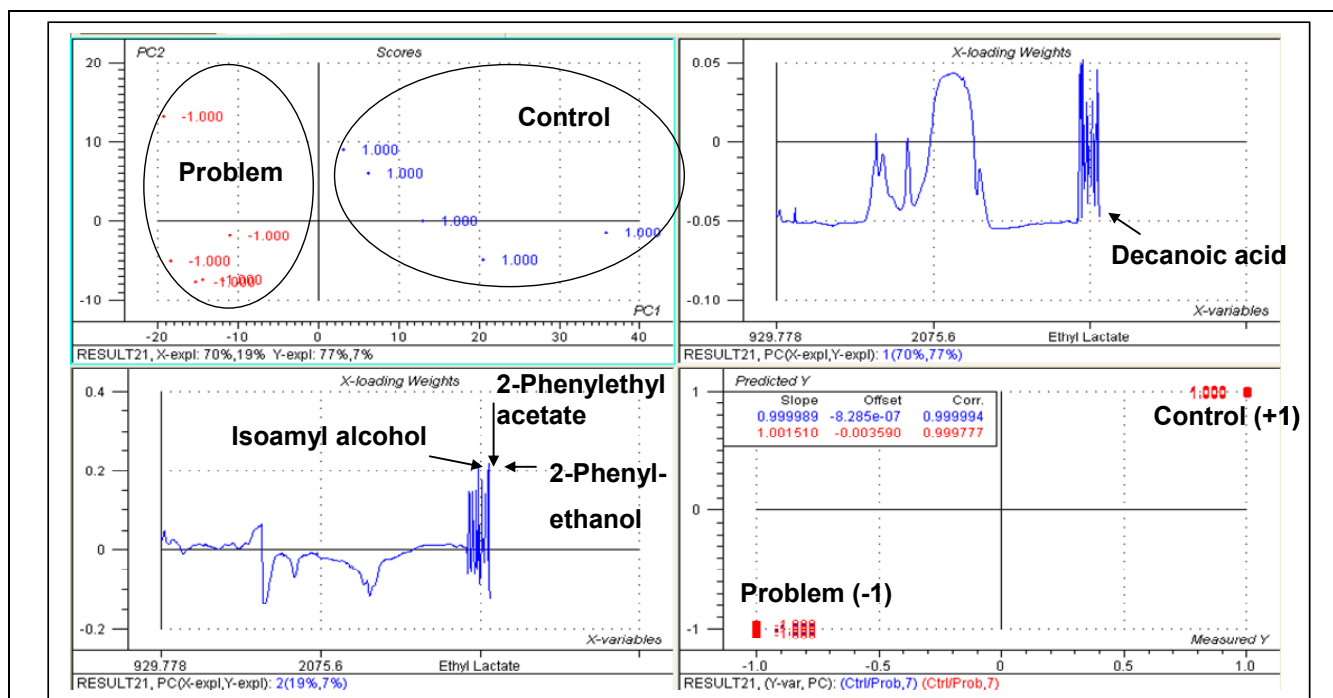


Figure 3.27 Score plot of PC1 vs PC2 indicates discrimination between problem (red) and control (blue) fermentation samples (matrix B: $\Delta = 0$). Only Viognier samples from the middle and late stages of fermentation were included in this model. PC1 modelled 70% of the X-variance and 77% of the Y-variance.

Although discrimination between problem and control fermentations was achieved with this DPLS model, no obvious differences between the spectra of the various samples could be observed by visual inspection alone (**Fig. 3.28**), apart from the changes related to fermentation progress. A similar effect was observed for a monitored Colombard fermentation, but no DPLS model was constructed due to the limited amount of samples for this specific cultivar.

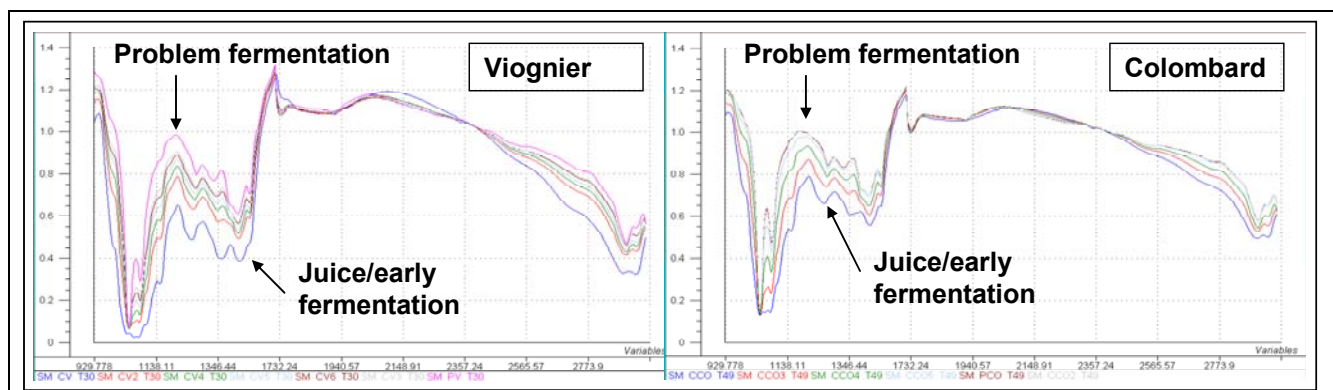


Figure 3.28 FT-IR spectra of control and problem fermentation Viognier and Colombard samples in the absorbance range 929 - 2966 cm^{-1} with the water absorbance regions (1543 - 1716 cm^{-1} and 2970 - 3626 cm^{-1}) excluded.

d) Merlot: Observation suggests clear discrimination between problem and control Merlot fermentations (**Fig. 3.29**). PC1 modelled 32% of the X-variance and 29% of the Y-variance. Four principal components modelled 96% of X-variance and 90% Y-variance. Isobutanol, diethyl succinate, acetic acid, volatile acidity and butyric acid were the variables with the highest contribution to the first component.

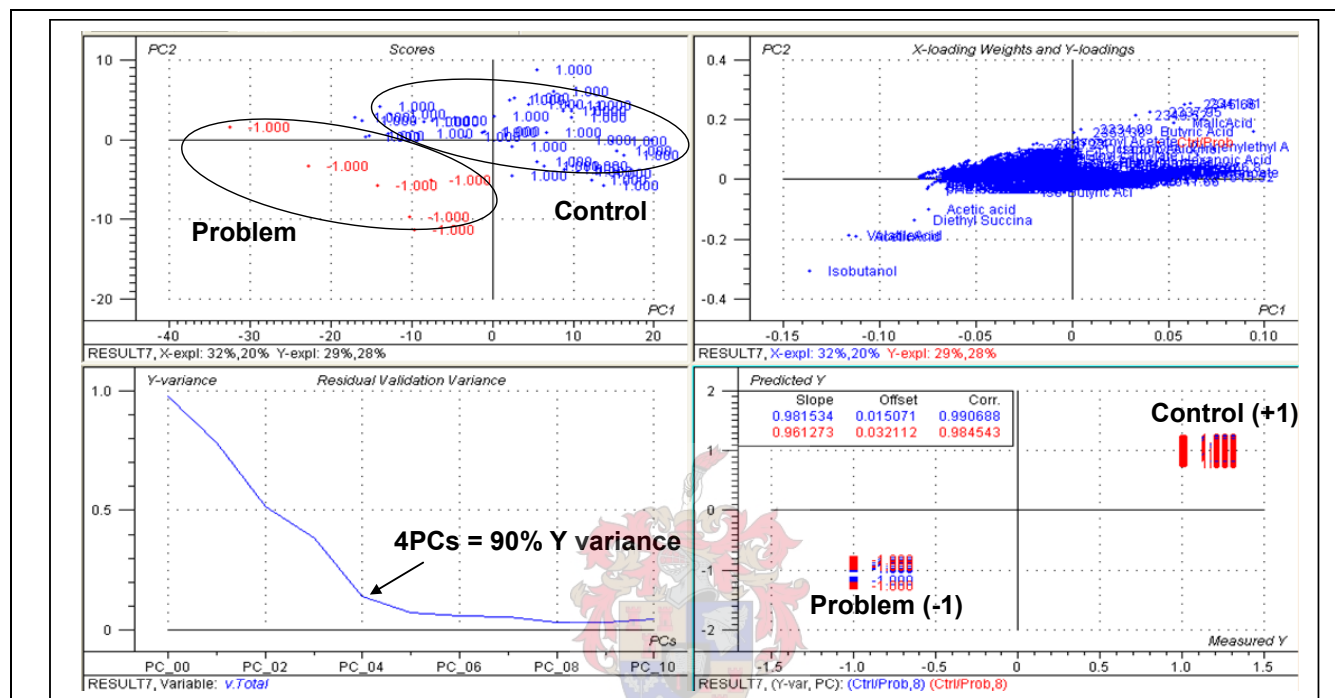


Figure 3.29 Score plot of PC1 vs PC2 for the DPLS model consisting of Merlot samples in the middle and late stages of fermentation. Discrimination between problem (red) and control (blue) fermentation samples was obtained. PC1 modelled 32% of the X-variance and 29% of the Y-variance.

e) Shiraz: Discrimination between the control and problem fermentation samples was obtained with PC1 modelling 45% of the X-variance and 58% of the Y-variance (**Fig. 3.30**). PC2 only explained 3% of the Y-variance and 44% of the X-variance. PC3, however, explained 3% of the X-variance and 18% of the Y-variance. The first three PCs modelled 92% of the X-variance and 79% of the Y-variance. Isobutanol, volatile acidity, acetic acid, methanol and pH were strongly negatively correlated with malic acid, isoamyl acetate, ethyl decanoate, ethyl octanoate, butyric acid, hexanoic, decanoic and octanoic acid on the loading weights plot.

f) Pinotage: Similarly, discrimination between control and problem fermentations was achieved with the first three PCs modelling 95% of the X-variance and 75% of the Y-variance (**Fig. 3.31**). The variables contributing most significantly to the first PCs are isobutanol, acetic acid, diethyl succinate, butyric acid, ethyl decanoate, ethyl octanoate and isoamyl acetate.

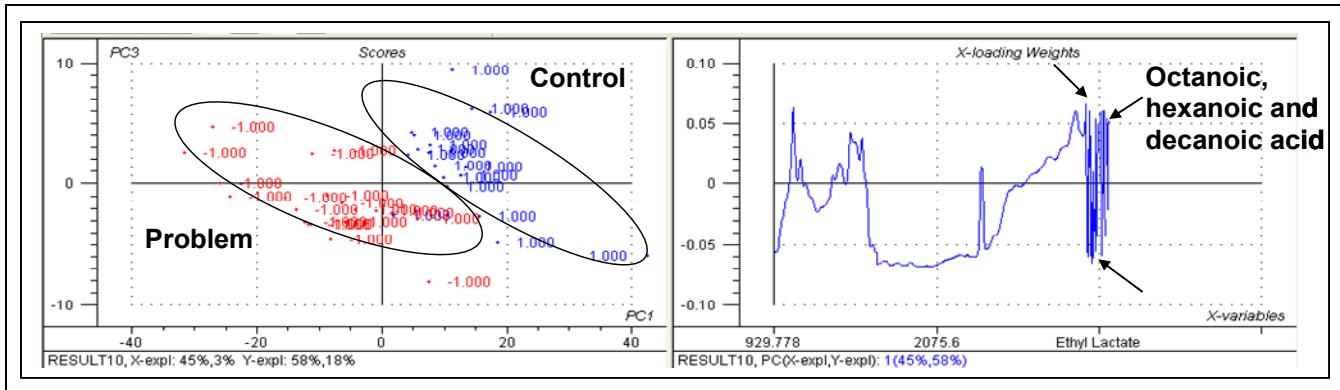


Figure 3.30 Score plot of PC1 vs PC3 for the DPLS model containing Shiraz samples from middle and late stages of fermentation. PC1 explained 45% of the X-variance and 58% of the Y-variance.

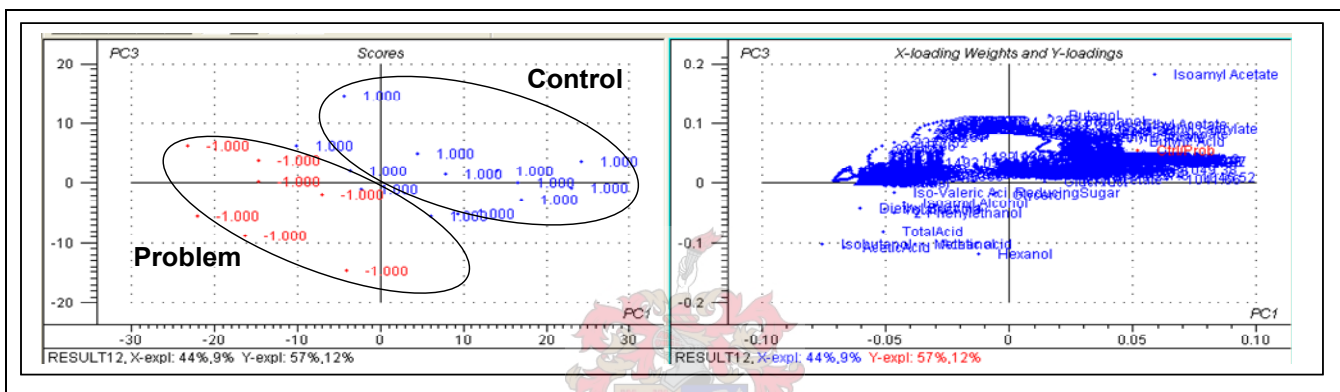


Figure 3.31 Score plot of PC1 vs PC3 for the DPLS model containing Pinotage samples from middle and late stages of fermentation. PC1 explained 44% of the X-variance and 57% of the Y-variance.

g) Cabernet Sauvignon: It appeared as if discrimination was achieved with this DPLS model (**Fig. 3.32**) but with closer inspection it was recognised that the control fermentation samples were in the middle stage of fermentation and the problem fermentation samples were closer to the end of fermentation. This discrimination was therefore purely based on different sugar concentrations, ethanol levels and associated fermentation products. This observation correlates with the high loading for reducing sugar on the loading weights plot. It is important to always keep the aforementioned effect in mind when the discrimination possibilities of DPLS models are evaluated. The necessity for fermentation samples to be in the same stage of fermentation when modelled is of extreme importance especially if a small dataset is used for modelling.

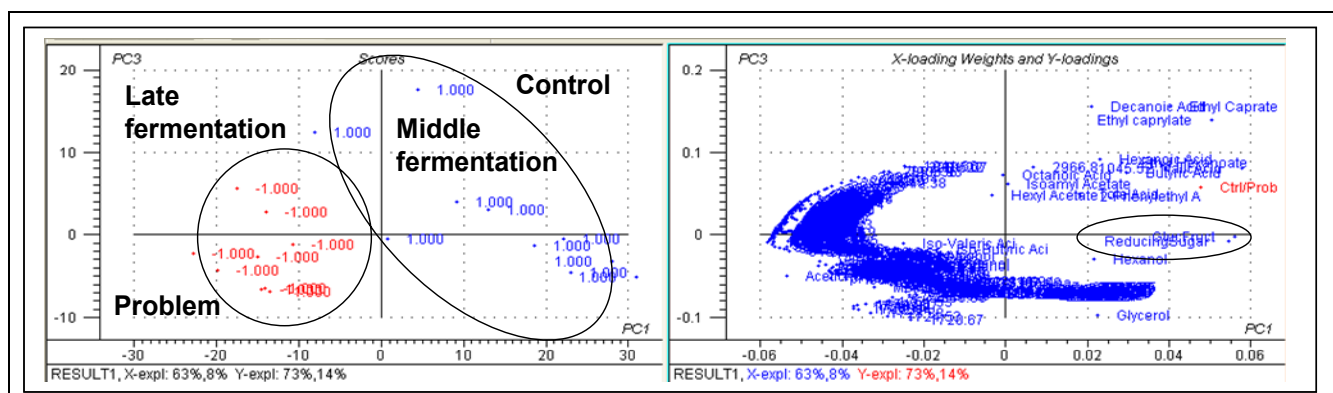


Figure 3.32 Score plot of PC1 vs PC3 for the DPLS model containing Cabernet Sauvignon samples from middle and late stages of fermentation. Discrimination observed due to different stages of fermentation and not necessarily between control and problem fermentations.

h) Pinot noir: samples from the middle and late stages of fermentation were modelled to investigate whether discrimination between control and problem fermentations were possible. Clear discrimination between problem and control fermentations was achieved (**Fig. 3.33**) with PC1 modelling 74% of the X-variance and 66% Y-variance.

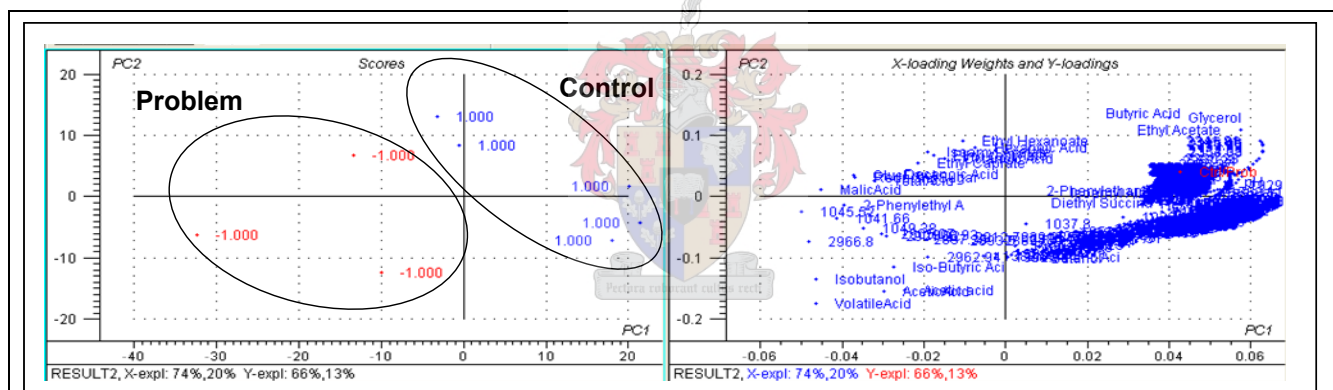


Figure 3.33 Score plot of PC1 vs PC2 for the DPLS model. Pinot noir samples in the middle and late stages of fermentation were modelled. Discrimination between problem (red) and control (blue) fermentations was achieved with PC1 modelling 74% X-variance and 66% Y-variance.

Separation along PC1 correlates with high loadings on the loading weights plot for numerous volatile components. It demonstrates that butyric acid, ethyl acetate, pH, glycerol ethanol and isoamyl alcohol are strongly correlated with the control samples. Volatile acidity, acetic acid, isobutanol, isobutyric acid, 2-phenylethyl acetate, butanol, isovaleric acid and propanol were negatively correlated with the above-mentioned components on PC1 and therefore correlated with the problem fermentations. Sugar had a relatively low loading on the loading weights plot and was negatively correlated with ethanol. Although the loading for this specific variable was not as significant as some of the loadings for the volatile compounds mentioned, it should be kept in mind when interpreting the data. PC2 accounts for 20% of the

X-variance and 13% of the Y-variance. The same variables also had high loadings on this PC. The first three PCs modelled 98% X-variance and 95% Y-variance. This set of Pinot noir samples is extremely inadequate in numbers for modelling or prediction purposes and should rather be implemented to indicate possible exploration areas for future research endeavours.

3.3.5 UNIVARIATE STATISTICS

The mean and standard deviation (SD) for the volatile chemical components, quantified chemical components from the FOSS Winescan, microbial analysis, FAN determinations and SO₂ determinations are given in **Tables 3.7** and **3.4 (section 3.2.9)**. Microbial populations are expressed as colony forming units per mL (cfu/mL). FAN and volatile component concentrations are given in mg/L. Chemical data derived from FOSS Winescan spectra are given in g/L and SO₂ determinations are given in mg/L (ppm).

Table 3.7 The minimum (min), maximum (max), mean, number of samples and standard deviation (SD) of measured parameters of the must samples.

| Parameter | Number | Min. | Max. | Mean | SD |
|-----------------------------------|--------|------------------------|------------------------|------------------------|-------------------------|
| Yeast (cfu/mL) | 64 | 2.38 x 10 ³ | 7.64 x 10 ⁸ | 2.54 x 10 ⁷ | 9.832 x 10 ⁷ |
| AAB (MRS-AAB media) (cfu/mL) | 64 | 1.00 x 10 ¹ | 6.80 x 10 ⁴ | 2.28 x 10 ³ | 9.13 x 10 ³ |
| AAB (GYC media) (cfu/mL) | 64 | 1.00 x 10 ¹ | 1.00 x 10 ⁴ | 4.84 x 10 ² | 1.79 x 10 ³ |
| LAB (cfu/mL) | 64 | 1.00 x 10 ¹ | 3.00 x 10 ⁶ | 1.57 x 10 ⁵ | 5.48 x 10 ⁵ |
| <i>Brettanomyces sp.</i> (cfu/mL) | 64 | 1.00 x 10 ¹ | 3.82 x 10 ³ | 8.08 x 10 ¹ | 4.79 x 10 ² |
| Total SO ₂ (ppm) | 64 | 6 | 109 | 27.8 | 20.8 |
| Free SO ₂ (ppm) | 64 | 3 | 76 | 17.7 | 9.9 |
| FAN (mg/L) | 310 | 103.6 | 308 | 190.8 | 33.5 |
| Glycerol (g/L) | 379 | 2.87 | 40.65 | 11.9 | 4.7 |
| Ethanol (g/L) | 379 | 0 | 17.64 | 9.8 | 4.5 |
| Sugar (glucose+fructose) (g/L) | 379 | 0.26 | 400 | 68.7 | 77.7 |
| Total acid (g/L) | 379 | 2.04 | 9.02 | 4.3 | 0.8 |
| Volatile acid (g/L) | 379 | 0.10 | 1.72 | 0.29 | 0.19 |
| pH | 379 | 2.66 | 4.40 | 3.5 | 0.24 |

Similarly to the multivariate analysis, no significant observations were made in terms of the microbial data and SO₂ determinations apart from the large standard deviations that indicate the variation amongst the samples. During the 2005 season 64 problem fermentation

samples were collected and microbial populations and SO₂ concentrations were determined. However, during this particular season no control samples were collected and in the following season these analysis were discarded due to their insignificance in comparison with less time consuming analysis (FT-IR spectral analysis) which exhibited more significant value. Therefore, comparison between control and problem fermentations for these particular two parameters was not possible.

The chemical parameters glycerol, ethanol, sugar (glucose+fructose), total acid, volatile acidity and pH indicated in **Table 3.7** and the volatile components in **Table 3.4 (section 3.2.9)** show the immense variation (indicated by the large standard deviations) amongst the samples in terms of their chemical composition. This observation was also made with multivariate analysis which indicated that samples from all stages of fermentation were represented in this dataset.

The FAN concentrations for the majority of the samples were sufficient for the completion of fermentation. This variable also had low loadings in the multivariate analysis for the discrimination between problem and control fermentations. Insufficient FAN concentrations are rarely considered a problem today because of the frequent addition of complex yeast nutrition by winemakers throughout fermentation.

3.4 CONCLUSIONS

Multivariate models were constructed to evaluate the possibility of discrimination between problem and control fermentation samples using multi-instrument data as well as FT-IR spectroscopy. A selection of these discriminant models showed definite potential to effectively distinguish between problem and control fermentations with the prerequisite that samples either have to be in the same stage of fermentation or that similar cultivars should be modelled. This result could definitely be used in the future for prediction purposes.

FT-IR spectroscopy showed promise as a rapid method for determining various compounds simultaneously in fermenting must following proper degassing. The raw spectra were extremely valuable in discriminating between control and problem fermentations. FT-IR spectroscopy has the potential to dramatically reduce analytical time and cost of monitoring wine fermentation for both research and commercial production applications. In addition, further studies are needed in order to improve the calibration specificity, accuracy and robustness for chemical parameters related to changes during fermentation with consideration of the constantly changing sample matrices during fermentation.

Due to the fact that this study relied completely on the cooperation of the wine industry for fermentation samples, there was no control over the number of problem fermentations reported and subsequent samples collected. However, this potential problem actually

strengthens the robustness and enormous potential of such a multivariate approach in monitoring fermentation. Although, this study might not be an accurate report of the frequency and character of industrial problem fermentations and participation from winemakers to similar projects needs to be further promoted in order to accurately monitor fermentation problems in industry, this study had some interesting outcomes. Most importantly, apart from the fact that there was no control over the amount of samples collected per cultivar and stage of fermentation leaning towards less than optimal sample sets, it was still possible to effectively discriminate between control and problem fermentations even under these robust conditions using multivariate data analysis. This multivariate approach shows potential for future research projects and industrial process monitoring as well as a definite potential for prediction of problem fermentations.

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4. RESEARCH RESULTS

Automated headspace solid-phase dynamic extraction (SPDE) coupled to GC-MS for cultivar discrimination and classification purposes

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ABSTRACT

The suitability of headspace solid-phase dynamic extraction (HS-SPDE) coupled with gas chromatography-mass spectrometry (GC-MS) technique for wine analysis was evaluated since this technique is relatively unknown and it has not yet been evaluated for the analysis of wine. Data showed that this technique was indeed suitable for the analysis of wine and the wealth of information generated with this technique was used for multivariate data analysis. The study further aimed at investigating the feasibility of this type of analysis to be possibly used in the discrimination between control and problem fermentations. In addition to the primary aim of this work, the possibility of constructing multivariate models to possibly discriminate between different varieties was also investigated. For this purpose the volatile composition (higher alcohols, fatty acids, ethyl esters and carbonyl compounds) was determined for 97 monovarietal wine samples consisting of red and white cultivars. Corrected peak area data for 68 analytes from the above-mentioned chemical groups were used for multivariate statistical purposes. Principal component analysis (PCA) was applied in order to determine the main sources of variability present in the data sets and to establish the relation between samples (objects) and volatile compounds (variables). This technique also identified clusters or groups of samples with similarities within the data structure. Discriminant partial least squares (PLS-discrim) models showed the potential to discriminate between control and problem fermentations and between various cultivars. Differentiation between different cultivars was achieved with fermentation products contributing most significantly to this discrimination. Although samples were collected from early till late fermentation, discrimination between cultivars was obtained through-out the fermentation progress. Observations suggest that the possibility to discriminate between problem and control fermentations with only the headspace data could possibly be pointed in the direction of prediction purposes in following studies.

4.1 INTRODUCTION

Volatile compounds play an important role in the organoleptic characteristic and quality of wines. Several hundred compounds have been identified as important contributors to wine aroma (Rapp *et al.*, 1988; Guth, 1997; López *et al.*, 1999; Ferreira *et al.*, 2000, 2001; Anzar *et al.*, 2001) although some of these are present only in trace amounts (Etiévant, 1991; Ebeler, 2001). These compounds belong to heterogeneous groups such as monoterpenes, alcohols, aldehydes, ketones, esters, organic acids and fatty acids. Some of the compounds originate from grapes and others are formed during fermentation processes or wine ageing (Nykänen, 1986; Vianna and Ebeler, 2001; Hernández-Orte *et al.*, 2002). The wine aroma composition also depends on yeast strain used (Soles *et al.*, 1982; Cabrera *et al.*, 1988; Lurton *et al.*, 1995; Antonelli *et al.*, 1999) and fermentation conditions such as pH of the must or wine, content and type of nitrogen available, sugars, fermentation temperature and aeration (Killian and Ough, 1979; Suomaleinen and Lehtonen, 1979; Suomaleinen, 1981; Mauricio *et al.*, 1995, 1997; Vianna and Ebeler, 2001). Fermentation compounds, both qualitatively and quantitatively, play a key role in wine aroma and include especially higher alcohols, fatty acids and esters. The compounds originating from grapes are typical of a specific variety and the amino acid profile would therefore be related to the aroma profile of the wine (Hernández-Orte *et al.*, 2002). Monitoring the quantities of volatiles during fermentation is very important in understanding their synthesis from yeast and the factors affecting their production. The analysis of the volatile fraction of wine is extremely challenging due to the complex nature of the wine matrix. The great variety of volatile compounds, with different polarities, volatilities and a wide range of concentrations, is responsible for the complexity of wine and ensures its specificity and character.

Before aroma components can be separated and analysed, they must be extracted from the wine matrix. Therefore, sample preparation (especially extraction and concentration of aroma compounds) remains one of the critical areas in aroma volatile analysis. Extraction and concentration techniques often used for this purpose include static headspace (Villén *et al.*, 1995), purge and trap (Zhang *et al.*, 1994), solid-phase extraction (Arrhenius *et al.*, 1996), solvent extraction (Vianna and Ebeler, 2001), simultaneous extraction and distillation (Blanch *et al.*, 1996), supercritical fluid extraction (Karásek *et al.*, 2003), liquid-liquid microextraction (Ortega *et al.*, 2001), ultrasonic-assisted extraction (Vila *et al.*, 1999), microwave extraction (Liu *et al.*, 2005) and stir bar sorptive extraction (Zalacain *et al.*, 2004), etc. Most of these techniques have several disadvantages, including extensive equipment requirements, significant quantities of expensive and toxic organic solvents, multiple-step procedures prone to analyte loss and is time consuming and labour-intensive.

In recent years, a new technique, solid-phase micro-extraction (SPME), was developed by Pawliszyn and co-workers (Arthur and Pawliszyn, 1990; Eisert and Pawliszyn, 1997; Pawliszyn, 1997). This technique offers two main advantages: no extraction solvent is required and it enables extraction, pre-concentration and sample introduction to be performed simultaneously without pre-treatment of the sample. SPME involves the partitioning of the analyte between a polymeric material coated onto the end of a fused silica fibre and the sample matrix (the headspace in the case of headspace extractions). Analytes are adsorbed onto the polymeric coating and this step is followed by desorption of the analytes into the gas chromatograph (GC) injector port (Pawliszyn, 1997). This traditional fibre solid-phase micro-extraction (SPME) technique has become very popular during the last years and SPME has been widely applied for the analysis of a broad range of flavour compounds in wine and other alcoholic beverages (De la Calle Garcia *et al.*, 1996, 1997, 1998; Evans, *et al.*, 1997; Jelen *et al.*, 1998; Mestres *et al.*, 1998; Vas *et al.*, 1998, 1999; Hayasaka and Bartowsky, 1999; Vas and Lorincz, 1999). One of the limits of SPME is its reduced concentration capability due to the small volume of polymer coating on the fibre (Bicchi *et al.*, 2004). Numerous attempts have been made to overcome some other minor disadvantages of SPME (fragility of the fused-silica, unprotected stationary phase coating, limited flexibility of surface area and film thickness) (Bicchi *et al.*, 2004) by coating the inside of a needle or capillary instead of the outside of a fibre (Murphy, 1996; McComb *et al.*, 1997).

The solid-phase dynamic extraction (SPDE) procedure is a modern enrichment method which presents a further development providing higher analytical efficiency for sorption and solvent-free extraction followed by gas chromatography-mass spectrometry (GC-MS) analysis (Lachenmeier *et al.*, 2003). The principles of this method are based on solid phase micro-extraction (SPME). For SPDE, an internally coated steel needle is used for the extraction and pre-concentration from the solution headspace (HS). A dynamic extraction is performed by repeated aspiration and dispensation of the syringe volume (Jochmann *et al.*, 2006). Consequently, analytes present in the sample are adsorbed onto the sorbent inside the needle. Analyte desorption into the GC injector port is induced by the rapid heating of the metal needle followed by GC-MS analysis. The entire procedure is fully automated. SPDE is not as widely applied as SPME and reference to the use of this technique is limited (Lachenmeier *et al.*, 2003; Jochmann *et al.*, 2006). Reported analysis is only on the headspace of samples which provides a longer lifetime for the coated needle (Lachenmeier *et al.*, 2003; Jochmann *et al.*, 2006). Results suggest that SPDE provide for a rapid and sensitive method with higher extraction rates, a faster automated operation and robustness of the capillary in comparison with SPME (Lachenmeier *et al.*, 2003).

The SPDE technique has not yet been evaluated for the analysis of wine. Therefore, the principal aim of the present work was to evaluate the suitability of this technique to

characterise the chemical composition of the aroma compounds and in addition to test the feasibility of these “headspace fingerprint” profiles to investigate the possibility to discriminate between control and problem fermentations. The sorptive extraction technique, solid phase dynamic extraction (SPDE), followed by GC-MS analysis was used for this purpose. Furthermore, since the evolution of higher alcohols, acetates, fatty acids, ethyl esters and carbonyl compounds is associated with fermentation progress, headspace volatile data of some of the more abundant cultivars including Chardonnay and Sauvignon blanc were used to construct multivariate models in order to investigate the objectives set.

4.2 MATERIALS AND METHODS

4.2.1 MUST SAMPLES

A total of 379 actively fermenting must samples of 500 mL each were collected from large scale fermentation tanks at various South African commercial wineries. The samples were collected from a sample tap situated a third from the bottom on the side of the tank (12 000 L, 25 000 L, 33 000 L) or at the top of the tank in the case of 100 000 L tanks after rinsing the sampling bottle with the respective must.

This work is part of a larger study and a subset of 97 of the samples was subjected to headspace SPDE GC-MS analysis. Samples were stored at -20°C until analysis. The cultivar distribution was as follows: Cabernet Sauvignon (6), Chardonnay (15), Chenin blanc (2), Malbec (4), Merlot (13), Muscat varieties (4), Pinot noir (7), Pinotage (9), Sauvignon blanc (18), Shiraz (13), Barbera (1), Viognier (1), Mouvedre (1), Shiraz Rosé (1), Petit Verdot (1) and one unknown white cultivar.

4.2.2 HEADSPACE SOLID-PHASE DYNAMIC EXTRACTION PROCEDURE

Headspace solid-phase dynamic extraction (HS-SPDE) was performed to avoid direct contact between the sample matrix and the needle. The volatile wine compounds were extracted after optimisation of the major parameters showing influence in the extraction process (Câmara *et al.*, 2004, 2006): time and temperature of adsorption, ionic strength and pH. Samples were defrosted and for each SPDE analysis, 10 mL of wine was transferred to a 20 mL headspace glass vial (La-Pha Pack, Langerwehe, Germany) containing 1 g of sodium chloride (NaCl) (Saarchem, Merck, Gauteng) and a small magnetic stir bar. The addition of NaCl facilitates maximum amounts of volatiles in the headspace by decreasing their water solubility. 100 µL of internal standard solution of 2-octanol (Sigma-Aldrich, Germany) was added, giving a final concentration of 2 mg/L. The internal standard solution was prepared by adding 50 mg 2-octanol to 250 mL of absolute ethanol (Sigma-Aldrich, Germany). The vials were tightly

sealed with screw-caps fitted with PTFE-Teflon septa (La-Pha Pack, Langerwehe, Germany). A 10 minute pre-equilibration step was sufficient for the sample and headspace to equilibrate completely (data not shown). For the extraction procedure, a 74 mm PDMS/AC (90% polydimethylsiloxane and 10% activated carbon) coated needle (Chromsys, Alexandria VA, USA), connected to a 2.5 mL gas-tight syringe, performed 50 aspiration repeats of 1000 μL each at 70 $\mu\text{L/s}$ while the sample was continuously agitated with a magnet at 750 rpm (bi-directional). The needle was then removed from the sample vial and immediately inserted into the sample port of the GC where 500 μL helium (Afrox, Cape Town) was pulled into the syringe. Desorption was achieved in the GC inlet (heated to 230°C, splitless mode) by pumping the helium through the needle into the inlet at 15 $\mu\text{L/s}$. Post-desorption bake-out of the needle at 270°C for 10 min was shown to ensure full desorption of all analytes from the needle coating and this was shown to avoid carry-over between injections.

4.2.3 GAS CHROMATOGRAPHY-MASS SPECTROSCOPY CONDITIONS

GC-MS analysis was performed using a gas chromatograph (Agilent Technologies, model 6890N, Network GC system, USA) coupled to a mass selective detector (Agilent Technologies, model 5973 *inert*, Network GC system, USA) and equipped with Enhanced Chemstation version D.01.02.16 software (Agilent technologies, Little Falls, Wilmington, USA). The GC was fitted with a CTC CombiPal autosampler (CTC Analytics, Switzerland) in SPDE mode. Compounds were separated on a J&W DB-WAX capillary column (Agilent technologies, model number 122-7033) with dimensions 30 m x 0.25 mm i.d. (inside diameter), 0.5 μm film thickness. Splitless injection mode was used with split vent closed for 2 minutes. The initial oven temperature was 35°C, held for 2 min and then increased to 220°C at 5°C/min and held for 6 min. Post-run time was 2 min at 220°C. The injector temperature was 230°C and the transfer line was held at 240°C. The carrier gas was helium at 0.8 mL/min, constant flow. The mass spectrometer was set in electron-impact (EI) mode at 70 eV covering a mass-to-charge ratio range (m/z) from 29 to 280 atomic mass units (amu). The ion source and quadrupole temperatures were set to 230°C and 150°C, respectively. The overall GC run time was 47 min.

Peak identification of the volatile components was achieved by comparison of mass spectra and confirmation with GC retention indices of standards (Sigma-Aldrich, Germany and Merck, Gauteng) and with spectral data from the Wiley 7th and NIST 98 mass spectral libraries. Separate ions for each component, usually the most prominent in the mass spectrum, were used for component integration. Components' ion chromatogram peak areas were measured and divided by the peak area of the internal standard to obtain the corrected peak areas. These corrected peak areas and not the actual concentrations were used for further data analysis.

4.2.4 CHEMOMETRICS AND DATA ANALYSIS

4.2.4.1 Data processing

The chemical data, consisting of corrected peak areas (peak area/internal standard area), obtained from the SPDE GC-MS analysis were imported into *The Unscrambler* software (version 9.2, CAMO ASA, Norway) for the purpose of principal component analysis (PCA) and partial least squares (PLS) regression. The objects were fermenting must samples from various cultivars and stages of fermentation. The data matrix with rows representing must samples (objects) and columns corresponding to volatile aroma compounds (variables) was used for computerised multivariate analysis. These techniques were applied to the scaled corrected peak areas of the volatile compounds. Scaling was performed as follows: the individual peak area for a specific compound was divided by the average peak area for that compound. The whole data matrix comprised 97 objects and 68 volatile variables.

4.2.4.2 Principal component analysis (PCA)

Principal component analysis (PCA) is an unsupervised technique frequently used to reduce the dimensionality and the complexity of the original data matrix whilst retaining the maximum amount of variability (Adams, 1995; Otto, 1999; Martens and Martens, 2001; Naes *et al.*, 2002). The projection of the samples in a multi-dimensional space allows for the identification of the main directions of variance, depicted by a principal component (PC). It is therefore possible to interpret the relationships between various samples in the score plot defined by the PC's and to study the relationship between variables and objects in the loadings plot. Samples with similar (sharing high loadings for some compounds in the loadings plot) aroma compositions cluster together and PCA allows for these possible sample groupings to be identified. Similarly, PCA also allows for the discrimination between samples which differ in aroma composition. In addition, variables which contribute the most to differences between samples could be identified and variables which are highly correlated with each other could also be identified.

4.2.4.3 Discriminant Partial Least Squares (DPLS or PLS-Discrim) regression

Partial least squares regression models were constructed for discrimination purposes by using a no metric dummy variable (Y-variable) as a reference value (Osborne *et al.*, 1993; Ding *et al.*, 1999; Naes *et al.*, 2002). This dummy variable is an arbitrary number for a sample belonging to a particular group. The discriminant PLS model (DPLS model) was developed by regression of the headspace SPDE GC-MS data (X-variables/matrix) against the assigned reference value (dummy variable). Two different types of discrimination models were constructed and tested. The ability of a model to discriminate between normal and problem

fermentations was tested by assigning a dummy variable (signified by -1 for stuck/sluggish samples and +1 for control fermentation samples) to the samples. Similarly, samples were also assigned a dummy variable (signified by -1 for red cultivars and +1 for white cultivars) to test the ability of another model to discriminate between red and white cultivars using only headspace data. The purpose of constructing these regression models was to investigate the potential of the models to differentiate between problem and control fermentations and between cultivar differences using only headspace analysis.

4.3 RESULTS AND DISCUSSION

4.3.1 OPTIMISATION OF METHODOLOGY FOR GC-MS

All experimental conditions (a selection of the results are summarized in **Table 4.1**) including extraction temperature and time, ionic strength, ethanol contents and pH (Câmara *et al.*, 2004, 2006) of the sample matrix as well as the settings and oven program for the GC-MS analysis were developed and optimised by Dr. Vivian Watts (KWV, Paarl).

Table 4.1 Method development results (with the permission of Dr. V. Watts).

| Parameter | Specific parameters tested | Optimum chosen |
|------------------------------------|---------------------------------------|----------------|
| Extraction temperature | 30°C, 50°C, 70°C | 40°C |
| Number of aspirations | 10, 25, 50, 75 | 50 |
| Aspiration/injection plunger speed | 30 µL/s, 50 µL/s, 70 µL/s, 100 µL/s | 70 µL/s |
| Type of salt | NaCl, Na ₂ SO ₄ | NaCl |
| Helium desorption volume | 500 µL, 1000 µL, 2500 µL | 500 µL |
| Desorption speed | 10 µL/s, 20 µL/s, 40 µL/s | 20 µL/s |
| Injection mode | Split/splitless | Splitless |

These conditions were optimised prior to the analysis of the 97 samples. The optimum conditions were chosen on the following criteria: peak shape and intensity and sensitivity. Some parameters (extraction temperature and injection type) were optimised were based on peak shape and overall chromatographic quality and not purely maximum sensitivity. A PDMS/AC coated needle was used to analyse fermentation products such as esters, higher alcohols, organic- and fatty acids. The repeatability of a selection of the wine aroma volatiles measured with this method is given in **Table 4.2**. Only the repeatability for some long-chain compounds (octanoic acid, decanoic acid, hexanoic acid, β-phenethyl alcohol, diethyl succinate and isobutyl decanoate) was in excess of 10% relative standard deviation (%RSD).

Table 4.2 Repeatability for a selection of the measured wine aroma volatiles (with the permission of Dr. V. Watts).

| Component | Ion | % RSD (n=6) | Component | Ion | % RSD (n=6) |
|------------------------|-----|-------------|----------------------------|-----|-------------|
| isobutyl acetate | 43 | 1.55 | ethyl octanoate | 88 | 2.40 |
| ethyl butyrate | 71 | 1.95 | acetic acid | 60 | 10.04 |
| n-propanol | 31 | 9.37 | isoamyl hexanoate | 70 | 4.03 |
| ethyl-2-methylbutyrate | 102 | 3.19 | propyl octanoate | 145 | 4.19 |
| ethyl-3-methylbutyrate | 88 | 3.08 | ethyl nonanoate | 88 | 4.51 |
| isobutanol | 43 | 7.05 | 1-octanol | 56 | 4.09 |
| isoamyl acetate | 70 | 0.69 | ethyl decanoate | 88 | 4.56 |
| n-butanol | 56 | 2.40 | isoamyl octanoate | 70 | 6.29 |
| ethyl-2-butenolate | 69 | 1.09 | diethyl succinate | 101 | 13.36 |
| amyl alcohols | 70 | 1.24 | TDN | 157 | 8.53 |
| isoamyl butyrate | 71 | 2.32 | β -phenethyl acetate | 104 | 4.99 |
| hexyl acetate | 43 | 1.13 | β -damascenone | 69 | 6.73 |
| trans-hexenyl acetate | 67 | 1.09 | ethyl dodecanoate | 88 | 9.77 |
| ethyl heptanoate | 88 | 2.39 | hexanoic acid | 60 | 16.99 |
| ethyl lactate | 45 | 6.58 | isobutyl decanoate | 70 | 10.77 |
| n-hexanol | 56 | 1.19 | β -phenethyl alcohol | 91 | 23.98 |
| cis-3-hexen-1-ol | 67 | 3.48 | octanoic acid | 60 | 20.90 |
| tran-3-hexen-1-ol | 67 | 1.96 | decanoic acid | 60 | 21.64 |
| 2-octanol (Int. Std) | 45 | 1.36 | | | |

Given the often voiced opinion that relative GC-MS peak areas should not be interpreted on its own, but rather be supported by a validated method such as GC-FID, it was shown that the peak areas obtained with SPDE for a white wine sample correlate well with the actual concentrations of the analytes as determined using a calibrated solvent extraction GC-FID method (**Fig. 4.1**). Since this method has been validated in the classical appropriate analytical chemistry, it is of great interest that there is such a good relative relationship between the two techniques.

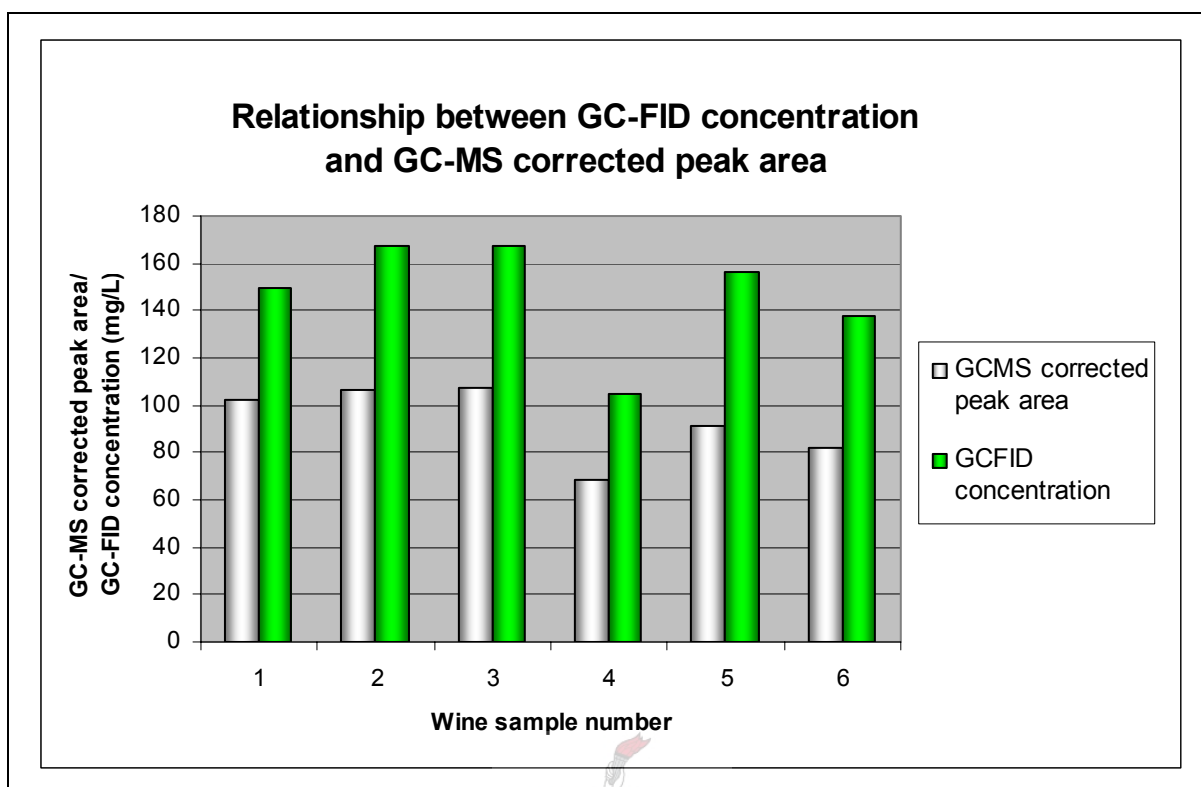


Figure 4.1 Peak areas obtained with SPDE for a white wine correlate well with the actual concentrations of the analytes as determined using a calibrated solvent extraction GC-FID method (results with permission of Dr. V. Watts).

Although method development is an ongoing process, it was found that this technique is suitable for wine analysis with excellent repeatability and lots of promise for future use since a wealth of information is determined with this technique.

4.3.2 PCA MODELLING

4.3.2.1 PCA as a tool to follow the evolution of compounds formed during fermentation

Samples were collected during various stages of fermentation and for this reason the possibility to follow the evolution of volatile compounds during the fermentation process using headspace data was investigated. Principal component analysis (PCA) was performed on 68 analytical compounds obtained from the HS-SPDE GC-MS analysis of fermenting must samples in order to identify the main sources of variation between the 97 different samples (**Fig. 4.2**). Separation along the first principal component (PC1) effectively reflects mainly the separation between red (indicated by blue coloured word “red”) and white (indicated by the red coloured word “white”) cultivars. This could be the result of two types of vinification practices, namely vinification with maceration (red cultivars) and vinification without maceration (white cultivars). This result was also observed in a study by Piñeiro *et al.* (2006)

which investigated the influence of winemaking practices on the volatile composition of monovarietal wines. However, this is not the only possible reason for such separations between these two groups of cultivars as it has been shown that various cultivars have characteristic precursor compounds (for example amino acids) present in the grape juice (Huang and Ough, 1991; Guitart *et al.*, 1999) which could be metabolised to volatile compounds (amongst other things) by yeast (Longo *et al.*, 1992).

Samples from the early stages of fermentation, with almost no alcohol and yeast fermentation products present did not form separate clusters for red and white cultivar groups. Observations suggest that the progression of red (indicated by arrow A) and white (indicated by arrow B) fermentations results in the increased differentiation observed between red and white cultivars. This is most likely due to the subsequent formation of fermentation products as the fermentation progresses. This observation is supported by inserting a category variable for stage of fermentation, and it is clear that separation along PC2 is possibly a result of the fermentation progress from early (indicated by blue coloured: “early”), middle (indicated by red coloured: “mid”) to late (indicated by green coloured: “late”) fermentation stages.

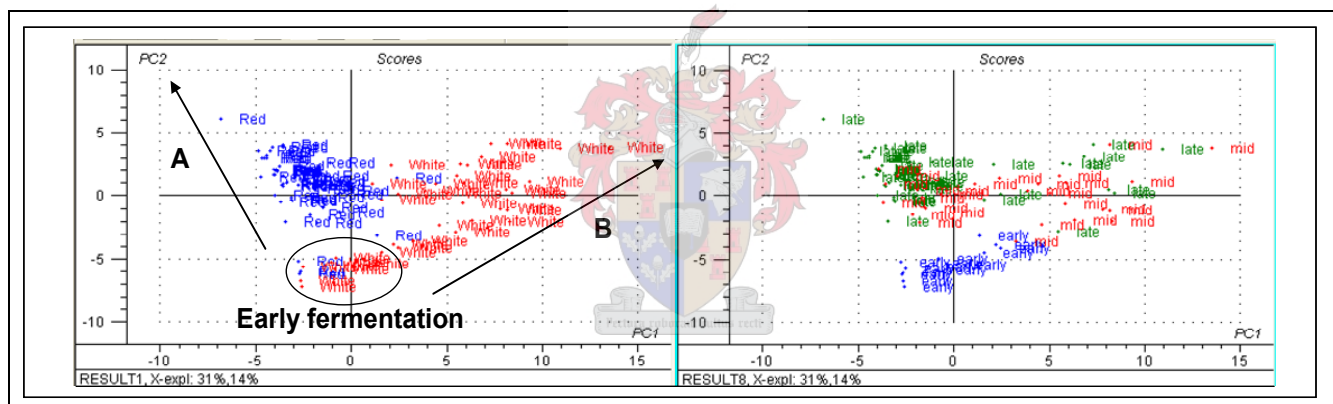


Figure 4.2 Score plot of PC1 vs PC2. PCA was performed on the volatile components and discrimination between red (indicated by blue) and white (indicated by red) cultivars were achieved along PC1. Separation along PC2 was assigned to fermentation stage: early (blue), mid (red) and late (green).

Variables with high loading weights (both negative and positive) represent those variables which significantly contribute to the separation between red and white cultivars along PC1 (loading weights for PC1 listed in **Table 4.3**). The group of variables with the highest negative loading weights (indicated by *) contributed most significantly in explaining the separation along PC1 and these variables are associated with the red cultivar grouping towards the left hand side of the graph (**Fig. 4.2**). The variables (*) include fusel alcohols (isoamyl, isobutanol, β -phenyl ethanol) with some acids (isobutyric) and minority alcohols (4-methyl pentanol and octanol). These are generated by the yeast amino acid metabolism (Nykänen, 1986).

Table 4.3 The loading weights (W) for each volatile component along the first principal component (PC1). High loadings (both negative and positive) indicate significant variables.

| Component | W-PC1 | Component | W-PC1 | Component | W-PC1 |
|--------------------------|-----------|------------------------------|----------|---------------------|-------|
| isobutanol* | -0.126 | ethyl nonanoate | 2.82E-02 | isobutyl hexanoate◇ | 0.188 |
| 4-methylpentanol* | -0.125 | β-damascenone | 4.43E-02 | isobutyl octanoate◇ | 0.190 |
| β-phenyl ethanol* | -0.124 | isoamyl butyrate | 4.78E-02 | ethyl octanoate◇ | 0.193 |
| n-butanol* | -0.109 | ethyl heptanoate | 4.95E-02 | hexanoic acid◇ | 0.196 |
| isobutyric acid* | -0.107 | isobutyl acetate | 5.52E-02 | decanoic acid◇ | 0.196 |
| ethyl-2-methylbutyrate* | -0.103 | ethyl-2-butenolate | 5.90E-02 | isoamyl hexanoate◇ | 0.199 |
| ethyl-3-methylbutyrate* | -0.103 | trans-3-hexen-1-ol | 5.96E-02 | hexyl acetate◇ | 0.201 |
| 1-octanol* | -0.102 | ethyl-2-hexenoate | 6.79E-02 | Octanoic acid◇ | 0.203 |
| active/isoamyl alcohols* | -0.1 | cis-3-hexen-1-ol | 7.47E-02 | isoamyl octanoate◇ | 0.208 |
| ethyl lactate* | -9.72E-02 | heptyl acetate | 8.40E-02 | isobutyl hexanoate◇ | 0.188 |
| pentyl acetate* | -9.62E-02 | ethyl-3/4-hexenoate | 8.46E-02 | | |
| vitispirane* | -8.77E-02 | ethyl butyrate | 9.21E-02 | | |
| diethyl succinate* | -8.33E-02 | acetoin | 9.28E-02 | | |
| benzyl alcohol° | -6.92E-02 | β-phenethyl acetate◇ | 0.118 | | |
| unknown succinate ester° | -6.88E-02 | ethyl-9-decenoate◇ | 0.121 | | |
| ethyl benzoate° | -6.26E-02 | propyl decanoate (both)◇ | 0.135 | | |
| n-propanol° | -5.34E-02 | octyl acetate◇ | 0.143 | | |
| 1-octen-3-ol° | -5.18E-02 | isoamyl acetate◇ | 0.153 | | |
| ethyl acetate° | -3.67E-02 | hexenyl acetate (cis/trans)◇ | 0.156 | | |
| benzaldehyde° | -3.38E-02 | methyl hexanoate◇ | 0.159 | | |
| acetic acid° | -3.08E-02 | propyl hexanoate◇ | 0.16 | | |
| n-hexanol° | -2.31E-02 | hexenyl acetate (cis/trans)◇ | 0.17 | | |
| ethyl pentanoate° | -2.23E-02 | methyl octanoate◇ | 0.174 | | |
| hexanal | -2.64E-03 | propyl octanoate◇ | 0.175 | | |
| linalool | 1.81E-03 | ethyl dodecanoate◇ | 0.176 | | |
| nerolidol | 4.93E-03 | isoamyl decanoate◇ | 0.18 | | |
| methyl salicylate | 6.15E-03 | ethyl decanoate◇ | 0.183 | | |
| n-butyric acid | 1.89E-02 | ethyl hexanoate◇ | 0.185 | | |
| TDN | 2.09E-02 | methyl decanoate◇ | 0.186 | | |

Variables also correlated to red wines but to a lesser extent are indicated by ($^{\circ}$). This is reflected by the lower loading weights (W) for these variables which indicates a less significant contribution to the separation along a specific PC, in this case PC1.

Variables which have the highest positive loading weights (indicated by \diamond) in **Table 4.3** significantly contribute in the separation along PC1 and these variables are strongly correlated with the white cultivars situated on the right hand side of the graph (**Fig. 4.2**). Hexanoic, octanoic and decanoic acids and their ethyl esters appear to be strongly correlated with each other and cluster together on the loading weights plot for PC1 (graph not shown). Therefore, it appears as if the compounds from yeast fatty acid metabolism (Nykänen, 1986) are grouped together and correlates with the white cultivars. Acetates of some fusel alcohols (isoamyl, hexanol and phenyl ethanol) appear next to them on the loading weights plot.

In order to establish which variables contributed significantly to the separation along PC2, which was associated with the progression of fermentation from early to mid and finally late fermentation, loading weights for PC2 were evaluated (**Table 4.4**). Interestingly enough, the majority of the volatile compounds had positive loading weights (\blacktriangle) relative to the percentage of variables with negative loading weights (\ominus). The positive loading weights relate to the mid and late fermentation stages situated more towards the middle and top half (positive) of the graph along PC2. The small amount of significantly low loading weights could be related to the negative half of the graph along PC2, indicating the early stages of fermentation with little or no formation of volatile compounds as a result of yeast metabolism.

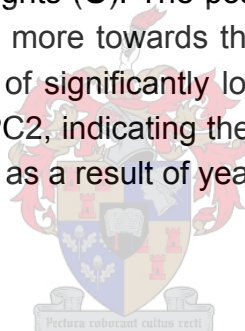


Table 4.4 The loading weights (W) for each volatile component along the second principal component (PC2). High loadings (both negative and positive) indicate the significance of variables.

| Component | W-PC2 | Component | W-PC2 | Component | W-PC2 |
|--|-----------|--------------------------------------|----------|--------------------------------------|-------|
| hexanal [⊖] | -0.116 | ethyl pentanoate [⊠] | 6.61E-02 | acetic acid [⊠] | 0.200 |
| benzaldehyde [⊖] | -8.91E-02 | isoamyl octanoate [⊠] | 6.68E-02 | isoamyl butyrate [⊠] | 0.213 |
| trans-3-hexen-1-ol [⊖] | -5.94E-02 | TDN [⊠] | 7.96E-02 | isobutanol [⊠] | 0.214 |
| acetoin [⊖] | -4.46E-02 | hexanoic acid [⊠] | 7.99E-02 | ethyl butyrate [⊠] | 0.215 |
| octyl acetate [⊖] | -3.21E-02 | ethyl-2-hexenoate | 8.29E-02 | ethyl-2-butenolate [⊠] | 0.219 |
| n-hexanol [⊖] | -3.10E-02 | β-phenethyl acetate [⊠] | 8.43E-02 | n-butanol [⊠] | 0.229 |
| β-damascenone [⊖] | -3.00E-02 | ethyl nonanoate [⊠] | 8.78E-02 | pentyl acetate [⊠] | 0.238 |
| decanoic acid [⊖] | -2.21E-02 | ethyl hexanoate [⊠] | 9.51E-02 | active/isoamyl alcohols [⊠] | 0.249 |
| ethyl-9-decenoate [⊖] | -9.45E-03 | ethyl dodecanoate | 9.97E-02 | ethyl acetate [⊠] | 0.258 |
| cis-3-hexen-1-ol [⊖] | -6.35E-03 | ethyl octanoate [⊠] | 0.1 | | |
| hexyl acetate [⊠] | 1.23E-03 | 1-octanol [⊠] | 0.101 | | |
| hexenyl acetate (cis/trans) [⊠] | 7.71E-03 | benzyl alcohol [⊠] | 0.102 | | |
| methyl octanoate [⊠] | 7.78E-03 | isobutyl acetate [⊠] | 0.104 | | |
| hexenyl acetate (cis/trans) [⊠] | 1.04E-02 | ethyl decanoate [⊠] | 0.11 | | |
| heptyl acetate [⊠] | 1.30E-02 | propyl decanoate (both) [⊠] | 0.111 | | |
| octanoic acid [⊠] | 2.25E-02 | isoamyl decanoate [⊠] | 0.112 | | |
| nerolidol [⊠] | 2.77E-02 | unknown succinate ester [⊠] | 0.114 | | |
| linalool [⊠] | 3.40E-02 | vitispirane [⊠] | 0.123 | | |
| propyl octanoate [⊠] | 3.53E-02 | ethyl-3/4-hexenoate [⊠] | 0.133 | | |
| propyl hexanoate [⊠] | 4.00E-02 | isoamyl acetate [⊠] | 0.139 | | |
| n-butyric acid [⊠] | 4.14E-02 | diethyl succinate [⊠] | 0.147 | | |
| methyl decanoate [⊠] | 4.56E-02 | ethyl lactate [⊠] | 0.156 | | |
| methyl hexanoate [⊠] | 4.66E-02 | ethyl-2-methylbutyrate [⊠] | 0.161 | | |
| methyl salicylate [⊠] | 5.53E-02 | ethyl-3-methylbutyrate [⊠] | 0.161 | | |
| ethyl heptanoate [⊠] | 5.62E-02 | β-phenethyl alcohol [⊠] | 0.175 | | |
| isobutyl hexanoate [⊠] | 5.99E-02 | isobutyric acid [⊠] | 0.182 | | |
| isobutyl octanoate [⊠] | 6.23E-02 | 4-methylpentanol [⊠] | 0.184 | | |
| isoamyl hexanoate [⊠] | 6.31E-02 | ethyl benzoate [⊠] | 0.184 | | |
| 1-octen-3-ol [⊠] | 6.44E-02 | n-propanol [⊠] | 0.195 | | |

In order to investigate the above-mentioned observation more clearly PCA (shown in **Fig. 4.3**) was performed on three subsets (early, middle and late fermentation) of samples respectively. The definitions of these descriptors are given in **Table 4.5**.

Table 4.5 Definitions of the fermentation progress descriptors.

| Descriptor | Definition |
|------------|---|
| early | less than 4% v/v alcohol |
| middle | more than 4% v/v alcohol; more than 20 g/L residual sugar |
| late | less than 20 g/L residual sugar |

PCA on the volatile data of the early stages showed no clear distinction between red and white cultivars (**Fig. 4.3**). Samples in the middle stages of fermentations showed increased differentiation between red and white cultivars (**Fig. 4.3**). The best distinction between red and white cultivars was achieved by modelling samples in the late stages of fermentation. This definitely points to the importance of the formation of volatile compounds by yeast during fermentation and that these type of observations could somehow be linked to yeast physiology. Additional experiments are however needed to further explore these observations and speculation.

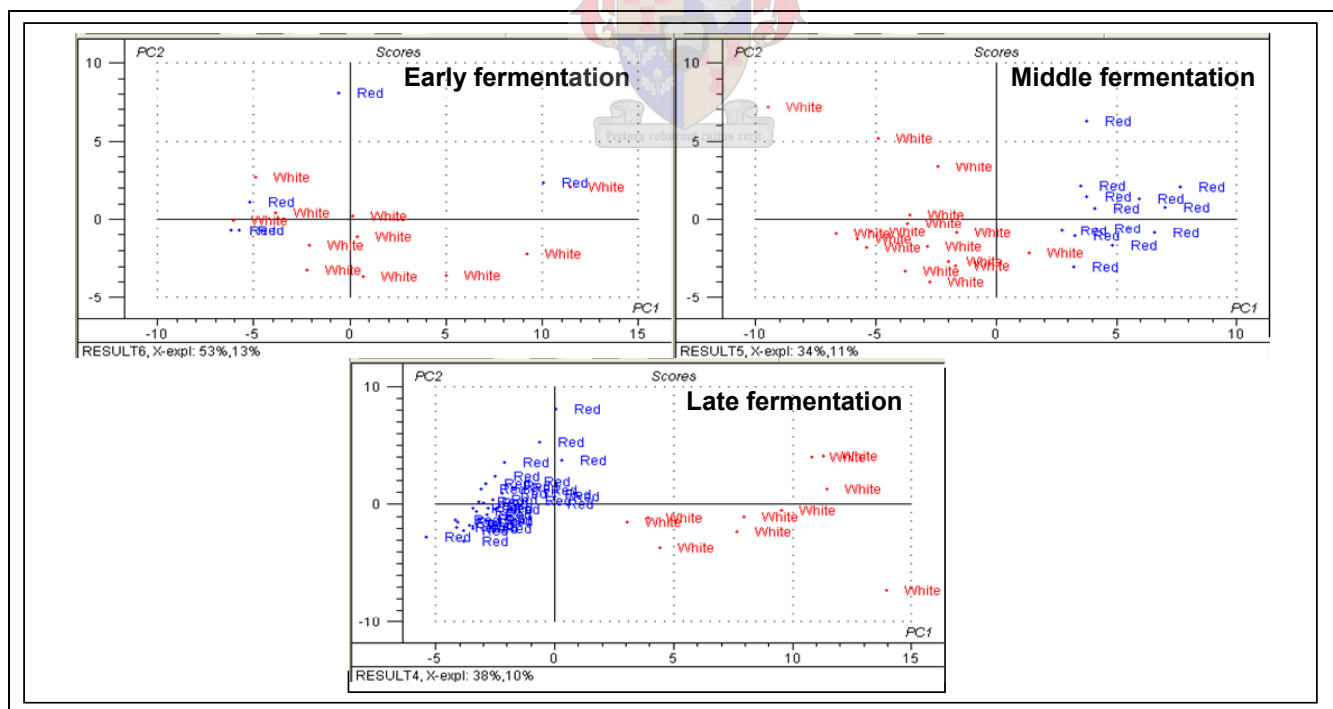


Figure 4.3 PCA score plot for samples in early fermentation, middle fermentation and late fermentation stages. Differentiation between red and white cultivars improved with fermentation progress.

The influence of oenological practices, including yeast and fermentation conditions, in the composition of the volatile profile of wines is therefore evident. Obvious differences in the preparation of white and red wines could possibly explain the observed results. Fatty acids and their ethyl esters are correlated with white wines possibly due to the lower fermentation temperatures used to prepare white wines in comparison to red wine. Vinification with maceration (red cultivars) resulted in the more abundant formation of ethyl esters of lactic, acetic and succinic acid. Vinification without maceration (white cultivars) showed an increase of fatty acids and their ethyl esters. Interestingly enough, red and white cultivars could be distinguished from each other with only fermentation products. No colour or phenolic compounds which are normally associated with this differentiation were taken into account in this analysis and this separation between red and white cultivars was based only on headspace analysis.

4.3.2.2 PCA used for pattern recognition

Based on the wealth of information captured in the headspace analysis, it was of interest to investigate whether the possibility to differentiate between control and problem fermentations within a cultivar was feasible. For this reason the most abundant cultivars, Chardonnay (n=14) and Sauvignon blanc (n=14), were chosen. PCA analysis was performed on the headspace analysis of these cultivars respectively to investigate the stated objective (**Fig. 4.4**). Differentiation between control and problem fermentations was achieved for Chardonnay and Sauvignon blanc samples. Control fermentation samples were collected throughout the fermentation (fermentation progress indicated by the arrow) and it shows that fermentation problems occurred from middle of fermentation onwards for both cultivars in this sample set.

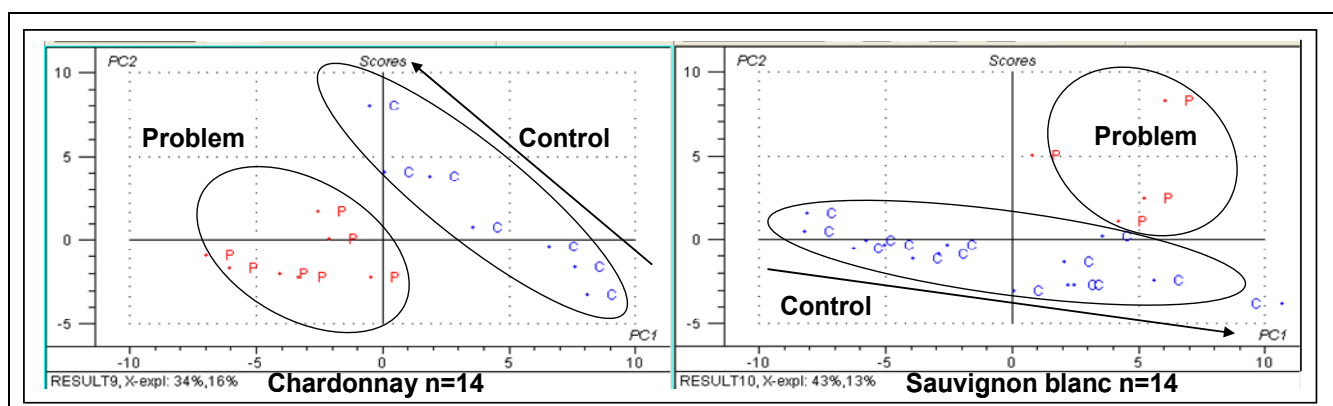


Figure 4.4 Score plot of PC1 vs PC2. PCA was performed on the headspace volatile components of Chardonnay and Sauvignon blanc datasets. Data shows the possibility of differentiation between control (blue C) and problem (red P) fermentations was achieved. Fermentation progress is indicated by the arrow in both figures.

4.3.3 PLS-DISCRIM

4.3.3.1 PLS-discrim as a tool for possible discrimination purposes

A PLS discriminant regression model was constructed to differentiate between red and white cultivars (**Fig. 4.5**). This was performed to evaluate the interaction between the X-variables (headspace analysis) and the desired Y-variable. In this case the Y-variable was the dummy variable assigned to each sample to test the discrimination ability of the model. 42% of the X-variance related to the headspace analysis (X-variables) modelled 78% of the Y-variance. It is clear from the regression plot that the samples in the early fermentation are the ones overlapping on the regression plot (predicted vs measured).

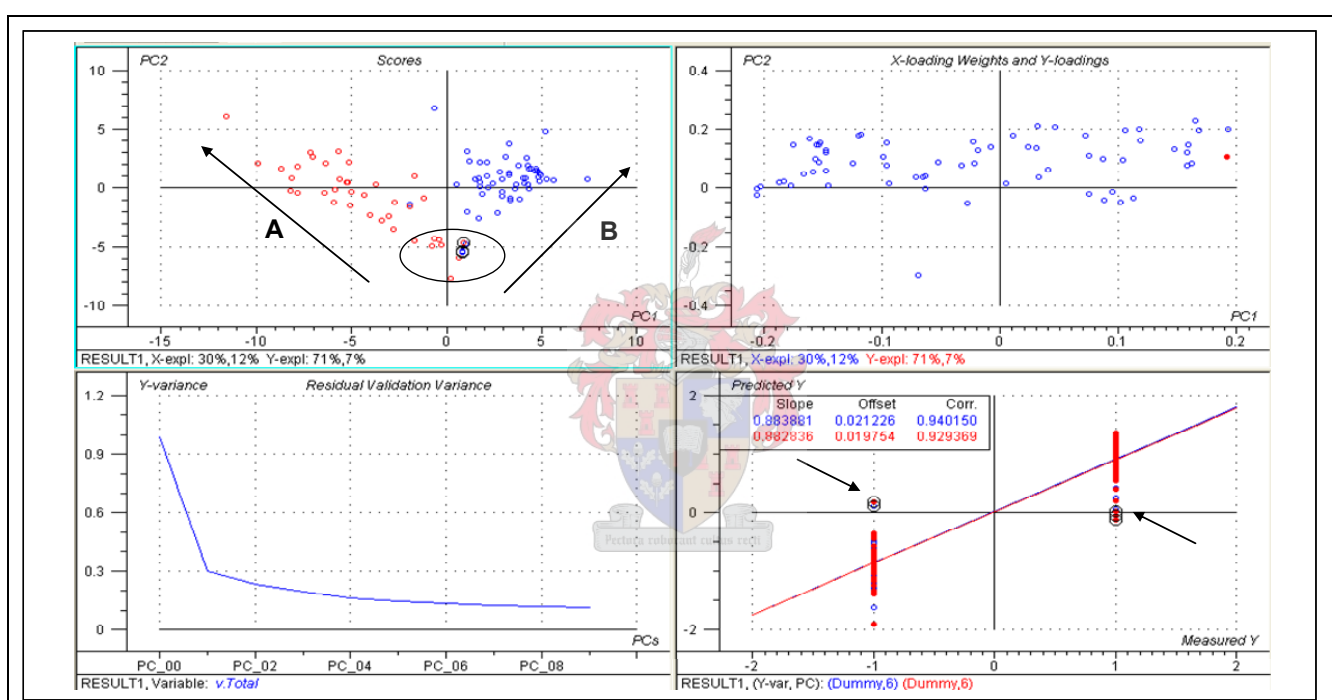


Figure 4.5 Overview of discrimination between red (blue circles) and white (red circles) cultivars. 42% of the X-variance modelled 78% of the Y-variance. Arrow A indicates the fermentation progress for white cultivars and arrow B for red cultivars. The black circles shows samples in the early stage of fermentation which could not be classified successfully into red or white cultivars.

Headspace data was used to investigate the feasibility of this data to discriminate between control and problem fermentations by constructing a PLS-disrim regression model (**Fig. 4.6**). All the samples (including both white and red cultivars) were used for this model. The first two PCs modelled 44% X-variance and 74% of the Y-variance. Due to the combination of red and white cultivars, observations also identified a secondary structure (indicated by the dashed ovals) within this model (**Fig. 4.6**). This implicated the ability to

potentially discriminate between problem and control fermentations for red and white cultivars, respectively.

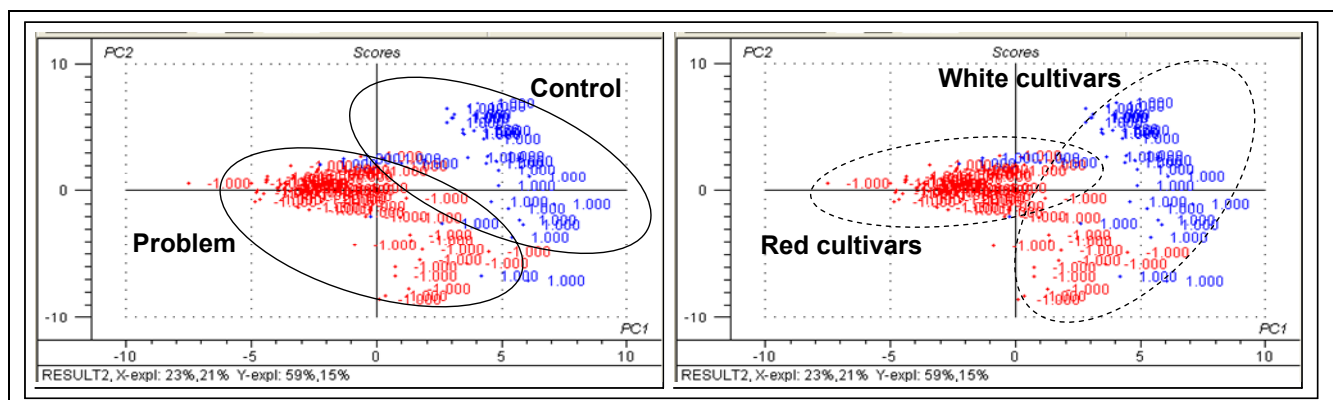


Figure 4.6 Score plot of PC1 vs PC2 indicates partial discrimination between problem (red) and control (blue) fermentation samples. A secondary discrimination structure (indicated by dashed ovals) differentiates between problem and control fermentations for red and white cultivars, respectively. PC1 modelled 23% of the X-variance and 59% Y-variance.

Subsequently, PLS models to discriminate between control and problem fermentations were constructed for red and white cultivars respectively (**Fig. 4.7**). Clear discrimination between control and problem fermentations was achieved for red cultivars (indicated by graph with solid ovals). The first two PCs modelled 21% X-variance and 84% Y-variance. High loadings for ethyl-3/4-hexenoate, acetic acid, isobutyric acid, isobutanol, pentyl acetate, n-butanol, active and isoamyl alcohols, n-butyric acid and ethyl pentanoate correlated with red cultivar problem fermentations. Methyl salicylate, 1-octanol, benzylaldehyde, benzyl alcohol, β -damascenone and to a lesser extent octanoic acid, hexanal, hexyl acetate, n-hexanol and octyl acetate had high loadings for red cultivar control fermentations. The latter was negatively correlated with variables associated with red problem fermentations.

Differentiation between control and problem fermentations was achieved for white cultivars (indicated by the graph with dashed ovals) (**Fig. 4.7**) with the first two PCs modelling 42% X-variance and 86% Y-variance. Ethyl-3-methylbutyrate, ethyl-2-methylbutyrate, ethyl hexanoate, ethyl-3/4-hexenoate, n-butyric acid, isobutanol, isoamyl butyrate, ethyl pentanoate and ethyl benzoate had high loadings for white problem fermentations. A discriminant PLS model using only these variables correlated with white problem fermentations as X-variables, could successfully discriminate between problem and control fermentations (data not shown). High loadings for hexenyl acetate, methyl octanoate, methyl hexanoate, hexyl acetate, β -phenethyl acetate, methyl salicylate and propyl octanoate correlates with white cultivar control fermentations. Decanoic acid, propyl hexanoate, propyl octanoate, octyl acetate and benzyl alcohol also correlates with white cultivar control fermentations, but to a lesser extent.

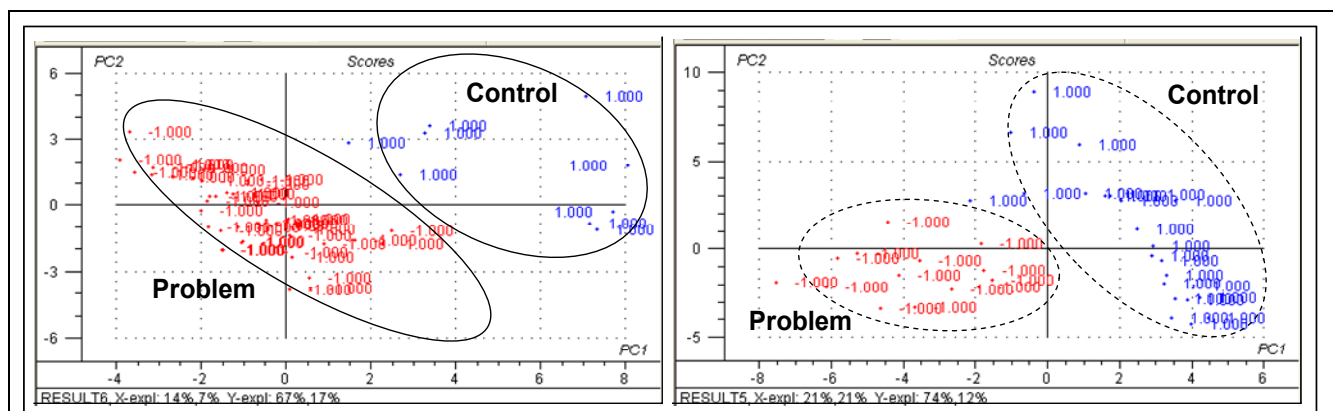


Figure 4.7 Score plot of PC1 vs PC2. Discrimination between problem (red) and control (blue) fermentation samples was achieved for red (indicated by graph with solid ovals) and white (indicated by graph with dashed ovals) cultivars respectively.

4.3.3.2 PLS-discrim as a tool for discrimination purposes within cultivar groups

PLS discriminant models were constructed for the most abundant cultivars in this sample set to investigate the feasibility of possible discrimination between control and problem fermentations within a specific cultivar. Differentiation between control and problem fermentations was achieved for Chardonnay samples with the first three PCs modelling 54% X-variance and 98% Y-variance (**Fig. 4.8**). Variables in group A correlated with problem fermentations. These include ethyl-3/4-hexenoate, ethyl-3-methylbutyrate, ethyl-2-methylbutyrate, ethyl hexanoate, ethyl benzoate, ethyl decanoate, ethyl octanoate, isoamyl butyrate, ethyl butyrate, ethyl-2-hexenoate, ethyl pentanoate, ethyl dodecanoate and ethyl-2-butenate. Ethyl acetate, isoamyl decanoate, hexanoic acid, isobutanol, active and isoamyl alcohols and isoamyl octanoate also forms part of this group of variables but with lower loadings.

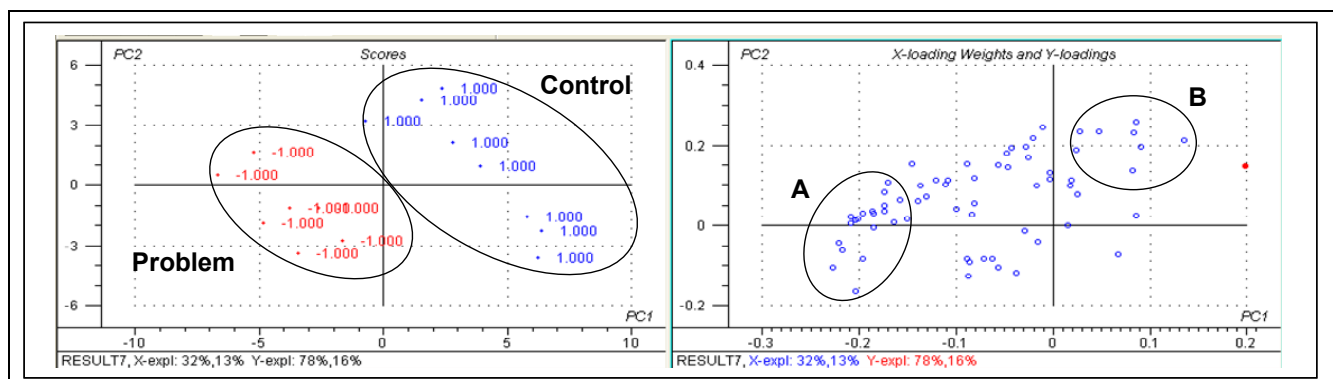


Figure 4.8 Score plot of PC1 vs PC2. Discrimination between control (blue) and problem (red) fermentations was achieved for Chardonnay samples. The loading weights plot show the variables associated with problem fermentations (group A) and control fermentations (group B).

The variables in group B correlate with control fermentations. These include acetoin, methyl hexanoate, methyl octanoate, octyl acetate, TDN, hexanal, hexyl acetate and propyl hexanoate. Similarly, discrimination between control and problem fermentations was also achieved for Sauvignon blanc samples with the implementation of a PLS model (**Fig. 4.9**).

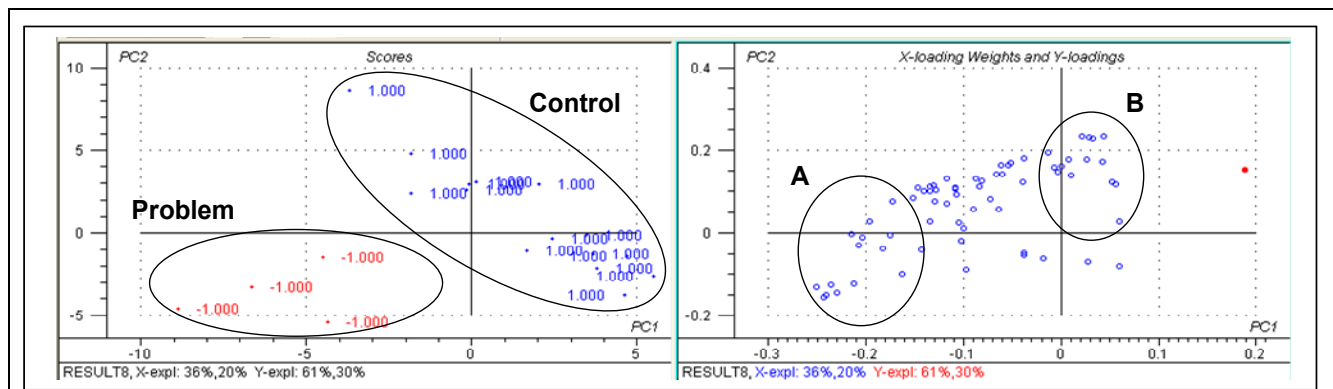


Figure 4.9 Score plot of PC1 vs PC2. 56% of the X-variance modelled 91% of the Y-variance. Discrimination between control (blue) and problem (red) fermentations was achieved for Sauvignon blanc samples. The variables associated with problem fermentations (group A) and control fermentations (group B) are shown on the loading weights plot.

Diethyl succinate, a succinate ester, ethyl lactate, ethyl-3-methylbutyrate, ethyl-2-methylbutyrate, n-butyric acid and 1-octanol had the highest loadings on the loading weights plot (indicated by group A) and correlate with problem fermentations. Additional variables which include ethyl-3/4-hexenoate, ethyl-2-hexenoate, ethyl pentanoate, ethyl heptanoate, isobutyric acid, isoamyl butyrate, *cis*-3-hexen-1-ol and ethyl hexanoate had lower loadings but also correlate with problem fermentations. Variables in group B had high loadings which correlate with control fermentations. This group of variables include hexenyl acetate, methyl octanoate, hexyl acetate, methyl hexanoate, isobutyl acetate and β -phenethyl acetate. Decanoic acid, ethyl-9-decenoate, propyl octanoate, benzyl alcohol, propyl hexanoate and octanoic acid forms part of this group of variables (group B) but contribute to a lesser extent (lower loadings).

In addition, the possibility of discriminating between different cultivars was investigated. Partial discrimination between Sauvignon blanc and Chardonnay samples was achieved (**Fig. 4.10**). The first two PCs modelled 40% of the X-variance and 70% Y-variance. Methyl salicylate, benzyl alcohol, hexenyl acetate, linalool, *trans*-3-hexen-1-ol, α -terpineol and octyl acetate had high loadings (group A) which correlate with Sauvignon blanc samples. Variables which correlate with Chardonnay samples include n-propanol, ethyl acetate, ethyl-2-butenate, ethyl dodecanoate, ethyl decanoate, ethyl butyrate, isobutanol, n-butanol, propyl decanoate, isoamyl decanoate, ethyl octanoate, ethyl hexanoate and pentyl acetate.

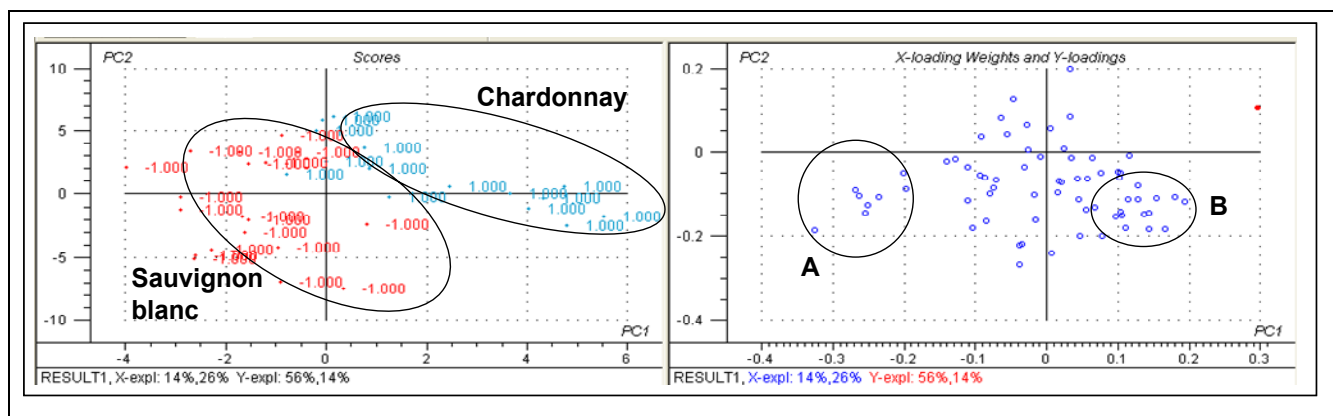


Figure 4.10 Score plot of PC1 vs PC2. Discrimination between Chardonnay (blue) and Sauvignon blanc (red) samples was achieved. Variables which correlate with Sauvignon blanc (group A) and Chardonnay (group B) are shown on the loading weights plot.

Partial discrimination between six red cultivars (Pinot noir, Pinotage, Shiraz, Cabernet Sauvignon, Merlot and Malbec) was achieved with a PLS2 model (**Fig. 4.11**). The most abundant cultivars in this particular sample set were used to evaluate the discrimination potential of this model. The first two PCs explained 38% X-variance and 23% Y-variance.

Additional cultivar discrimination models were constructed to evaluate the relationship between specific cultivars and variables. Discrimination between Cabernet Sauvignon and Pinotage, Shiraz, Malbec, Pinot noir and Merlot respectively, was achieved (**Fig. 4.12**). Ethyl lactate, benzyl alcohol, β -phenethyl alcohol, 4-methylpentanol, diethyl succinate and a succinate ester, n-butanol, pentyl acetate, linalool, β -damascenone, ethyl-2-butenate, ethyl-2-methylbutyrate and ethyl-3-methylbutyrate had high loadings which correlate with Cabernet Sauvignon. The formation of ethyl lactate might be due to the high concentration of lactic acid present in the wine after malolactic fermentation as well as the presence of ethanol (Moreno and Azpilicueta, 2006). A few of the Cabernet Sauvignon samples were in the malolactic fermentation stage and this could explain the high loading for ethyl lactate.

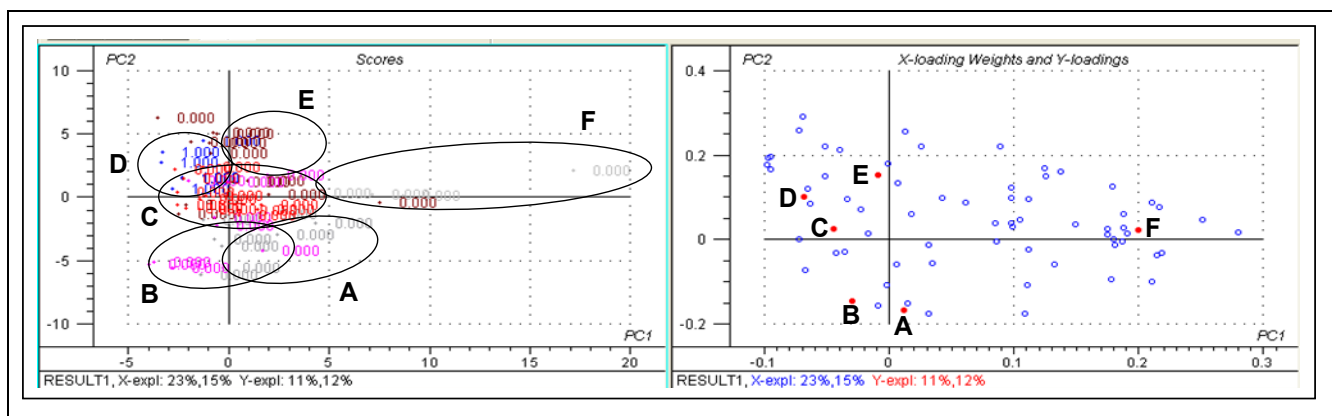


Figure 4.11 Score plot of PC1 vs PC2. Partial discrimination between six red cultivars was achieved. (A) Pinot noir, (B) Pinotage, (C) Shiraz, (D) Cabernet Sauvignon, (E) Merlot and (F) Malbec. The loading weights plot indicates the positions of the various cultivar Y-variables (indicated by the red dots).

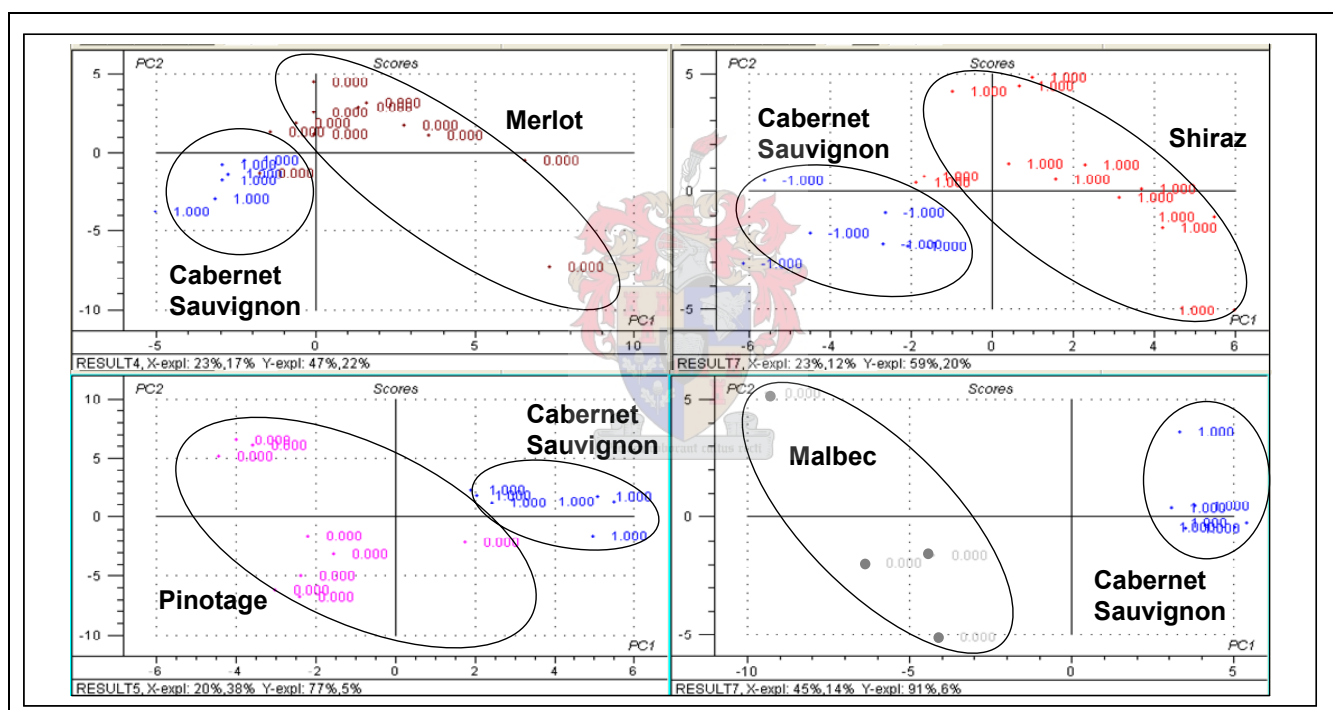


Figure 4.12 Score plot of PC1 vs PC2. Discrimination between Cabernet Sauvignon (blue) and Merlot (brown), Shiraz (red), Pinotage (pink) and Malbec (grey) was achieved.

Discrimination between Merlot and Shiraz, Pinot noir, Pinotage and Malbec respectively, was achieved (**Fig. 4.13**). Isobutyric acid, isobutanol, benzyl alcohol, β -phenethyl alcohol, active and isoamyl alcohols, ethyl-2-methylbutyrate, ethyl-3-methylbutyrate, methyl hexanoate, ethyl benzoate, 4-methylpentanol, propyl decanoate, hexanal, isoamyl decanoate and n-hexanol had high loadings on the loading weights plot and correlate with Merlot

samples. Ethyl pentanoate, n-hexanol, n-butyric acid, ethyl heptanoate, ethyl-9-decenoate and isoamyl decanoate correlate with Malbec samples.

The following cultivar combinations were used to construct discrimination PLS2 models (**Fig. 4.14**): Pinotage, Pinot noir and Shiraz; Shiraz, Pinotage and Merlot; Cabernet Sauvignon, Shiraz and Merlot; Pinotage and Pinot noir. Ethyl-3/4-hexenoate, *trans*-3-hexen-1-ol, 1-octanol, ethyl heptanoate and isoamyl butyrate correlate to Shiraz samples in the discriminant models. Benzyl alcohol, *cis*-3-hexen-1-ol, benzaldehyde, n-hexanol, ethyl heptanoate, 1-octanol, 1-octen-3-ol, methyl salicylate and n-butyric acid correlate to Pinot noir samples. The variables which contribute most significantly to the discrimination of Pinotage samples from the rest of the cultivars are isobutyl acetate, acetoin, propyl octanoate, ethyl hexanoate, isoamyl acetate, isoamyl butyrate, isoamyl decanoate, octyl acetate, n-propanol, propyl decanoate, ethyl decanoate and methyl decanoate.

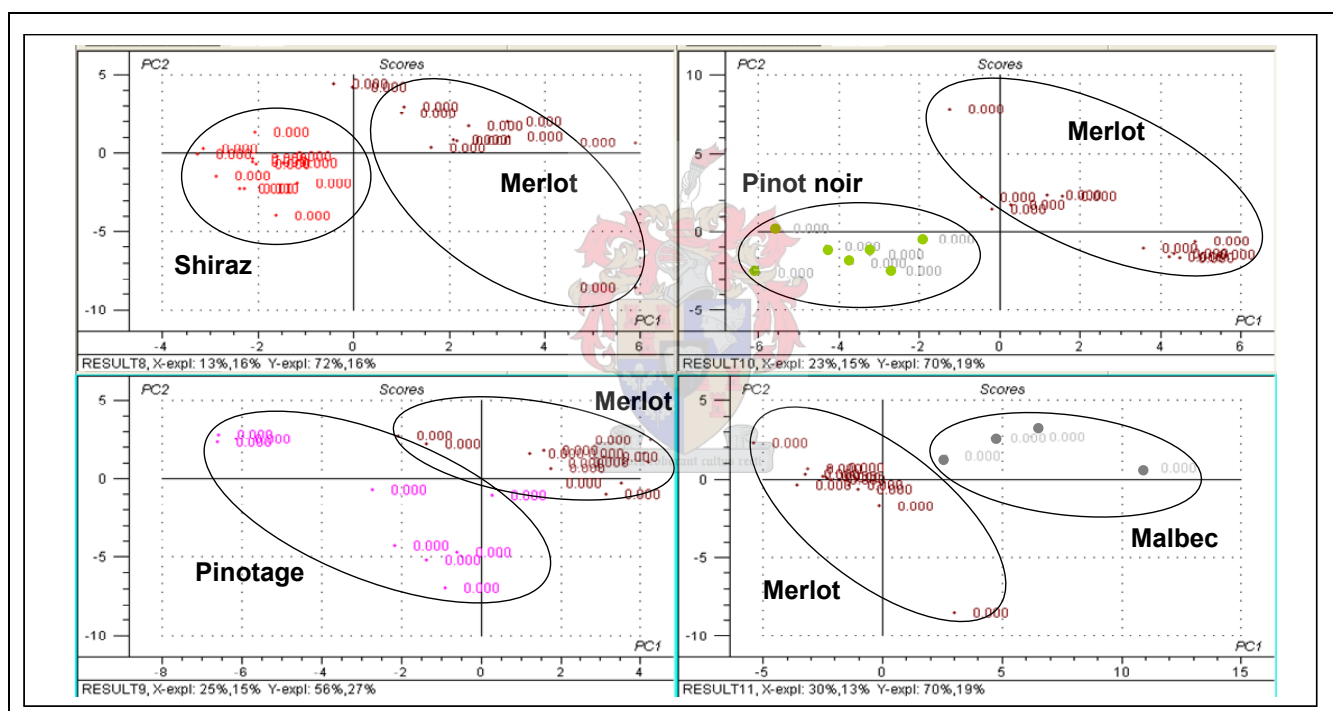


Figure 4.13 Score plot of PC1 vs PC2. Discrimination between Merlot (brown) and Shiraz (red), Pinot noir (green), Pinotage (pink) and Malbec (grey) was achieved.

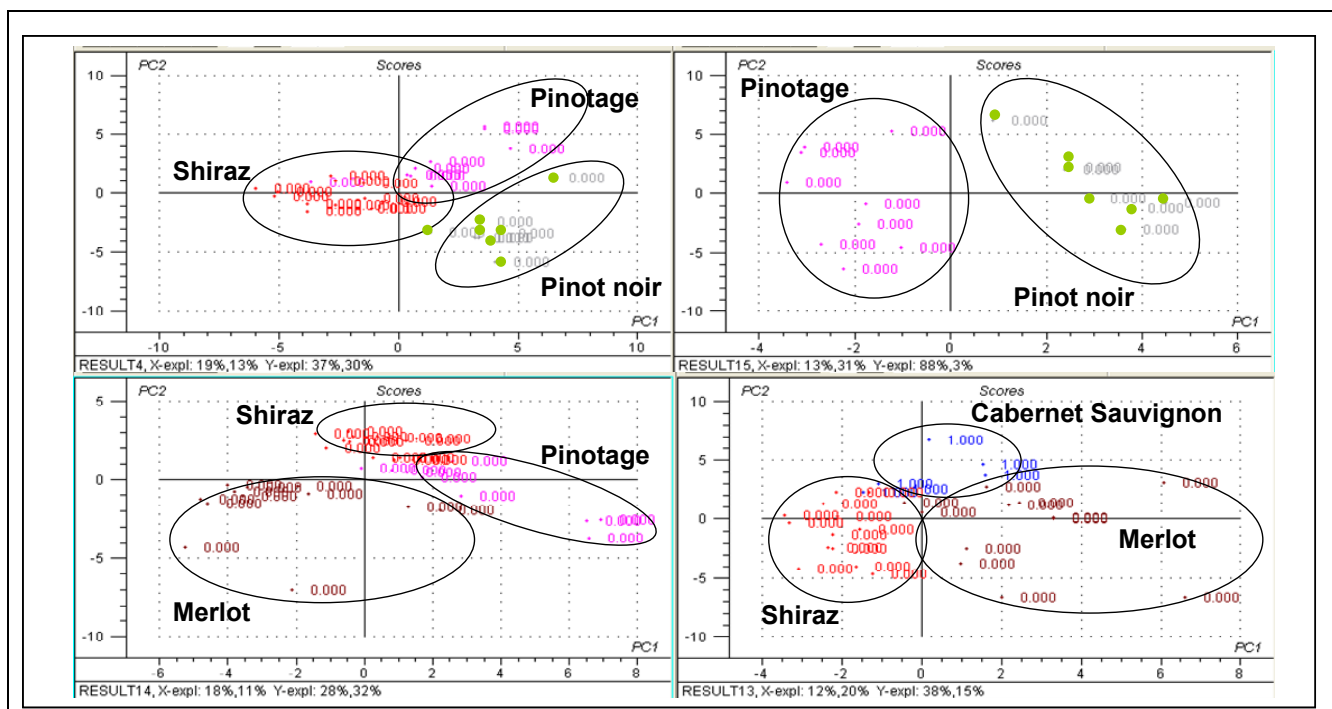


Figure 4.14 Score plot of PC1 vs PC2. Discrimination between the cultivar combinations Shiraz (red), Pinot noir (green) and Pinotage (pink); Shiraz (red), Merlot (brown) and Pinotage (pink); Shiraz (red), Cabernet Sauvignon (blue) and Merlot (brown); Pinotage (pink) and Pinot noir (green) was achieved.

4.4 CONCLUSIONS

This study has shown that headspace analysis with the use of the SPDE technique coupled to GC-MS is an effective analytical method to generate a wealth of information regarding the volatile composition of fermenting must and wine samples. The data were successfully implemented for multivariate data analysis. Satisfactory differentiation between problem and control fermentations and in addition between various cultivars was achieved with the use of headspace volatile components. The majority of the multivariate models explained more than 50% of the Y-variance with less than 50% X-variance.

The volatile compounds generated during alcoholic fermentation present relatively distinct profiles depending on the fermentation conditions. Vinification practices could also have an influence on these profiles. In the case of vinification without maceration (white varieties), there is a relative increase of fatty acids and their ethyl esters, whereas in vinification with maceration (red varieties), the ethyl esters of lactic, acetic and succinic acids are the compounds that are relatively more abundant. However, this is not the only possibility because the varietal juice composition may also influence the volatile compounds formed by yeast during fermentation.

Discrimination between subsets of cultivars was achieved. Cabernet Sauvignon was differentiated from Merlot, Shiraz, Pinotage and Malbec, respectively. Additional models could discriminate Merlot from Shiraz, Pinotage, Pinot noir and Malbec, respectively. Shiraz, Pinotage and Pinot noir were differentiated from each other with the Pinotage and Pinot noir samples clustering together and negatively correlated to the Shiraz samples. This is explained by the fact that Pinotage is a cross between the Pinot noir and Cinsaut cultivars. However, when only Pinotage and Pinot noir samples were included in the model, this analytical technique combined with chemometrics could discriminate between these two cultivars. This data clearly showed that this analytical technique also has the capacity to possibly profile different cultivars as successful discrimination between red and white cultivars and between different cultivars within one of these cultivar groups was achieved with the use of only headspace analysis.

The ability to discriminate between problem and control fermentations could possibly be a result of the following. Each grape variety has a more or less specific amino acid profile with possible influence by the geographic area, vintage and vinification practices. This profile could therefore have an influence on the amino acid metabolism of the yeast and consequently could influence the ratio of secondary metabolites produced by the yeast. Therefore, the amino acid profile of a grape variety could be related to the aroma profile of the wine (Hernández-Orte *et al.*, 2002). However, in the case of problem fermentations, it could be hypothesized that these musts (which are usually deficient in some amino acids/nitrogen compounds) would have a different amino acid profile than the control fermentations of the same cultivar. In principle, this difference in amino acid profile could result in a different aroma profile of the must samples which was possibly detected with the headspace SPDE analysis. This could possibly be the reason for the discrimination between problem and control fermentation samples obtained with the various PLS discriminant models constructed in this study. However, this is only speculative and needs further investigation in future studies.

This initial study therefore showed the feasibility of this analytical technique to be applied for wine analysis in future work. Although the individual cultivar sample set sizes were relatively small, the constructed models were still powerful enough to illustrate the possibilities of discrimination as set out by the objectives. These initial models are therefore crucial to identify and critically evaluate possible applications of this analytical technique combined with chemometrics for future research work. The ability of this technique to indicate possible fermentation products involved in cultivar discrimination and the possibility to use these types of multivariate models in predicting problem fermentations will be investigated in future work.

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5. GENERAL DISCUSSION AND CONCLUSIONS

Changes in the international wine market continuously demand higher quality products in order to meet consumers' expectations (Bisson *et al.*, 2002). In order to be competitive in the international wine market, winemakers are therefore obligated to produce wines of high quality. Success in this endeavour necessitates a sound understanding of the factors which influence the wine making process positively or negatively and to optimise their management. Depreciation of product quality could be the result of the occurrence of stuck and sluggish fermentations (Ingledew and Kunkee, 1985; Henschke, 1997; Alexandre and Charpentier, 1998; Bisson, 1999, 2005). Stuck and sluggish fermentations often have an immense practical impact for winemakers which could result in economic loss.

Modern winemaking provides mechanisation options, improved cellar technology and increased fermentation control. However, success of any fermentation process is never guaranteed, be it alcoholic or malolactic fermentation. This is a result of the complexity associated with the winemaking process as a whole and the large number of factors influencing fermentation efficiency. Several of the factors influencing fermentation directly reflect management strategies, including viticultural practices, harvesting procedures and fermentation conditions. This in turn affects yeast metabolism (Alexander and Charpentier, 1998; Bisson, 1999). A better understanding of every step of the process and proper management could limit the effect of some of the avoidable and other unavoidable factors that causes fermentation problems.

Stuck and sluggish fermentations remain a serious problem to wine industries worldwide despite extensive research efforts. Studies generally monitor one or two well-controlled factors under laboratory conditions. Although valuable information has been gathered from such studies, these conditions are not truly the same as in industrial cellars where synergy between factors results in more complex problems.

Therefore, the principal aim of this study was to evaluate the feasibility of an alternative holistic approach to investigate problem fermentations and attempt to address the above-mentioned weaknesses. For this purpose a database consisting of a diverse set of information related to problem fermentations was established. A non-directed approach of investigation was followed. The combination of analytical techniques with multivariate data analysis provided powerful technologies to extract significant information from this database. Furthermore, the possibility to discriminate between problem and control fermentations with the use of these technologies was investigated and this could allow for the construction of predictive models.

This study was unique in the sense that problem fermentations from industrial cellars were studied. This decision to study industrial problem fermentations presented the first

challenge of obtaining collaboration from commercial wineries in South Africa. Since the topic of stuck and sluggish fermentation is rather sensitive and secretive in the wine industry, the task was more challenging than initially thought. A total of 400 questionnaires (in 2 official languages, Afrikaans and English) were distributed and a promotional article was published in a local wine magazine (Wineland Magazine, Jan 2006). Of the original contact group, 55 winemakers agreed to participate in this project. Samples were however only collected from 36 cellars during the two harvest seasons.

The nature of this study therefore allowed no control over the amount, cultivar and origin of problem fermentation samples received. Consequently, the samples do not necessarily represent the occurrence of fermentation problems in the South African wine industry. This is a potential disadvantage of this approach. Nevertheless, the data could be used for comparative studies between the control and problem fermentations received.

An additional challenge to this project was to obtain control fermentations. The definition of control or normal fermentations under industrial conditions are rather controversial and differ between cultivars, grapes from different vineyards, vintages, cellars and area of origin. The ideal control fermentation for a specific stuck fermentation would entail the use of the same yeast strain, cultivar, grapes from the same vineyard and the fermentation should be at the same stage of fermentation. This particular situation is not encountered in industrial conditions. Therefore, in order to have some robust form of control fermentations for the purpose of this study, all samples collected from fermentations not reported as slow, sluggish or stuck were defined as control fermentations. This includes fermentation samples originating from a variety of cultivars, starter cultures used, oenological practices and different geographical origins.

Various analytical techniques were successfully used in this study. A part of this study focussed on using Fourier transform infrared (FT-IR) spectroscopy to quantify a number of wine components. Wine analysis for these chemical compounds using classical/traditional methods is time consuming and costly, especially with the large numbers of samples obtained in an industrial study and therefore FT-IR was evaluated. The use of Fourier transform infrared (FT-IR) spectroscopy has received much attention in recent years as a high-throughput analytical screening method used for the fast quantification (30s) of various wine compounds with minimal sample preparation (Patz *et al.*, 1999, 2004; Dubernet and Dubernet, 2000; Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003; Nieuwoudt *et al.*, 2004).

The data showed FT-IR spectroscopy could be used for quantification purposes and additional hidden information could be extracted from the absorbance spectra of grape must and presented visually with the aid of chemometrics (sophisticated statistical techniques). Multivariate techniques such as principal component analysis (PCA) and partial least squares

discriminant (PLS-discrim) regression were used to identify the main sources of variation between the samples and evaluate the possibility of discrimination between problem and control fermentations, respectively. The preliminary models constructed shows great potential to discriminate between control and problem fermentations and will definitely aid in the construction of predictive models. The data also indicated that problem fermentations occurred from the middle of fermentation onwards in this dataset.

This study has also shown that headspace analysis with the use of the SPDE technique coupled to GC-MS is an effective analytical method to generate a wealth of information regarding the volatile composition of fermenting must and wine samples. The data were successfully implemented for multivariate data analysis. Preliminary results indicate the potential of headspace data to be used in multivariate models for the discrimination between problem and control fermentations. However, this was an initial study to test the feasibility of this approach for discrimination purposes. The possibility to differentiate between problem and control fermentations could be further expanded for prediction purposes in future studies. The majority of the multivariate models explained more than 50% of the Y-variance (the ability to discriminate between problem and control fermentations) with less than 50% X-variance (headspace data).

In addition, preliminary results suggest that the use of multivariate models from headspace data has the capacity to discriminate between different cultivars. This observation needs further investigation in future research work but could possibly be linked to the grape juice composition of these specific cultivars (Hernández-Orte *et al.*, 2002). Specific yeast physiology could also be of importance in the case of problem fermentations but these aspects both need further investigation with the use of larger data sets.

The results obtained with this study indicate that the initial models constructed are an excellent base to identify and critically evaluate possible applications by combining multiple instrument data and performing multivariate data analysis for the study of problem fermentations in future research. This multivariate approach revealed interesting results which could be further explored and possibly used for future prediction purposes. The possibility to discriminate between control and problem fermentations could be used for early identification of problem fermentations with the use of these multivariate prediction models. Such models would be extremely useful to identify possible problem fermentations and could guide the winemakers in taking preventative measures. The importance of this prediction aspect to the wine industry is evident and needs further development.

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6. ADDENDUMS

A: QUESTIONNAIRE TO INDUSTRY



Industry-wide investigation into the causes of problem fermentations Institute for Wine Biotechnology, Stellenbosch University

As you are aware, problem fermentations (specifically sluggish or stuck fermentations) are a major technical challenge to the international wine industry, including South Africa.

However, few scientific approaches have been implemented to assess the causes of problem fermentations in the SA industry, and no data is available to assess the suitability of specific yeast strains for specific wine making conditions.

This Winetech sponsored project proposes to investigate the link between grape must composition, the use of specific commercial yeast strains and the occurrence of stuck fermentations. Such data will be of great scientific and industrial interest while also allowing wine makers to identify potentially risky fermentations before the problem occurs, and to take preventative measures.

For this reason we would be extremely pleased if you could provide us with samples of stuck/sluggish fermentations from the industry to investigate the matter. If you are experiencing any unexpected type of fermentation behaviour or even only minor fermentation problems, **PLEASE CONTACT US!** All information provided by you will be handled **strictly confidential**. **No work is required** from you, **ONLY a phone call!** If you are willing to participate in this project by providing us with wine samples, we would appreciate it if you could complete the questionnaire and return it as soon as possible.

Thank you. Your cooperation will be appreciated!

| Questions | Answers | | | | | | |
|---|--|-----------|--|-------|--|-----|--|
| Are you willing to participate in this project? | | | | | | | |
| Have you experienced any problem fermentations before? If yes, was it technical, yeast or MLF related? Please indicate (x) your choice. | <table border="1" style="width: 100%;"> <tr> <td style="width: 70%;">Technical</td> <td style="width: 30%;"></td> </tr> <tr> <td>Yeast</td> <td></td> </tr> <tr> <td>MLF</td> <td></td> </tr> </table> | Technical | | Yeast | | MLF | |
| Technical | | | | | | | |
| Yeast | | | | | | | |
| MLF | | | | | | | |
| Any comments, suggestions or other ideas? | | | | | | | |
| Contact details | Name of cellar: _____ Contact person: _____ Tel: _____ Fax: _____ E-mail: _____ | | | | | | |

6. ADDENDUMS

B: WINELAND ARTICLE (JAN 2006)

Wynboer

A Technical Guide
for Wine Producers

Industry-wide investigation into the occurrence and causes of problem fermentations

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*This research is sponsored by **Winetech***



**Sulette
Malherbe**

Introduction

Stuck and sluggish fermentations remain a major oenological concern for winemakers because of the economic impact of fermentation problems. Incomplete or "stuck" fermentations are defined as those leaving a higher than desired residual sugar content at the end of alcoholic fermentation, while slow or sluggish fermentations are characterised by a low rate of sugar utilisation (Bisson, 1999). Although these terms are often associated with alcoholic fermentation, the same problems might also occur in the case of malolactic fermentation. The focus of this article will be on alcoholic fermentation problems.

The current literature related to stuck or sluggish fermentation is substantial. A significant amount of information about the metabolism, physiology, cell biology, and stress adaptation in *Saccharomyces cerevisiae* has been generated under laboratory conditions. However, extrapolation from such studies has to be done with caution since these conditions do not exactly mimic industrial winemaking conditions.

Nevertheless, those studies have been useful in identifying numerous factors that may affect fermentation rate and cause sluggish or stuck fermentations, including high initial sugar content, nutrient, and in particular nitrogen limitation, ethanol toxicity, organic and fatty acid toxicity, presence of killer factors or other microbially-produced toxins, cation imbalance, temperature extremes, pesticide, and fungicide residues, microbial competition and poor oenological practices (Henschke, 1997; Ingledew and Kunkee, 1985). These factors may induce decreases in the metabolism of the yeast cell and consequently decreases in biomass production, cell viability and fermentation rate. Fermentation problems are rarely due to one single factor in isolation but usually the result of a combination of several factors resulting in a complex problem. The possibility of a

synergistic effect amongst these factors renders problem fermentations a rather challenging subject to investigate (Alexandre and Charpentier, 1998).

Metabolic basis of stuck and sluggish fermentations

The metabolic basis of stuck and sluggish fermentation has been fairly well established. The decrease in rate of sugar consumption is correlated with a decrease in sugar uptake capacity. Glucose and fructose consumption are reduced in response to various environmental or cellular stress conditions. Nutrient limitation (macronutrient and micronutrient), low pH, lack of oxygen, lack of adequate agitation, temperature extremes, presence of toxic substances, presence of other micro-organisms, imbalance of cations, and poor strain tolerances (particularly to ethanol or acetaldehyde). All of these have been associated with stuck and sluggish fermentations and have an impact on glucose and fructose transporter expression and activity (Alexandre and Charpentier, 1998).

Causes of stuck alcoholic fermentation

Glucose/fructose ratio

- According to literature, fructose levels in stuck wine are found to be 10 times higher than the glucose concentration. Stuck fermentation can therefore be expected for wines with glucose/fructose ratio smaller than 0.1 (Gafner and Schütz, 1996).

Nutritional limitation

- Nitrogen deficiency: A low initial level of nitrogen acts by limiting growth rate and biomass formation of yeast, resulting in a low rate of sugar catabolism.
- Oxygen deficiency: Responsible for sluggish fermentation as a consequence of inhibition of lipid biosynthesis which results in decreased ergosterol and unsaturated fatty acid content ('survival factors'), decreased biomass production and yeast viability. Consequently the ethanol tolerance in the yeast cell also changes. Toxic fatty acid production (octanoic and decanoic acid) is also affected by oxygen deficiency.
- Mineral deficiency: Magnesium plays a key role in metabolic control, growth and cell proliferation, glycolytic pathway and subsequently ethanol production. A deficiency in magnesium availability results in decreased yeast growth and fermentative activity.
- Vitamin deficiency: In some cases insufficient availability of vitamins (essentially thiamine) may lead to sluggish fermentations. In the presence of high concentrations of sulphur dioxide thiamine is cleaved, its biological activity is destroyed and fermentation difficulties may occur.

Inhibitory substances

- Ethanol: ethanol affects yeast cells in numerous ways resulting in subsequent growth inhibition/decrease in fermentation rate (inhibiting sugar transport activity).
- Toxic acids:
 - Medium chain fatty acids: decanoic and octanoic acid are intermediates in the biosynthesis of long chain fatty acids produced during alcoholic fermentation. Both inhibit hexose transporter system resulting in reduced fermentation rate.
 - Acetic acid: toxic to the cell and also enhances the ethanol toxicity.
- Effects of sulphites: highly toxic to micro-organisms. Molecular SO₂ is more active at low pH. Thus molecular SO₂ is extremely active against yeasts in low pH (3-3.5) must.

- Zymocidal or killer toxins.
- Fungicides/Pesticide residues can act directly or indirectly to inhibit yeast growth during fermentation (Specht, 2003).

Physical factors

- Excessive must clarification can often cause sluggish fermentation due to the loss in fatty acid content, sterol content and macromolecules (Alexandre and Charpentier, 1998).
- Temperature extremes could severely affect yeast growth and metabolism (Specht, 2003).
- Excessive use of SO₂ is toxic to yeast cells (Alexandre and Charpentier, 1998).

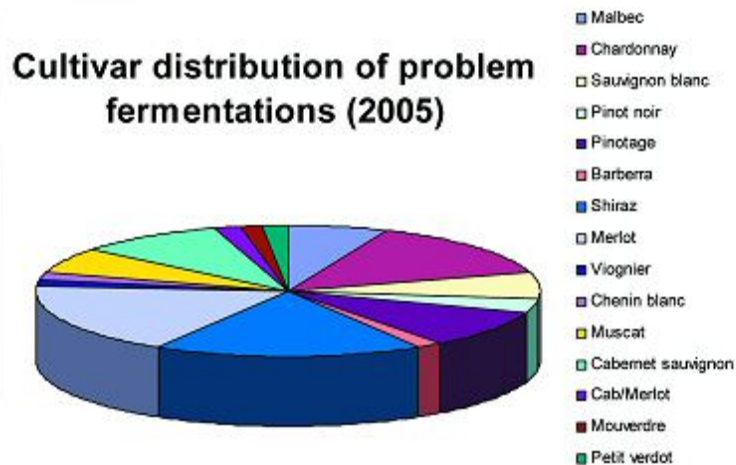
A new project on stuck and sluggish fermentation: What makes this project different?

As part of a Winetech research programme on improving the fermentation performance of yeast (project number IWBT 5/02), this project involves an industry-wide investigation into the occurrence and causes of problem fermentations.

Due to the multiple factors which can lead to slow or incomplete fermentation, previous studies on problem fermentations were mostly limited to investigating one or two factors.

This project is designed to overcome this weakness and to use industry data to assess the causative factors responsible for stuck fermentations in the South African context. The project will aim at establishing a database containing information on the occurrence, all parameters assessed (physical/chemical etc.) and the combination of causative factors. Chemometrics will be used for the statistical multifactorial analysis of this data. With the use of chemometric techniques it is possible to extract maximum information from analytical, spectral and sensory data. Careful application of these chemometrics could lead to the identification of valuable relationships amongst stuck fermentation samples that have gone unnoticed before. This novel approach to problem fermentations will therefore allow the holistic investigation of numerous factors simultaneously.

As conventional chemical analysis is both time consuming and expensive, especially in winery environments that operate hundreds of fermentation tanks simultaneously, infrared spectroscopy could offer an alternative to conventional chemical analysis. Many applications of IR to wine analysis have been found, however references to the monitoring of large scale wine fermentations are scarce (Urtubia et al., 2004), and the monitoring of problem fermentations nearly non-existent. Infrared spectroscopy (IR) can consequently be used for monitoring fermentations since many compounds can be measured quickly from a single sample without prior treatment. During the 2005 harvest season problem fermentation wine samples were collected or received from various South African wine regions and the cultivar distribution was as follows (see graph):



In order to establish a representative dataset of problem fermentations, as much samples as possible are needed for this study, originating from all wine regions. This is where we need your co-operation in 2006.

The project is therefore industry related (in other words, without the help and involvement of the commercial sector there is no project!). Previous studies on problem fermentations were done in experimental cellars or in synthetic media. Although these studies generated valuable information on the subject, the information is mostly of scientific value and not forcibly related to real industrial conditions. For the project described here the samples are collected directly from commercial cellars and analysed. This is therefore research IN the industry FOR the industry.

The accuracy of the laboratory analysis of a wine sample is only a true reflection of what is happening in a wine if the sample was collected in the correct manner.

The same goes for this project, the results obtained from the data generated is only as representative as the quality and number of samples that were taken. Therefore it is of utmost importance for the significance of this project to have as much samples as possible from all South African cellars.

In the unlikely event that one of your fermentations proceeds slower than normal (sluggish) or gets totally stuck, please contact me to collect a sample before you re-inoculate. This is purely for research purposes and all samples will be handled confidentially.

Any questions or further suggestions for this project are welcome.

Thank you to everyone who already participated during the 2005 season, it was greatly appreciated.

Please contact Sulette at (021) 808-4867 or 084 591 2112 or e-mail sulette@sun.ac.za.

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6. ADDENDUMS

C: PRELIMINARY RESEARCH AND DEVELOPMENT STRATEGY FOR EVALUATING FT-IR SPECTROSCOPY FOR QUANTIFICATION PURPOSES IN FERMENTING MUST

Fermenting must is a complex medium that constantly changes composition during the course of the fermentation and several analytical methods for the quantification of the diverse classes of chemical components in one sample are frequently required. Nevertheless, time and cost restrictions necessitate that the maximum amount of reliable data is obtained with the methods used, especially when large numbers of samples are analysed. The applications of Fourier transform infrared (FT-IR) spectroscopy to monitor industrial processes have increased in recent years. This technology can be used to quantify and monitor quality control parameters and extensive use of this technique for wine analysis has been reported (Patz *et al.*, 1999; Dubernet and Dubernet, 2000; Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003; Patz *et al.*, 2004).

FT-IR spectroscopy relies on the principle of detection of molecular vibrational frequencies in the mid infrared region of the electromagnetic spectrum. Each organic molecule has a characteristic absorbance in the mid infrared (MIR) region. The detection of molecular vibrations related to covalent bonds such as C-C, C-H, O-H, C=O, N-H present in the organic molecules, results in characteristic absorption spectra in the MIR region. The region 929 cm^{-1} to 1600 cm^{-1} captures a significant amount of this chemical information and is referred to as the “fingerprint” region (Smith, 1999). Since the MIR absorbance spectrum contains information about the chemical composition of a substance, calibrations to quantify specific individual components were developed. A Winescan FT120 spectrometer (Type 77110 and 77310 Reference manual, FOSS Analytical, Denmark, 2001) was developed specifically for industrial use and is equipped with ready-to-use calibrations, also referred to “global calibrations” that facilitate quantification of important wine parameters. The successful implementation of FT-IR spectroscopy for quantitative analysis requires the development of calibration algorithms that yield accurate data. These algorithms are developed using the FT-IR spectra of samples and the corresponding reference values to predict the concentrations of components of interest in future unknown samples (Naes *et al.*, 2002).

In a complex matrix such as wine or fermenting must, the more samples used for the establishment of the calibration, the better the expected prediction accuracies (normally). Calibration samples must be analysed with appropriate reference methods, and time and cost implications for handling such large volumes of analytical work restrict the number of samples that can be used. Judicious selection of calibration samples providing the maximum variation in the spectral properties (related to the X-variables), and variation in the concentration range

of the components of interest (Y-variables) is therefore very important (Naes *et al.*, 2002). Calibration algorithms are developed with the aim of obtaining accurate prediction data with the lowest possible prediction errors. The global calibrations were developed in Europe using samples processed with European winery conditions and validation of these calibrations under South African conditions was therefore necessary.

The main objective of this study was to do preliminary work related to optimisation of quantitative calibrations for fermenting must and wines undergoing malolactic fermentation using a WineScan spectrometer. This work forms part of a larger project to be initiated during 2007 at the Institute for Wine Biotechnology, Stellenbosch University, South Africa that will focus on an in-depth investigation into these aspects amongst others. The components analysed in this preliminary investigation were glucose, fructose, glycerol, ethanol, free assimilable nitrogen (FAN) and the main organic acids (citric acid, tartaric acid, malic acid, lactic acid, succinic acid and acetic acid). The specific aims of the work were: (i) evaluation of the prediction accuracies of the global calibrations when applied to South African fermenting must and malolactic fermentation samples; (ii) the evaluation of a strategy for the selection of a representative calibration sample set using the minimum number of samples, and (iii) the evaluation of the suitability of two reference methods, namely high performance liquid chromatography (HPLC) and enzymatic analysis for routine analysis. The data were used to establish preliminary partial least squares (PLS) regression calibration algorithms for the respective components.

6.1 MATERIALS AND METHODS

6.1.1 MUST SAMPLES

Small scale experimental Chenin blanc fermentations were set up and sampled during the course of the fermentations on a regular basis. The commercial yeast starter culture *S. cerevisiae* VIN 13 (Anchor yeast, South Africa) was used to ferment Chenin blanc juice (kindly provided by ARC Infruitec, Nietvoorbij, Stellenbosch) in four different fermentation canisters (25 L capacity) fitted with airlocks. Samples (50 mL) were removed from the canisters on a daily basis and analysed using a Winescan FT120 instrument (FOSS Analytical, Denmark, 2001). A 2 mL aliquot of each sample was stored at -20°C until reference analysis was performed. Although the storage of samples prior to reference analysis is not optimal, and can lead to precipitation of individual components, the availability of apparatus and time constraints necessitated this. With this experiment 70 samples were collected.

In order to expand this dataset and include variation in terms of origin and cultivar, samples were also collected from industrial wine cellars in the Stellenbosch winemaking area

during vintages 2005 and 2006. Fermenting must samples (500 mL quantities) were collected from large scale industrial fermentation tanks from a sample tap situated a third from the bottom on the side of the tank (12 000 L, 25 000 L, 33 000 L) or, in the case of 100 000 L tanks, from the top of the tank. All collection vials were first rinsed with wine before sampling. These samples were analysed with FT-IR spectroscopy as described before and 2 mL aliquots were frozen at -20°C for subsequent reference analysis. A total of 315 industrial fermentation samples were collected. The cultivar distribution was as follows: Cabernet Sauvignon (18), Chardonnay (31), Chenin blanc (37), Malbec (3), Merlot (45), Pinot noir (7), Pinotage (19), Sauvignon blanc (57), Shiraz (38), Viognier (11), Cabernet Franc (4), Colombard (6), Gamay noir (1), Gewürztraminer (8), Muscat Ottonel (2), Nouvelle (3), Petit Verdot (3), Pinot blanc (2), Semillon (7), Sangiovese (1), Weisser Riesling (2) and Rosé samples (10).

6.1.2 CALIBRATION SET SELECTION STRATEGY

All the small-scale experimental Chenin blanc fermentation samples were used for calibration purposes. In order to expand this calibration set in terms of cultivar and origin, a calibration set selection strategy (Naes *et al.*, 2002) was used to select a subset of samples from the samples collected from industry. This calibration sample set was selected to represent the range of concentrations needed for calibration as well as expanding the calibration in term of robustness with respect to different cultivars. As a result of time and cost constraints it is often necessary to select a particular calibration sample set providing all the necessary variation in the spectral properties (X-variables) and variation in the property of interest (Y-variable) (**Fig. 6.1**).

Principal component analysis (PCA) was performed on the FT-IR spectra of 304 must samples to identify the main directions of variance. A subset of 104 calibration samples was then identified in the score plot based on the following criteria: (i) retention of as wide as possible spread in the main directions of variance; and (ii) an even distribution of the samples in all four quadrants of the score plot. These samples are referred to as “fermenting must samples” in the text. Apart from the fermenting must samples, additional calibration samples for malolactic fermentation were selected from a PCA score plot containing samples undergoing malolactic fermentation. The cultivar distribution of the calibration samples are shown in **Table 6.1**.

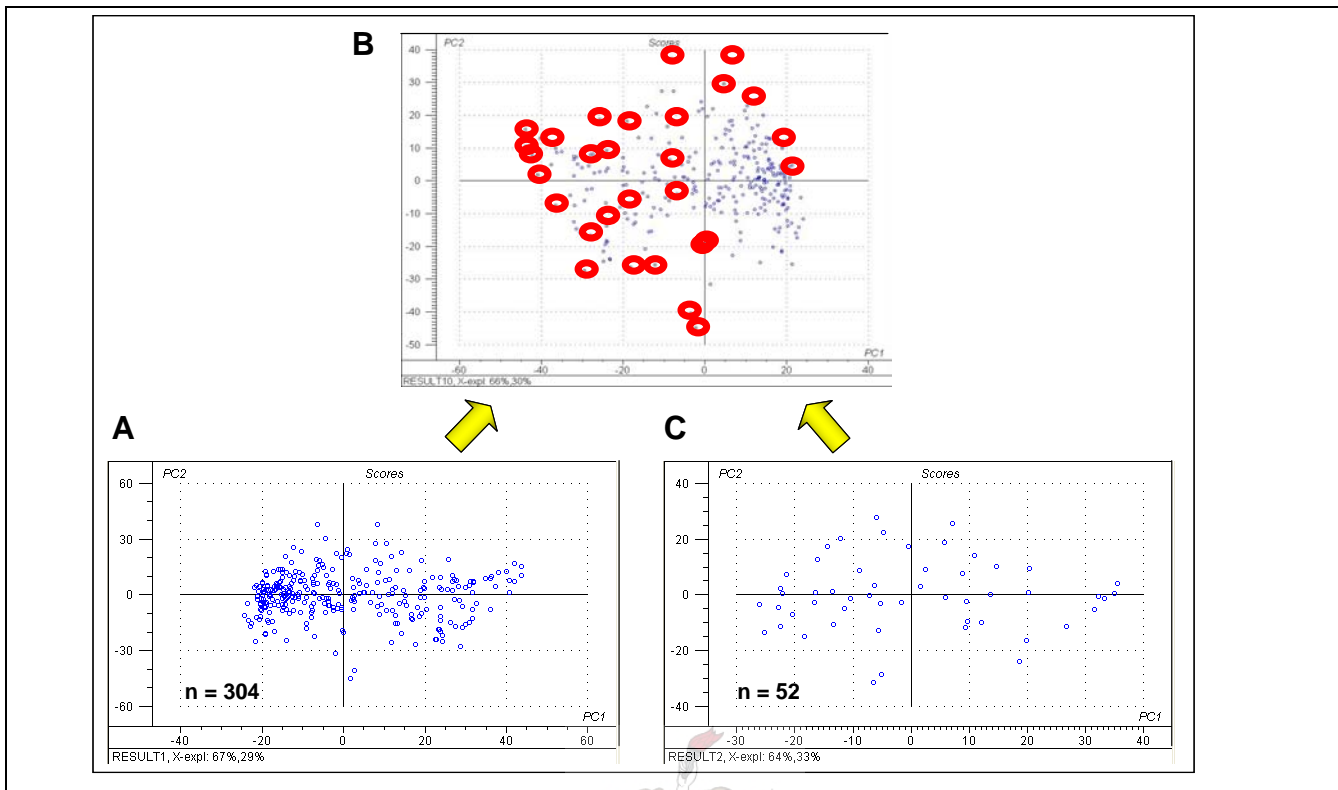


Figure 6.1 (A) Principal component analysis (PCA) was performed on the FT-IR spectra of 304 must samples to identify the main directions of variance. (B) A subset of 104 calibration samples (indicated with red circles) was then selected in the PCA score plot. (C) The PCA score plot of the FT-IR spectra of the calibration subset shows that the directions of variance in the original sample set were retained in the selected calibration subset.

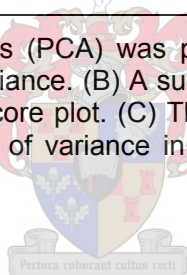
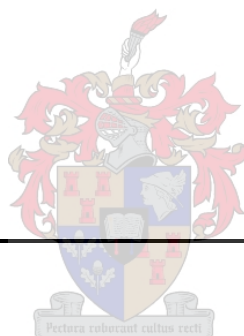


Table 6.1 Cultivar distribution of the initial calibration sets (MUF = must under fermentation and MLF = malolactic fermentation) selected from PCA score plots.

| MUF calibration set | | MLF calibration set | |
|---------------------|-----|---------------------|-----|
| Cultivar | Nr. | Cultivar | Nr. |
| Chardonnay | 10 | Cabernet Sauvignon | 25 |
| Chenin blanc | 11 | Pinotage | 9 |
| Colombard | 6 | Shiraz | 11 |
| Cabernet Sauvignon | 6 | Cinsaut | 1 |
| Merlot | 14 | Sangiovese | 1 |
| Malbec | 2 | Merlot | 5 |
| Nouvelle | 1 | | |
| Pinotage | 7 | | |
| Pinot noir | 1 | | |
| Petite Verdot | 2 | | |
| Shiraz | 14 | | |
| Sauvignon blanc | 19 | | |
| Viognier | 6 | | |
| Semillion | 2 | | |



6.1.3 REFERENCE ANALYSIS

The accuracy of the reference method was expressed as the standard error of laboratory (SEL) and calculated as:

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

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

6.1.3.1 Glucose, fructose and glycerol enzymatic determinations

Glucose and fructose concentrations were measured enzymatically (Roche, catalogue number 10139106035) with a 1 mL total assay volume in disposable cuvettes measured spectrophotometrically at 340 nm. Similarly, glycerol was measured enzymatically (Roche, catalogue number 10148270035).

6.1.3.2 Main organic acids, glucose, fructose, glycerol and ethanol determinations using high performance liquid chromatography (HPLC)

The main organic acids in wine, ethanol, glucose, fructose and glycerol were quantified with high performance liquid chromatography (HPLC). Liquid chromatography (LC) analysis was performed on a Waters 717 WISP plus autosampler (Waters Chromatography Division, Millipore Corp., Milford, MA) HPLC instrument. Separation of the components was achieved using 5 mM H₂SO₄ at a flowrate of 0.6 mL/min in isocratic separation on a resin-based Biorad Aminex HPX-87H column (300 mm x 7.8 mm, Biorad Laboratories, Richmond, CA, USA). The column temperature was 45°C and detection was achieved by combining refractive index (RI) and photo diode array (PDA) detectors (**Fig. 6.2**). The overall HPLC run time was 30 min. Target analytes were identified by comparison of retention times with commercial standards. Quantification was achieved by using external calibration curves for the following concentrations: 0.5 g/L, 5 g/L, 10 g/L, 15 g/L and 20 g/L, respectively.

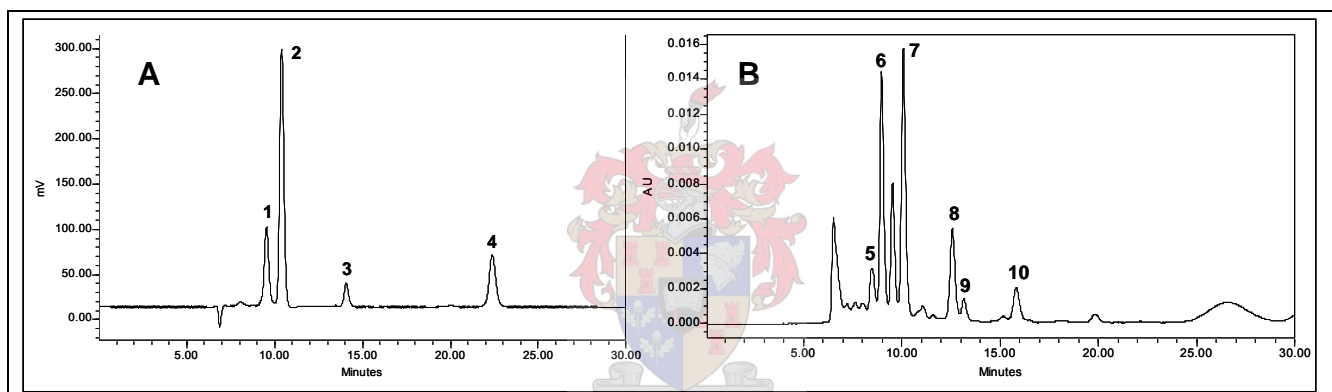


Figure 6.2 (A) Separation of glucose (1), fructose (2), glycerol (3) and ethanol (4) was achieved using 5 mM H₂SO₄ in isocratic separation on a resin based column at 45°C detection by refractive index (RI). (B) Separation of the organic acids, citric (5), tartaric (6), malic (7), succinic (8), lactic (9) and acetic (10) acid was achieved as mentioned in (A). PDA detection was used for the organic acids in the ultraviolet ($\lambda = 220$ nm) region.

Fructose and malic acid co-eluted when RI detection was used. Therefore, PDA detection in the ultraviolet region ($\lambda=220$ nm) was used to determine the malic and other organic acid concentrations. The malic acid concentration obtained with PDA was used to determine the RI_{area} (malic acid) and subtracted from the RI_{area} (fructose+malic acid) to obtain the RI_{area} (fructose) and consequently the fructose concentration. Gluconic acid co-elutes with tartaric acid and was not quantified in this analysis due to its association with low quality grapes irrelevant to this study (**Fig. 6.3**).

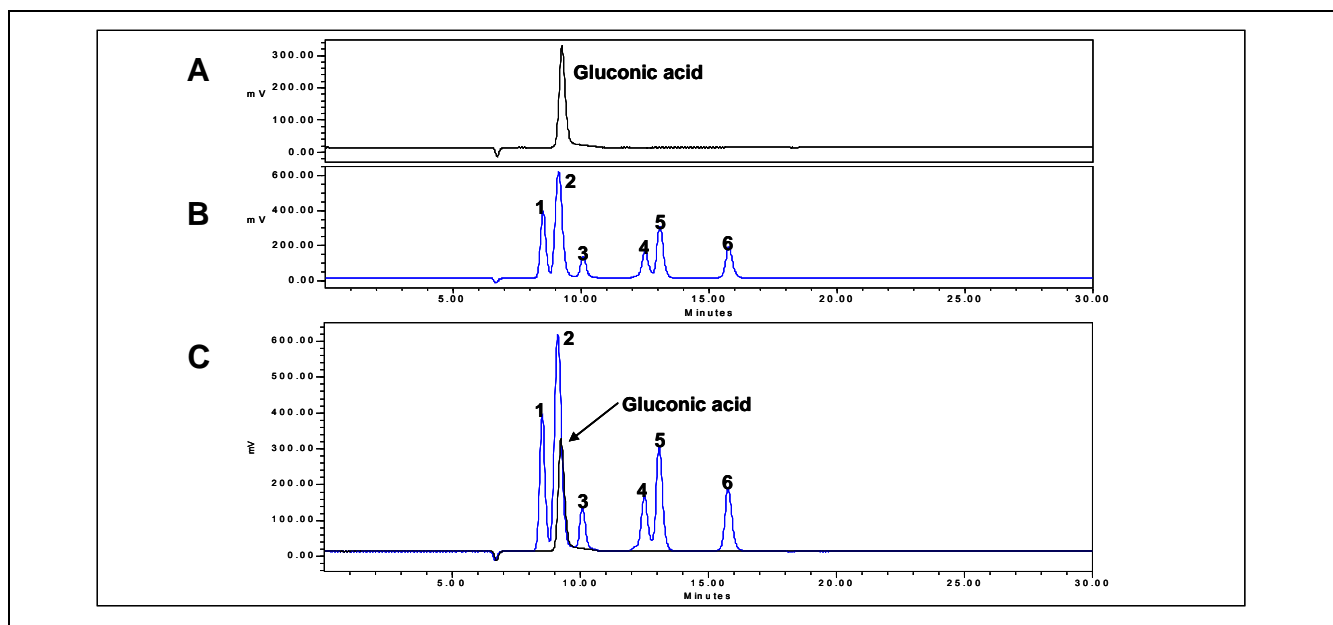


Figure 6.3 (A) Gluconic acid standard solution (B) Organic acids: citric acid (1), tartaric acid (2), malic acid (3), succinic acid (4), lactic acid (5) and acetic acid (6) could be separated. (C) Overlapping the chromatograms indicate the co-elution of gluconic and tartaric acid.

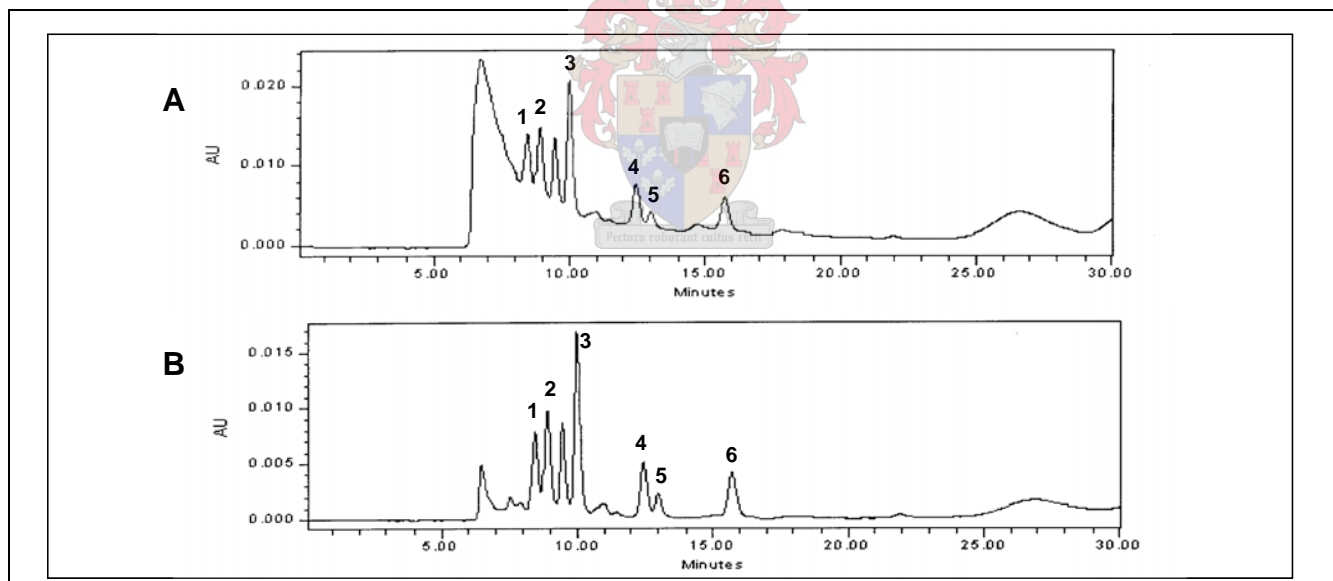


Figure 6.4 Sep-Pak C18 cartridges were used to purify the red must samples prior to HPLC analysis. (A) Shows the interference of phenolic compounds with the detection of organic acids: (1) citric acid, (2) tartaric acid, (3) malic acid, (4) succinic acid, (5) lactic acid and (6) acetic acid in the case of direct injection. (B) Shows the result after the used of Sep-Pak C18 cartridges which retains the interfering phenolic components.

Sample preparation by means of solid phase extraction (SPE) (Sep-Pak C18 classic cartridges, catalogue number WAT051910, Waters) was necessary for red wine samples to

remove the large polymeric phenolic compounds interfering in the separation of organic acids (**Fig. 6.4**). No additional sample preparation other than dilution with water and filtration (0.22 μm) was necessary for the white wines. The SPE cartridges were firstly conditioned with 5 mL methanol (Merck, Gauteng) followed by washing with 5 mL MilliQ water prior to use. The diluted red wine sample (2 mL) was purified with the cartridge. The first 1 mL was discarded and the following 1 mL was used for HPLC analysis. All analysis was performed in duplicate.

The reproducibility of the SPE sample preparation method was tested with six repeats of this procedure (**Fig. 6.5**). Red wine was spiked with known concentrations of standard solutions. Samples (spiked red wine) were injected directly and additional samples were prepared with the SPE procedure using Sep-Pak C18 cartridges. The respective results were used to determine the percentage recovery for each individual component (**Table 6.3**) and the reproducibility of this method. The recovery for the components was satisfactory with the exception of acetic acid, possible due to its volatility.

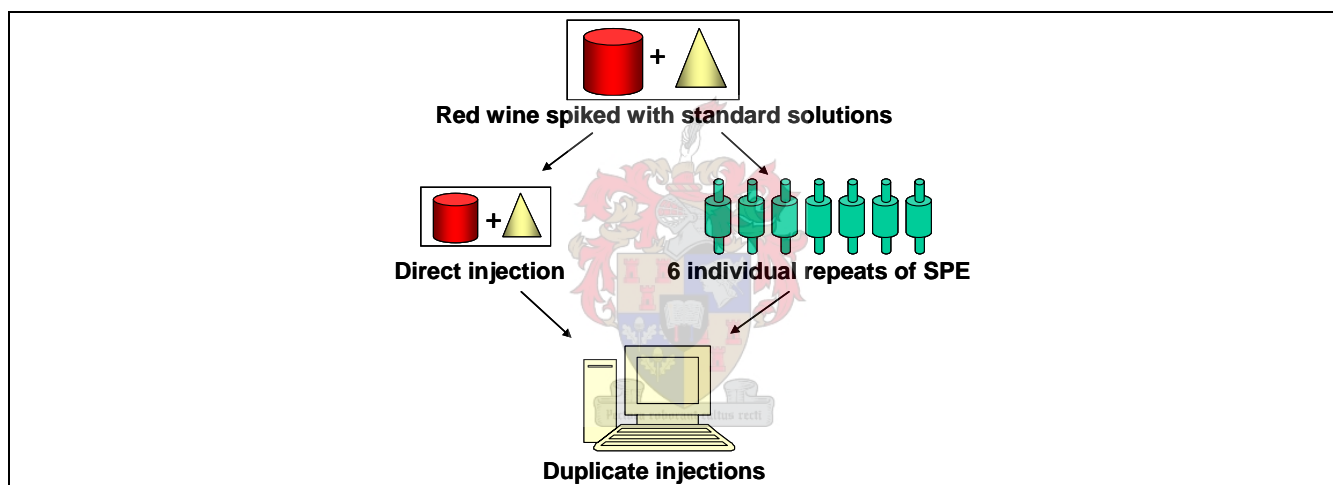


Figure 6.5 Six repeats of the SPE procedure using Sep-Pak C18 cartridges tested the reproducibility of this sample preparation method. Red wine was spiked with known concentrations of standard solutions. Samples were analysed directly and these results combined with the results obtained from the samples prepared with the SPE procedure was used to determine the percentage recovery for each individual component.

Sample preparation by means of SPE with Sep-Pak C18 cartridges was reproducible with an average coefficient of variance (%CV) of less than 0.5% for all individual components between the six repeats except for acetic acid (%CV=2.28) (**Table 6.3**).

Table 6.3 The average percentage recovery for each individual component for red wine samples analysed with six individual cartridges.

| Component | % Recovery |
|---------------|------------|
| citric acid | 67±0.27 |
| tartaric acid | 73±0.39 |
| malic acid | 60±0.33 |
| succinic acid | 77±0.23 |
| lactic acid | 69±0.44 |
| acetic acid | 38±2.28 |
| glucose | 90±0.14 |
| fructose | 89±0.22 |
| glycerol | 88±0.36 |
| ethanol | 72±0.10 |

6.1.3.3 Free assimilable nitrogen (FAN) determinations using formol titration

The formol titration method was used to estimate the assimilable nitrogen concentration in the must samples. The sample was neutralized with 1N NaOH to pH 8.5, followed by the addition of an excess of neutralized formaldehyde (pH 8.5) and re-titration of the solution to the endpoint. Both the NH_4^+ and FAN alpha-amino acids concentrations were determined with this method (Zoecklein *et al.*, 1995). The descriptive statistics including the concentration range, average, relative standard deviation (%RSD) and standard error of laboratory (SEL) are given in **Table 6.4**.

Table 6.4 The descriptive statistics for the FAN determinations.

| Component | Nr. | Range (mg/L) | Average | %RSD | SEL |
|-----------|-----|--------------|-------------|------|-----------|
| FAN | 310 | 103.6 - 308 | 190.76 mg/L | 0.45 | 1.62 mg/L |

6.1.4 FT-IR SPECTROSCOPY

The Winescan FT120 spectrometer (software version 2.2.1) equipped with a purpose built Michelson interferometer (FOSS Analytical, Denmark) was used for spectral acquisition of samples. Duplicate scans were obtained of each must sample immediately after sample preparation. Samples were centrifuged to eliminate particles that could cause blockages (Patz *et al.*, 2004) and disturbances in the optical path length of the cuvette (Nieuwoudt *et al.*, 2004)

and degassed by means of vacuum. Various instrument settings have been pre-selected by the manufacturer and can not be changed by the user. Samples (7-8 mL) are pumped through the CaF₂-lined cuvette situated in the heater unit of the instrument and heated to exactly 40°C before analysis. The samples are scanned from 5011 to 929 cm⁻¹ at 4 cm⁻¹ intervals which results in 1056 data points per spectrum. A small section of the near-IR region is included in this spectrum. The frequencies of the IR beam transmitted by a sample are recorded at the detector and used to generate an interferogram that is calculated from a total of 20 scans. The interferogram is processed by Fourier transformation to generate a single beam transmittance spectrum. A FOSS Zero liquid S-6060 (Winescan FT120 Type 777110 and 77310 Reference manual, FOSS Analytical, Denmark, 2001), which is scanned prior to the wine sample, is used to correct for the background absorbance (which include the absorbance of water). The single beam transmittance spectrum of the zero liquid is used to calculate the ratio of the sample spectrum to the zero liquid spectrum. This ratio is used to generate the final transmittance spectrum at each recorded data point. The transmittance spectra are finally converted to linearized absorbance spectra through a series of mathematical procedures (Winescan FT120 Type 777110 and 77310 Reference manual, FOSS Analytical, Denmark, 2001).

6.1.4.1 Wavenumber selection

Although the whole spectral range (929 – 5011 cm⁻¹) is stored for each calibration sample, certain ranges of frequencies are eliminated to prevent noise being included in the calculations. The Advanced Performance Software Module (version 2.1.0), which is an extension of the basic software of the Winescan FT120 instrument, allows wavenumber selection from only three spectral ranges: 964 – 1543 cm⁻¹, 1716 – 2732 cm⁻¹ and 2434 - 2970 cm⁻¹. The regions 1543 – 1716 cm⁻¹ and 2970 – 3626 cm⁻¹ contain strong water absorption areas preventing any energy passing through the cell (Patz *et al.*, 2004; Nieuwoudt *et al.*, 2004). The region from 3626 - 5011 cm⁻¹ is eliminated because it contains very little useful information (Winescan FT120 Type 777110 and 77310 Reference manual, FOSS Analytical, Denmark, 2001). A maximum of 15 “filters” (wavenumbers or small groups of wavenumbers) can be defined (by default) for calibration purposes. The wavenumbers (“filters”) at which the correlation between the measured absorbance and the corresponding reference value was best described were selected. Possible overfitting of calibration models (which introduces noise and uninformative variation into the calibration) was evaluated. Filters were deselected to identify the minimum number of filters and the lowest possible root mean square error of calibration (RMSEC), referred to as standard error of cross validation (SECV) on the WineScan software. RMSEC and bias (indication of systematic error) were

used to evaluate the calibration models. The calculation of these parameters is standard statistical operations and has been described (Naes *et al.*, 2002)

6.1.4.2. Global calibrations

Advanced mathematical techniques are used to generate calibration equations for individual parameters in a complex medium (such as wine) with extremely multivariate spectra. The Winescan calibration software uses partial least squares (PLS) regression to generate an equation which best describes the relationship between the absorptions at selected frequencies (cm^{-1}) or groups of frequencies (the “filters”) and the reference values in the data set (Patz *et al.*, 2004). Calibrations for specific components are constructed using samples of specific concentration ranges (Table 6.5). In order to adhere to good laboratory practices (GLP), global calibrations need to be validated prior to implementation. Results from global calibrations can be adjusted by means of slope and intercept adjustments, if necessary.

Table 6.5 Component range of the data sets used in the global calibrations for the major wine parameters for the Winescan FT120 must under fermentation (MUF) and finished wine (FW) products.

| Component | FOSS product | Average | Min. | Max. | Unit | Reference |
|------------------|--------------|---------|------|-------|-------|----------------------|
| glucose+fructose | MUF | 5.66 | 0 | 175 | g/L | Appl. Note 158, 2001 |
| ethanol | MUF | 11.5 | 0 | 14.14 | % v/v | Appl. Note 157, 2001 |
| acetic acid | MUF | 0.37 | 0 | 2.90 | g/L | Appl. Note 154, 2001 |
| malic acid | MUF | 1.25 | 0 | 13.2 | g/L | Appl. Note 159, 2001 |
| lactic acid | FW | 1.16 | 0 | 3.87 | g/L | Appl. Note 135, 2001 |
| glycerol | FW | 6.79 | 3.6 | 14.57 | g/L | Appl. Note 191, 2001 |
| citric acid | FW | 0.27 | 0.05 | 1.00 | g/L | Appl. Note 188, 2001 |
| tartaric acid | FW | 2.02 | 0.84 | 3.86 | g/L | Appl. Note 189, 2001 |
| malic acid | FW | 0.82 | 0 | 5.2 | g/L | Appl. Note 136, 2001 |
| ethanol | FW | 12.12 | 8.5 | 14.75 | % v/v | Appl. Note 131, 2001 |

6.1.5 CHEMOMETRICS AND DATA ANALYSIS

6.1.5.1 Principal component analysis (PCA)

FT-IR spectra were exported to *The Unscrambler* software (version 9.2, CAMO ASA, Norway) for the purpose of PCA. Duplicate spectra were averaged. The data matrix, defined by variables in the columns (1056 wavenumbers) and samples in the rows, was autoscaled through mean-centering by column (subtracting the average value from a variable, for each data point). In order to make variable variances more comparable, the data matrix was scaled of weighted using the inverse of the standard deviation (Esbensen, 2002) as follows:

$$x^{\text{scaled}} = x^* \frac{1}{\text{SDev}}$$

The maximum directions of variance in a data set is modelled by PCA with the projection of the objects (FT-IR spectra) as a swarm of data points in a space defined by principal components (PC's). Each PC is a linear function of a number of variables and contains in decreasing order the main information among the objects. PC's can be interpreted independently since they are calculated to be orthogonal to one another. 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 identify 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 error matrix is dependent on the problem definition and represents the variation not explained by the extracted PC's. This decomposition is described by the following algorithm:

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

where X is the independent variable matrix, T is the scores matrix, P is the loadings matrix, E is the error matrix, n is the number of objects, m is the number of variables and k is the number of PC's used (Esbensen, 2002; Eriksson *et al.*, 1999).

6.1.5.2 Partial least squares regression 1 (PLS1-R)

The complexity and extremely multivariate nature of wine spectra necessitates the use of advanced mathematical techniques to generate the calibration equations for the individual parameters (glucose, fructose, glycerol, ethanol, citric acid, tartaric acid, malic acid, succinic acid, acetic acid and lactic acid). The Winescan calibration software uses partial least squares (PLS1) regression, a bilinear regression modelling method, to project the original x variables onto a smaller number of PLS components (Esbensen, 2002). These components 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 (the reference data for each individual parameter) by generating a mathematical equation that best fits the reference values in the data set. The relationship between the y and x variables are given 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. The objective of the regression is to predict y variables using the most relevant PLS components.

PLS calibrations for the above-mentioned parameters were made using *The Unscrambler* software (version 9.2, CAMO ASA, Norway) as well as the Advanced Performance Module (version 2.1.0) which is an extension of the basic software of the Winescan FT120 instrument.

The filters selected by the Winescan FT120 software for the calibrations of individual parameters were used as selected variables in the PLS regression calibration model for calibrations in *The Unscrambler* software. The X-Y relation outliers plot (T vs. U scores) in the PLS-R was investigated for each data set to identify and confirm the outliers detected in PCA. Each sample has a score along each model component. These scores represent information of several variables condensed into a few underlying variables and can be used to detect sample patterns, groupings, similarities or differences. T-scores are found in the X-matrix and U-scores in the Y-matrix. The plot of T scores vs. U scores gives an overview of the relationship between the X- and Y-matrix for each particular PC and directly shows how the regression works. The location of predicted values on the regression line is interpreted as follows: extreme samples (very high or very low concentrations of the analyte) lie at the ends of the line and outliers protrude orthogonally from the line. The loading weights (W) are the effective loadings directly related to the regression relationship between X (spectra) and Y (reference value) and were investigated for each PC (Høskuldsson, 1996).

The strategy for the development and validation of a calibration model is shown in **Figure 6.6**). The validation of the calibration was attempted by an independent test set (validation set). The samples of the test set (sample set size a third to half of the calibration set) were in the same range as the calibration set, representing the same cultivars and from early, middle and late fermentation stages. This process of validation is preferred to full cross validation. The sub-models are too identical to the calibration set and the cross validation error is not based on the full model in the case of full cross validation. However, in this study the sample set was relatively small and contained huge variation in terms of cultivar and stage of fermentation, which made the use of test set validation less successful. For this

reason, cross validation was used in this study. Future work will expand the sample set in order to permit test set validation of the calibration models.

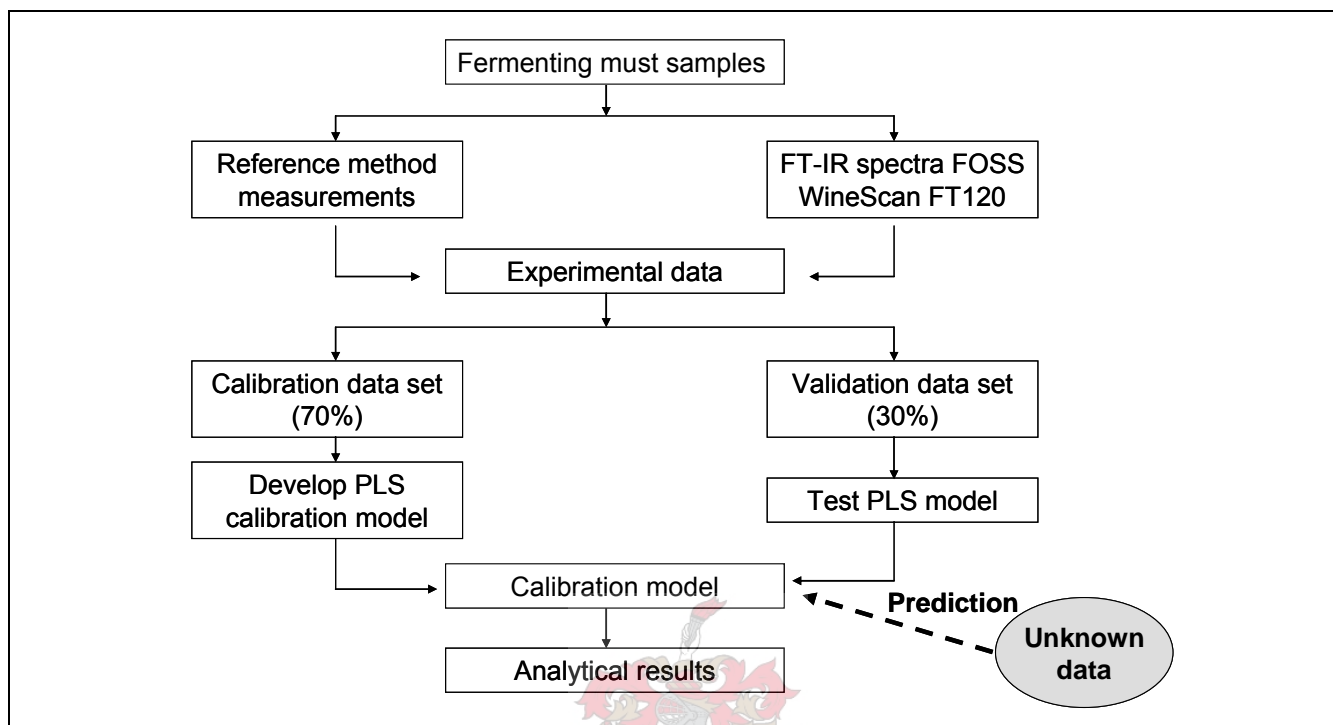


Figure 6.6 Diagram of the strategy followed to develop a calibration model.

6.1.5.3 Performance evaluation of the calibration sets using statistical indicators

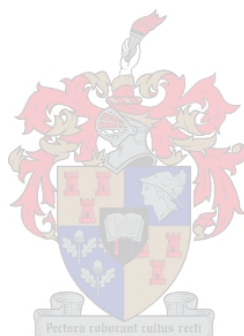
The Advanced Performance Module provided with the Winescan FT120 instrument calculated the statistical indicators bias, SECV (standard error of cross validation) and SEP (standard error of prediction) for evaluating the performance of the calibration models. Bias, calculated as the difference between the reference values and the predicted values, gives an indication of a systematic error in the predictive values (Esbensen, 2002). SECV expressed the accuracy of the predictive ability of the calibration model, relative to the reference data, when based on the calibration samples and using cross validation. Cross validation was automatically performed by the software by keeping out successive groups of samples from the calibration set (10% of the total number of calibration samples at a time) and using these subsets for the prediction. This is continued until all samples have been kept out once. This cross validation selection strategy has been set by the manufacturer and can not be changed by the instrument user. SEP expressed the prediction accuracy of the calibration model relative to the reference data based on an independent validation set. The calculation procedures of these standard statistical indicators are described by other authors (Eriksson *et al.*, 1999; Esbensen, 2002; Winescan FT120 type 77110 and 77310 reference manual).

6.2 RESULTS AND DISCUSSION

6.2.1 EXPLORATORY ANALYSIS OF FT-IR SPECTRA

A FT-IR spectrum of wine or fermenting must provides the collective absorbance of all the IR-active components, including those corresponding to the vibrations of the C-O, C-C, C-H and C-N bonds present in the sample (Smith, 1999). Two wavenumber regions 1543 - 1716 cm^{-1} and 2970 – 3626 cm^{-1} respectively, contain significant contribution of water absorption (**Fig. 6.7A**) to the spectra (Patz *et al.*, 2004; Nieuwoudt *et al.*, 2004) and an additional region, the “fingerprint” region, wavenumbers 929-1600 cm^{-1} (Smith, 1999) contains information about the chemical composition of wine samples.

Pinotage must samples were collected throughout the fermentation process ranging in sugar concentration from 277 g/L to 1 g/L residual sugar and FT-IR spectra were obtained for each of these samples. Variation in the fingerprint region was observed and these spectral differences can be related to different stages of fermentation as a result of sugar and ethanol concentration changes (**Fig. 6.7B**).



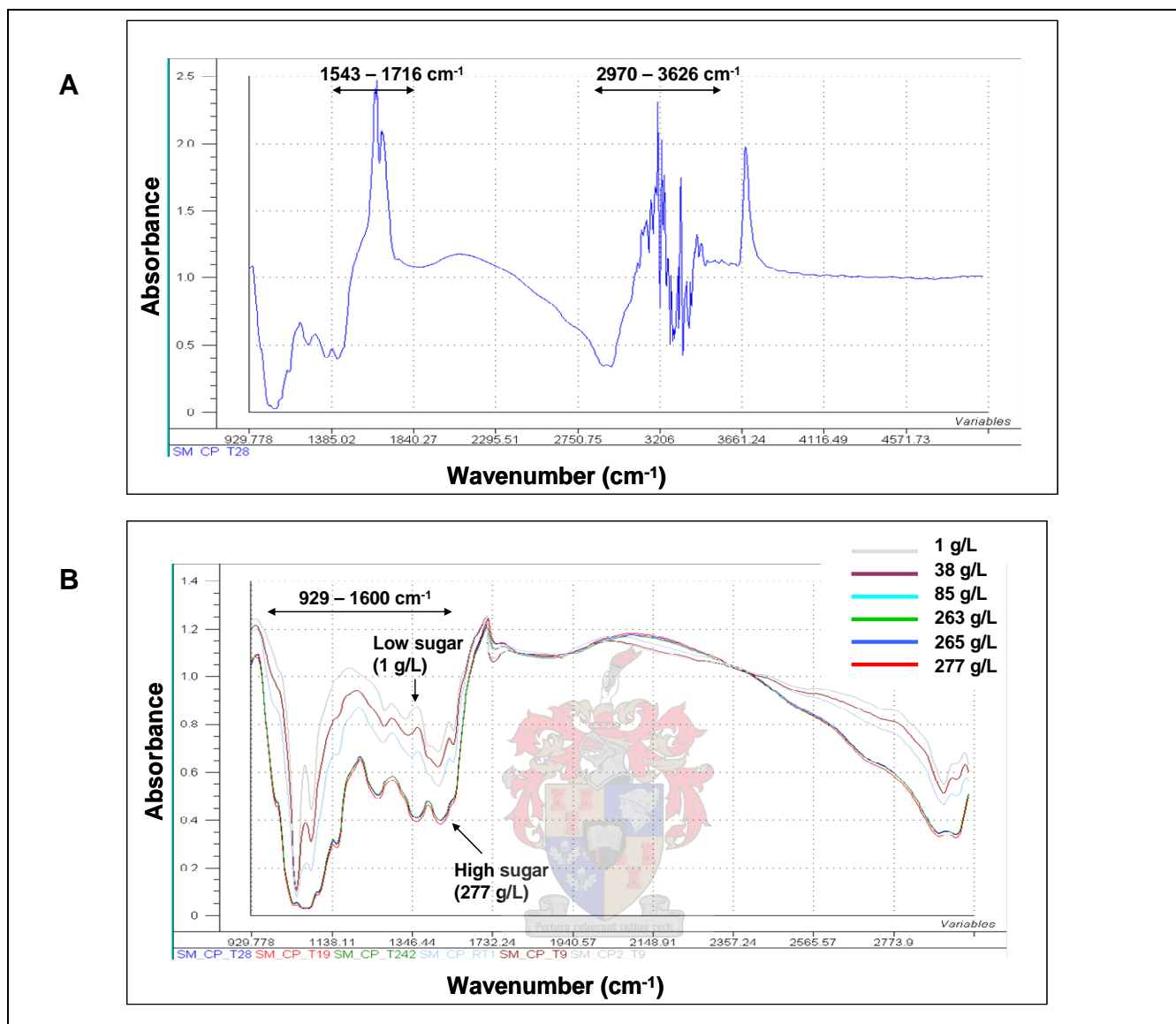


Figure 6.7 (A) FT-IR spectra of a Pinotage must sample. The two water absorption regions are indicated. (B) Spectral variation between high sugar and low sugar must of Pinotage samples in the region 929 – 1600 cm^{-1} . Samples are from different stages of fermentation. (red = 277 g/L, blue = 265 g/L, green = 263 g/L, light blue = 85 g/L, purple = 38 g/L, grey = 1 g/L residual sugar)

6.2.2 PCA MODELLING OF THE CALIBRATION SAMPLES

PCA modelling was used to identify the main sources of variation amongst the different calibration samples and to investigate the general data structure. PCA on all the spectral wavenumbers allowed for the identification of various stages of fermentation. However, because the noise was still included in these modelling stages, PC1 only modelled 42% of the variance between the samples.

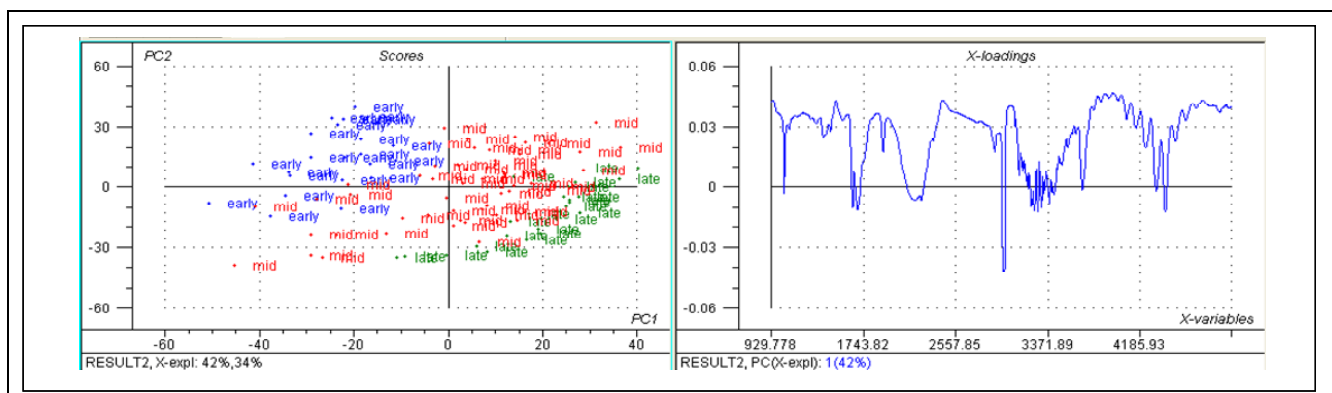


Figure 6.8 PCA modelling of the calibration sample set using all the wavenumbers (PC1 vs PC2). The calibration sample set clearly consists samples from all stages of fermentation: early (blue), mid (red) and late (green) fermentation. PC1 modelled 42% of the variance between the samples.

In addition, PCA was performed on the spectra with the exclusion of the water absorbance regions. Better differentiation between the different stages of fermentation was visible and PC1 modelled 64% of the variance amongst the samples. This is most probably the result of fermentation progress accompanied by a decrease in sugar concentration and a subsequent increase in ethanol concentration along PC1.

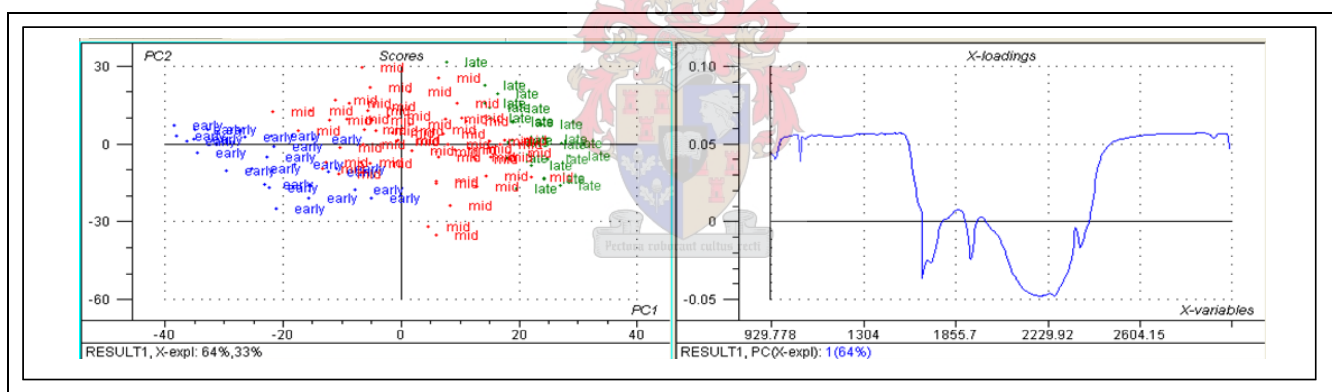


Figure 6.9 PCA modelling of the calibration sample set using all the wavenumbers with the exclusion of the water absorbance regions. Distribution between the samples along PC1 accounts for 64% of the variance and could be related to stage of fermentation: early (blue), mid (red) and late (green) fermentation.

6.2.3 PRELIMINARY CALIBRATION STRATEGY AND RESULTS

The initial calibration sets consisting of only Chenin blanc samples were used for calibration purposes in order to evaluate the potential of such glucose and fructose quantification using FT-IR spectroscopy. These initial calibrations showed promise although expansion in a few concentration ranges is needed (**Fig. 6.10**). However, it should be kept in mind that these calibrations were constructed using only a single cultivar, fermented with a single yeast starter culture in four separate canisters.

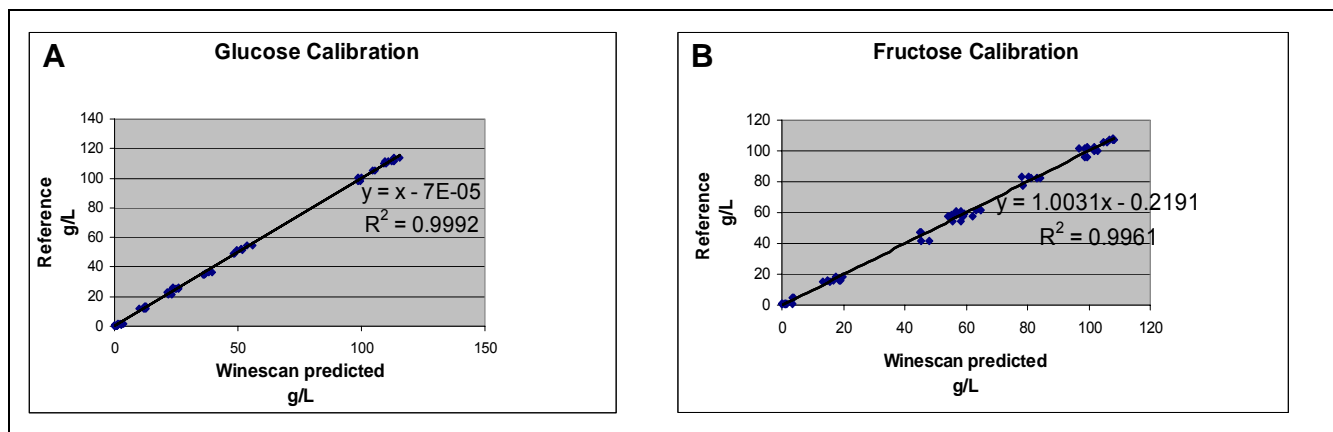


Figure 6.10 Preliminary calibration for glucose (A) and fructose (B) using only Chenin blanc samples fermented under controlled laboratory conditions.

Table 6.6 Calibration statistics for the initial glucose and fructose calibration using only Chenin blanc fermentations conducted under controlled laboratory conditions.

| Component | FOSS product | Observations | Conc. range | SEC | Meanbias | R ² |
|-----------|--------------|--------------|-----------------|------|----------|----------------|
| glucose | MUF | 70 | 0.03-114.22 g/L | 1.16 | 0 | 0.9992 |
| fructose | MUF | 66 | 0.47-107.58 g/L | 2.39 | 0 | 0.9960 |

In addition to the first calibration attempts the sample set was expanded to include industrial samples collected from commercial cellars to widen the cultivar distribution and robustness of the calibrations. Our interest was primarily in evaluating the suitability of the commercially available calibrations of the FOSS Winescan FT120 for fermenting must and attempt to make valuable recommendations for the following calibration strategies. The selection of a sample set consisting of finished wine samples (samples finished with alcoholic fermentation) was only included to illustrate the enormous difference between these matrices and to emphasize the importance of critically investigating the calibration efficiency for fermenting must. This point is illustrated by **Fig. 6.11** and **Fig. 6.12**. It is clear that the MUF sample set contains large variation as a result of the enormous change in the matrix during fermentation (**Fig. 6.11**). In comparison, the MLF sample set consisting of samples in a more homogeneous state (e.g. finished with alcoholic fermentation) shows much less variation. The impact of this variation on the calibration process is clearly showed in **Fig. 6.12**. Due to the amount of variation in the MUF calibration set the variance was poorly explained by the PLS factors. The homogeneity of the MLF calibration set allowed the filters to effectively explain the variance in this calibration set in terms of malic acid. This is clear from the definite decrease in the cross validation vs factors plot.

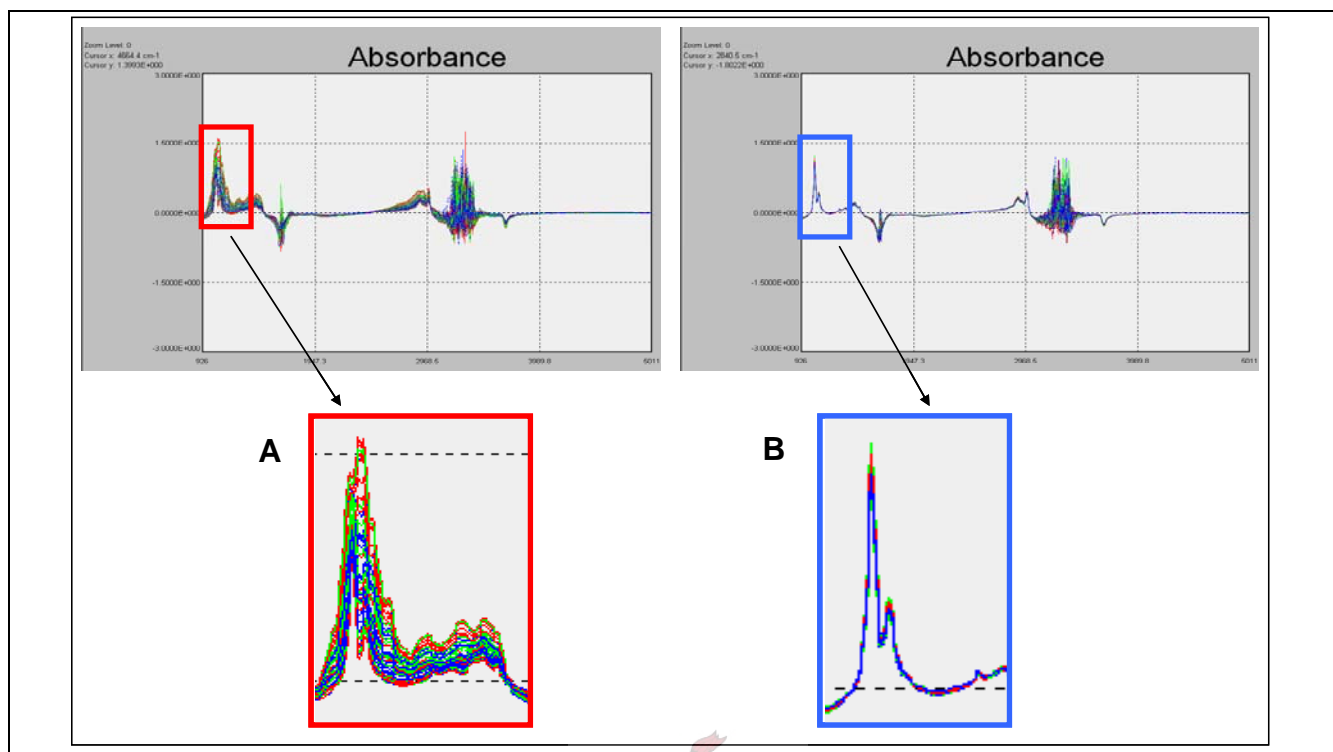


Figure 6.11 (A) **MUF** sample set contains large variation due to the enormous change in matrix during fermentation. This can be seen in the FT-IR spectra of the samples. Contrary to this variation are the spectra of the **MFL** sample set (B). Since all these samples have completed alcoholic fermentation the variation in terms of sugar concentration is far less.

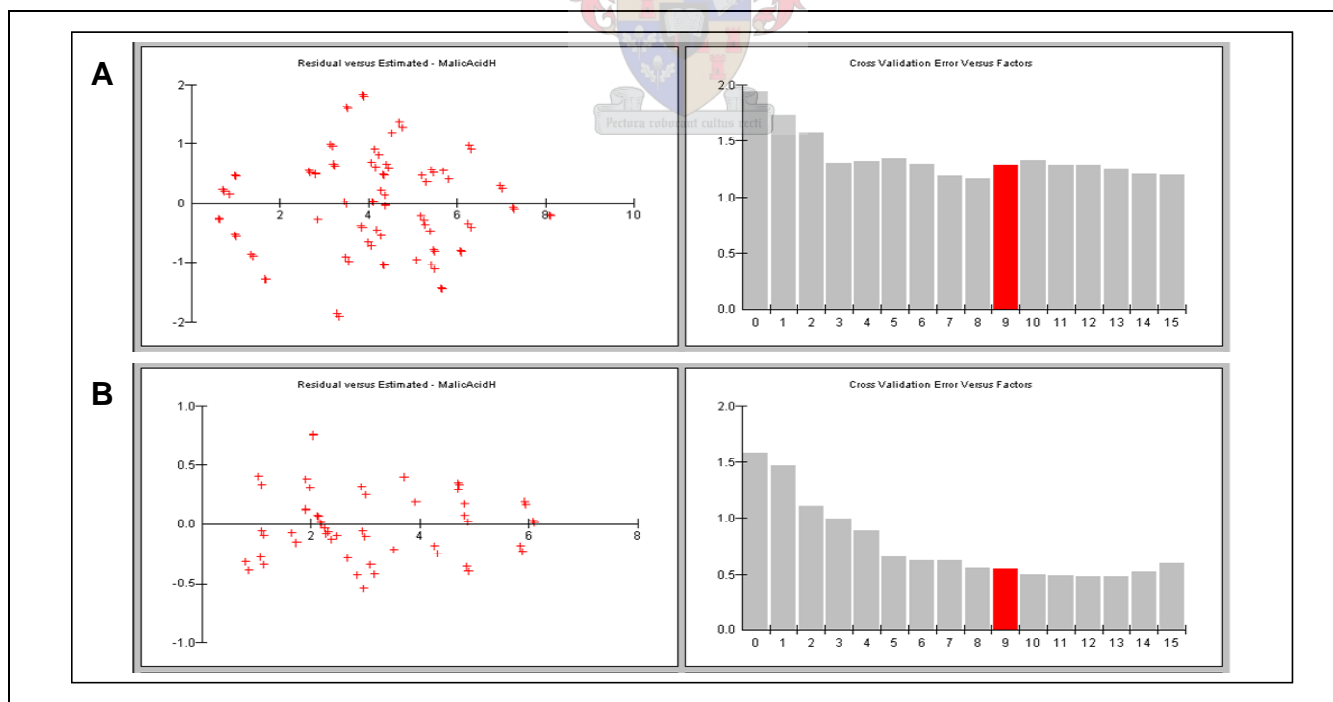


Figure 6.12 Due to the amount of variation in the **MUF** calibration set the variance was poorly explained by the PLS factors (A). The homogeneity of the **MFL** calibration set allowed the filters to effectively explain

the variance in this calibration set in terms of malic acid (B). A definite decrease in cross validation error vs factors plot is observed (B).

The preliminary calibration attempt for free assimilable nitrogen (FAN) showed that the change in the matrix during fermentation is so enormous that the PLS factors explains the variation poorly if all the samples collected throughout fermentation is modelled simultaneously (**Fig. 6.13A**). The correlation coefficient increased with the separation of samples into different stages of fermentation (**Fig. 6.13B, C, D**).

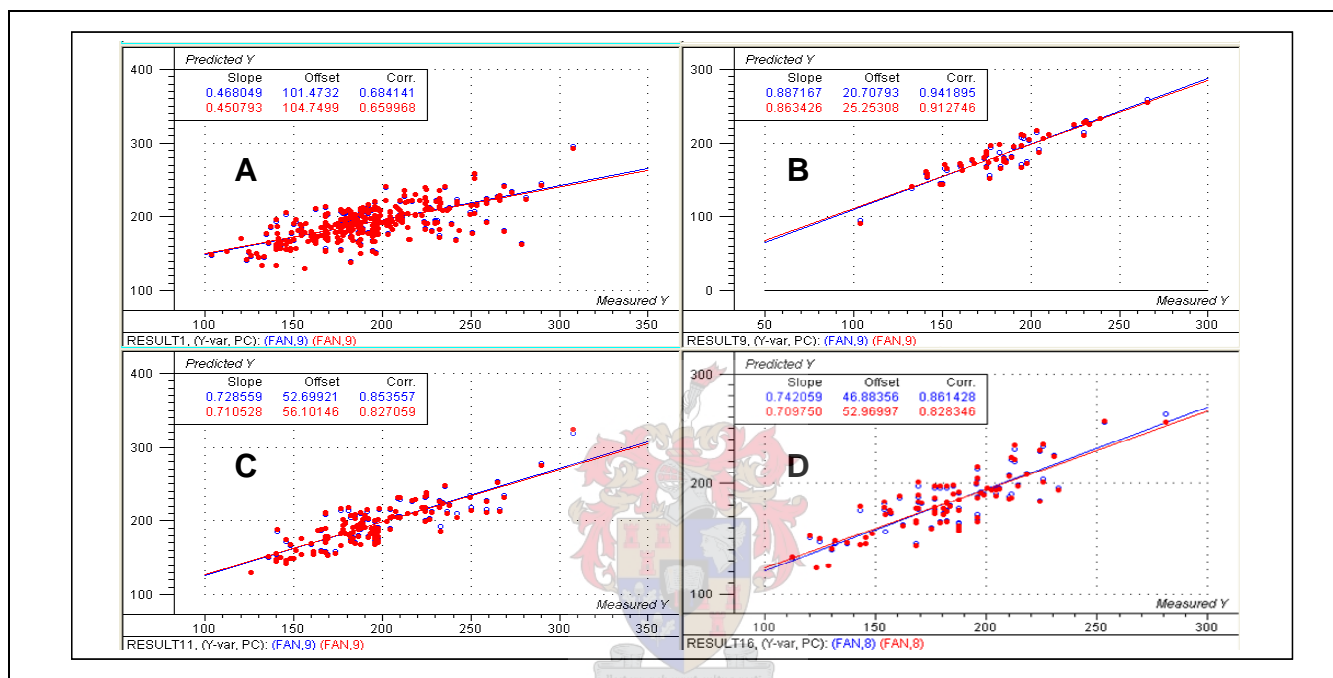


Figure 6.13 Preliminary calibration (blue) for FAN determination using FT-IR spectroscopy (A) all samples (B) samples from the early stages of fermentation (C) samples from middle stages of fermentation and (D) samples from late stages of fermentation. Validation results are indicated (red). Better calibration statistics (higher R^2) was obtained with the separation of fermentation stages (B, C, D) than with the combined effort (A). The influence of the enormous matrix change occurring during fermentation and its effect on calibration is evident.

An initial validation of the current fructose calibration resulted in the following (**Fig. 6.14**): (A) the present calibration for fructose has a RMSEC of 16.499 and a mean bias of 4.25. The proposed slope and intercept adjustment would therefore lower the RMSEC to 15.15 with a R^2 of 0.806 (indicated by **Fig. 6.14A**); (B) The slope and intercept adjustment was made and the RMSEC was 13.45 and the mean bias decreased to 0 (indicated by **Fig. 6.14B**). This is a clear indication that the current global calibration is possibly not optimal for the analysis of fermenting must and needs further development for South African conditions. Observations also suggest that the calibration could be split above 75 g/L fructose into a separate calibration since some of the samples are poorly predicted above 75 g/L (**Fig. 6.14B**).

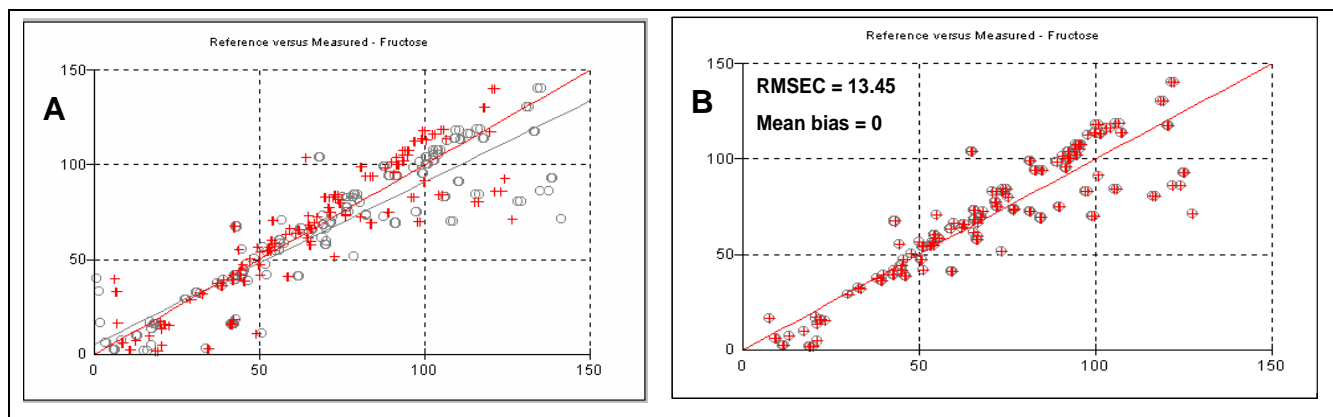


Figure 6.14 (A) The proposed slope and intercept adjustment (red) versus the present calibration (grey). The accepted slope and intercept adjustment showed a decrease in the RMSEC from 16.499 to 13.45 and a resultant mean bias of 0 (B).

In addition to the above-mentioned approach, the same sample set was used to construct a preliminary new calibration (**Fig. 6.15**) for fructose. A lower RMSEC of 10.75 and a mean bias of 0 was obtained in comparison with the original calibration (RMSEC = 16.499 and mean bias = 4.25) indicating once again the necessity of further calibration development.

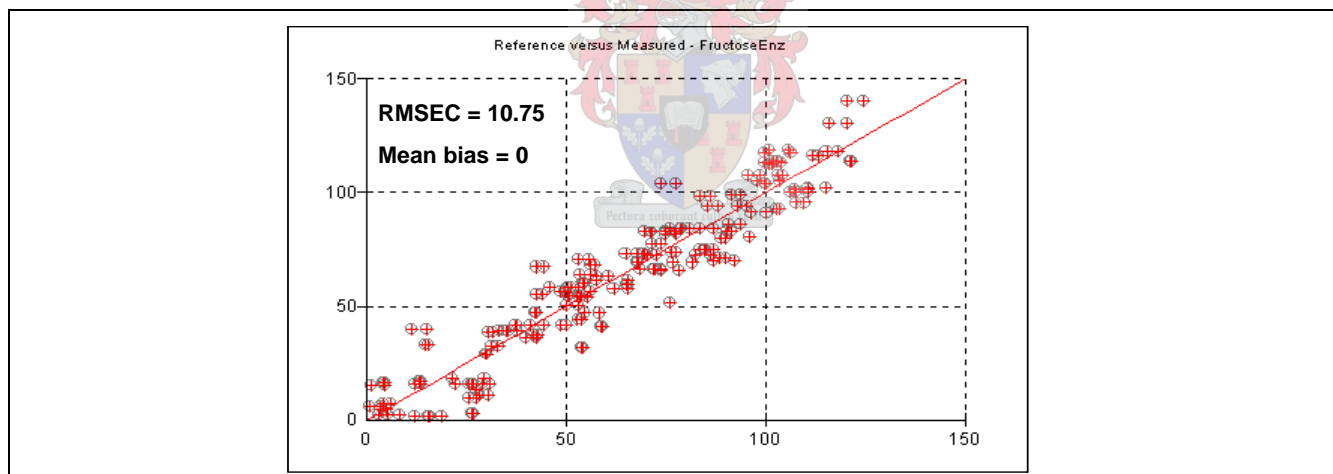


Figure 6.15 The preliminary slope and intercept adjustment decreased RMSEC from 16.499 to 10.75 and a mean bias of 0. Possible spilt in the calibration above 75 g/L should be investigated.

A preliminary attempt for the quantification of glycerol in fermenting must with the use of FT-IR spectroscopy (**Fig. 6.16**). Although this initial calibration shows promise, expansion of the data set is needed before any conclusion or adjustments to the current calibration should be considered.

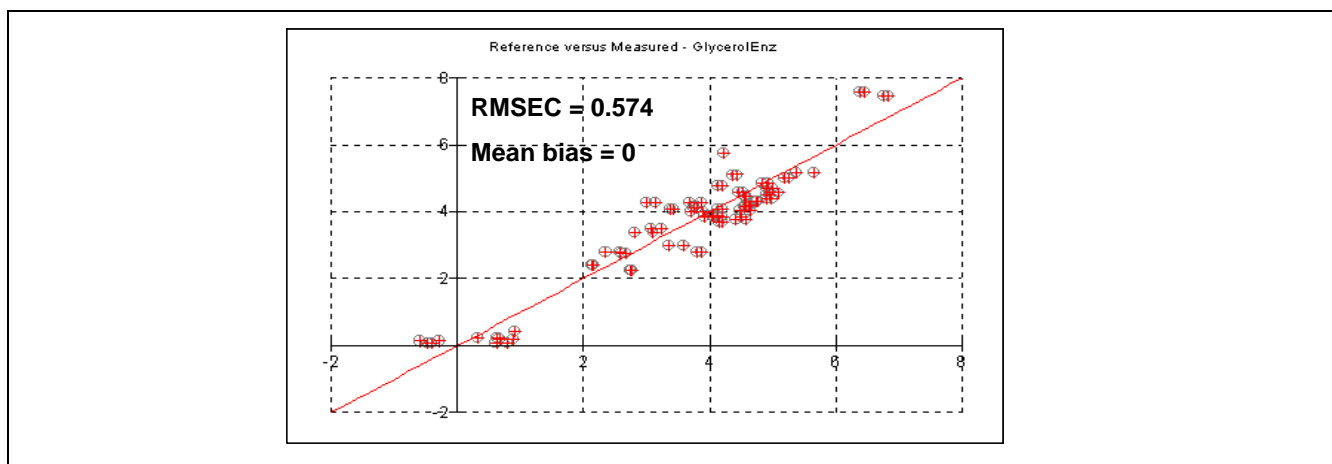


Figure 6.16 Preliminary glycerol calibration for fermenting must shows promise, however expansion of the dataset is needed.

It is clear from both the calibration and validation attempts that the development of specific calibrations using FT-IR spectroscopy is definitely necessary. In order to increase the accuracy of these prediction models, new or additional modelling is needed to decrease the RMSEC and increase the correlation coefficient of specific calibrations.

6.C.3 CONCLUSIONS

In this study preliminary work was done to evaluate the commercial calibrations, so-called “global calibrations” of the WineScan instrument for accuracy of prediction of selected chemical components in actively fermenting must, as well as in wines undergoing malolactic fermentation. Furthermore, a selection strategy was successfully implemented to obtain representative calibration sample sets under the restriction of keeping the number of samples as low as possible. Enzymatic and HPLC reference methods were used and evaluated for suitability in routine analysis of high numbers of samples. In order to more effectively generate accurate reference values for calibration purposes additional reference methods could be investigated. The possibility to automate some of the reference methods (enzymatic) could also be investigated.

Multivariate regression models were constructed between the FT-IR spectra and the reference values using the FOSS Advanced Module software. This software uses 10% fractions of data for cross validation purposes. *The Unscrambler* software was also used to construct regression models and this software allows the selection of full cross validation (take-one-out) to test the model. Although full cross validation is not the preferred method of validation, it is accepted in this case due to the small size of the calibration sample set. Further studies on the calibration of these chemical parameters will expand the sample set to permit test set validation.

The results obtained from this initial study show that a dedicated effort at validation of commercial calibrations for actively fermenting must samples is urgently needed. Validation of commercial calibrations for finished wine has been performed by the South African Winescan User group (personal communication, H el ene Nieuwoudt, Institute for Wine Biotechnology, Stellenbosch University). However, the validation of commercial calibrations and the establishment of new calibrations for fermenting must, present an enormous challenge as a result of the change in matrix. Results in this preliminary study visually displayed the impact that spectral variation throughout the fermentation has on the calibration itself, compared to the calibration process of finished wine. As a result of the variation in sugar concentration, cultivar and wine origin, all reflected in the spectra, the selected filters (group of wavenumbers) are unable to use a regression algorithm to describe the relationship between the spectra and the desired analyte (e. g. malic acid concentration). This effect might be eliminated by expanding the size of the calibration set and including larger numbers of specific cultivars or eliminating those cultivars present in small numbers that might affect the calibration.

Fourier transform infrared spectroscopy has showed promise as a rapid method for determining various compounds simultaneously finished wine. Followed by proper sample preparation and validation of commercial calibrations FT-IR spectroscopy has the potential to dramatically reduce analytical time and cost of monitoring wine fermentation for both research and commercial production applications. Further studies are needed in order to improve the calibration specificity, accuracy and robustness for chemical parameters related to changes during fermentation with consideration of the constantly changing sample matrices during fermentation.

6.C.4 LITERATURE CITED

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