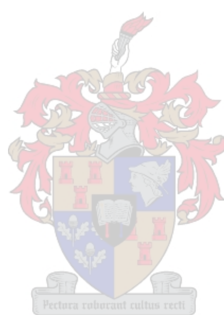
The evaluation of Fourier transform infrared (FT-IR) spectroscopy for quantitative and qualitative monitoring of alcoholic wine fermentation

by

Cynthia M Magerman



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Declaration

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Summary

Fermentation is a complex process in which raw materials are transformed into high-value products, in this case, grape juice into wine. In this modern and economically competitive society, it is increasingly important to consistently produce wine to definable specifications and styles. Process management throughout the production stage is therefore crucial to achieve effective control over the process and consistent wine quality. Problematic wine fermentations directly impact on cellar productivity and the quality of wine. Anticipating stuck or sluggish fermentations, or simply being able to foresee the progress of a given fermentation, would be extremely useful for an enologist or winemaker, who could then take suitable corrective steps where necessary, and ensure that vinifications conclude successfully. Conventional methods of fermentation monitoring are time consuming, sometimes unreliable, and the information limited to a few parameters only. The current effectiveness of fermentation monitoring in industrial wine production can be much improved. Winemakers currently lack the tools to identify early signs of undesirable fermentation behaviour and to take preventive actions.

This study investigated the application of Fourier transform mid infrared (FT-IR) spectroscopy in transmission mode, for the quantitative and qualitative monitoring of alcoholic fermentation during industrial wine production. The major research objectives were firstly to establish a portfolio of quantitative calibration models suitable for quantification of the major quality determining parameters in fermenting must. The second major research objective focused on a pilot study aimed at exploring the use of off-line batch multivariate statistical process control (MSPC) charts for actively fermenting must. This approach used FT-IR spectra only, for the purpose of qualitative monitoring of alcoholic fermentation in industrial wine production. Towards these objectives, a total of 284 industrial-scale, individual, actively fermenting tanks of the seven major white cultivars and blends, and nine major red cultivars, of Namaqua Wines, Vredendal, South Africa, were sampled and analysed with FT-IR spectroscopy and appropriate reference methods during vintages 2007 to 2009.

For the quantitative strategy, partial least squares regression (PLS1) calibration models for determination of the classic wine parameters ethanol, pH, volatile acidity (VA), titratable acidity (TA) and the total content of glucose plus fructose, were redeveloped to provide a better fit to local South African samples. New PLS1 models were developed for the must components glucose, fructose and yeast assimilable nitrogen (YAN), all of which are frequently implicated in problem fermentations. The regression statistics, that included the standard error of prediction (SEP), coefficient of determination (R^2) and bias, were used to evaluate the performance of the redeveloped calibration models on local South African samples. Ethanol (SEP = 0.15 %v/v, R^2 = 0.999, bias = 0.04 %v/v) showed very good prediction and with a residual predictive deviation (RPD) of 30, rendered an excellent model for quantitative purposes in fermenting must. The models for pH (SEP = 0.04, R^2 = 0.923, bias = -0.01) and VA (SEP = 0.07 g/L, R^2 = 0.894, bias = -0.01 g/L) with RPD values of 4 and 3 respectively, showed that the models were suitable for screening purposes. The calibration model for TA (SEP = 0.35 g/L, R^2 = 0.797, bias = -0.004 g/L) with a RPD of 2, proved unsatisfactory for quantification purposes, but reasonable for screening purposes. The calibration model for the total content of glucose plus fructose (SEP = 0.6.19 g/L, R^2 = 0.993, bias = 0.02 g/L) with a RPD of 13, showed very good prediction and can be used to quantify total glucose plus fructose content in fermenting must. The newly developed calibration models for glucose (SEP = 4.88 g/L, R^2 = 0.985, bias = -0.31 g/L) and fructose (SEP = 4.14 g/L, R^2 = 0.989, bias = 0.64 g/L) with RPD values of 8 and 10 respectively, also proved fit for quantification of these important parameters. The new calibration models of ethanol, total

glucose plus fructose; and glucose and fructose individually, showed an excellent relation to local South African samples and can be easily implemented by the wider wine industry.

Two calibration models were developed to determine YAN in fermenting must by using different reference methods, namely the enzyme-linked spectrophotometric assay and Formol titration method, respectively. The results showed that enzyme-linked assays provided a good quantitative model for white fermenting must (SEP = 14.10 mg/L, R^2 = 0.909, bias = -2.55 mg/L, RPD = 6), but the regression statistics for predicting YAN in red fermenting must, were less satisfactory (data not shown). The Formol titration method could be used successfully in both red- and white fermenting must (SEP = 16.37 mg/L, R^2 = 0.912, bias = -1.01 mg/L, RPD = 4). A minor, but very important finding was made with respect to the storage of must samples that were taken from tanks, but that could not immediately be analysed with FT-IR spectroscopy or reference values. Principal component analysis (PCA) of frozen samples showed that must samples could be stored frozen for up to 3 months and still be used to expand the calibration sample sets when needed. Therefore, samples can be kept frozen to a later stage if immediate analyses are not possible.

For the purpose of the pilot study that focused on the use of FT-IR spectroscopy for qualitative off-line monitoring of alcoholic fermentation, a total of 21 industrial-scale fermentation tanks were monitored at 8- or 12-hourly intervals, from the onset of fermentation to complete consumption of the grape sugars. This part of the work excluded quantitative data, and only used FT-IR spectra. MSPC charts were constructed on the PLS scores of all the FT-IR spectra taken at the various time intervals of the different batches, using time as the *y*-variable. The primary aim of this research objective was to evaluate if the PLS batch models could be used to discriminate between normal and problem alcoholic fermentations. The models that were constructed clearly showed the variations in patterns over time, between red- and white wine alcoholic fermentations. One Colombar tank that was fermented at very low temperature in order to achieve a specific wine style, was characterised by a fermentation pattern that clearly differed form the rest of the Colombar fermentations. This atypical fermentation was identified by the batch models constructed in this study. PLS batch models over all the Colombar fermentations clearly identified the normal and problem fermentations.

The results obtained in this study showed that FT-IR spectroscopy showed great potential for effective quantitative and qualitative monitoring of alcoholic fermentation during industrial wine production. The work done in this project resulted in the development of a portfolio of calibration models for the most important quality determining parameters in fermenting must. The quantitative models were subjected to extensive independent test set validation, and have subsequently been implemented for industrial use at Namaqua Wines. Multivariate batch monitoring models were established that show good discriminatory power to detect problem fermentations. This is a very useful diagnostic tool that can be further developed by monitoring more normal and problem fermentations. Future work in this regard, will focus on further optimisation and expansion of the quantitative and qualitative calibration models and implementation of these in the respective wineries of Namaqua Wines.

Opsomming

Fermentasie is ‘n komplekse proses waartydens rou material getransformeer word na produkte van hoë waarde, in hierdie geval, druiwesap na wyn. In die huidige ekonomies-kompeterende samelewing, is dit al hoe meer belangrik om volhoubaar wyn te produseer wat voldoen aan definieerbare spesifikasies en style. Goeie prosesbestuur tydens die wynproduksie stadium is baie belangrik om herhaalbaarheid en gehaltebeheer te verseker. Problematiese wynfermentasies het ’n direkte impak op beide kelderproduktiwiteit en wynkwaliteit. Die voorkoming van slepende- of steekfermentasies, of selfs net om probleme te voorsien, sou uiters bruikbaar wees vir ‘n wynkundige of wynmaker, wat dan die toepaslike regstellende stappe kan neem waar nodig, om te verseker dat die wynbereiding suksesvol voltooi word. Konvensionele metodes van monitering van alkoholiese fermentasie is tydrowend, soms onbetroubaar en die inligting beperk tot ‘n paar parameters. Die huidige effektiwiteit van fermentasie monitering in industriële wynproduksie kan heelwat verbeter word. Wynmakers ervaar tans ’n behoete aan tegnologië wat die vroeë tekens van ongunstige fermentasiepatrone kan identifiseer, en hul doeltreffendheid om moontlike regstellende aksies te neem, is dus beperk.

Hierdie studie het die toepassing van Fourier transformasie mid-infrarooi (FT-IR) spektroskopie in transmissie, ondersoek met die oog op kwantitatiewe en kwalitatiewe monitering van alkoholiese gisting tydens industriële wynproduksie. Die vernaamste navorsingsdoelwitte was eerstens om ’n portefeulje van kwantitatiewe kalibrasiemodelle te vestig, wat geskik is om die belangrikste kwaliteitsbepalende parameters in gistende mos te kwantifiseer. Die tweede hoofnavorsingsdoelwit was ’n loodsstudie wat ondersoek ingestel het na die opstel van multiveranderlike statistiese proseskontrole grafieke van aktief-gistende mos, met die oog op aflyn-kwalitatiewe monitering van alkoholiese gisting in industriële wynproduksie. Hiervoor is slegs FT-IR spektra gebruik. Vir die doel van hierdie studie is monsters van ’n totaal van 284 individuele, aktief-gistende tenke van die sewe hoof wit kultivars en hul versnydings en nege hoof rooi kultivars van Namaqua Wyne, Vredendal, Suid Afrika, geneem. Al die monsters is met toepaslike chemiese metodes en FT-IR spektroskopie analiseer tydens die parsseisoene van 2007 tot 2009.

Vir die kwantitatiewe strategie is parsiële kleinste kwadraat (PKK1) kalibrasiemodelle vir die bepaling van die klassieke wynparameters etanol, pH, vlugtige suur (VS), titreerbare suur (TS) en die totale konsentrasie van glukose plus fruktose herontwikkel, om beter te pas op plaaslike Suid-Afrikaanse monsters. Nuwe PKK1 kalibrasiemodelle is ontwikkel vir die komponente glukose, fruktose en gis-assimileerbare stikstof, aangesien hierdie komponente gereelde aanduidings van probleemgisting is. Die regressiestatistieke het die standaardvoorspellingsfout (SVF), bepalingskoëffisiënt (R^2) en sydigheid ingesluit en was gebruik om die prestasie van die herontwikkelde kalibrasiemodelle vir plaaslike Suid-Afrikaanse monsters te evalueer. Etanol (SVF = 0.15 %v/v, R^2 = 0.999, sydigheid = 0.04 %v/v) het baie goeie regressiestatistiek getoon en met ‘n relatiewe voorspellingsafwyking (RVA) van 30, was dit ‘n uitstekende model vir kwantifisering in gistende mos. Die modelle vir pH en VS met RVA waardes van 4 en 3 onderskeidelik, is geskik vir semi-kwantitatiewe toepassings. Die kalibrasiemodel vir TS met ‘n RVA waarde van 2, was nie geskik vir akkurate kwantifisering nie, maar wel vir semi-kwantitatiewe analises. Die kalibrasiemodel vir die totale glukose plus fruktose inhoud in gistende mos, met ‘n RVA waarde van 13, het uitstekende regressiestatistiek gegee en is geskik vir akkurate kwantifiseringsdoeleindes. Die nuut-ontwikkelde kalibrasiemodelle vir glukose en fruktose, met RVA waardes van onderskeidelik 8 en 10, is geskik vir akkurate

kwantifisering van hierdie belangrike parameters. Die kalibrasiemodelle vir etanol, totale glukose plus fruktose, en glukose en fruktose afsonderlik, het uitstekende korrelasies getoon met plaaslike Suid-Afrikaanse monsters en is gereed om toepassing te vind in die wyer wynindustrie.

Twee kalibrasiemodelle is ontwikkel om gis-assimileerbare stikstof in gistende mos te bepaal, deur gebruik te maak van verskillende verwysingsmetodes van analise; hierdie metodes was ‘n ensiem-gekoppelde spektrofotometriese toets en die Formoltitrasie metode. Resultate het getoon dat goeie regressiestatistiek vir FT-IR spektroskopie-gebaseerde kalibrasiemodelle waar data wat met die ensiem-gekoppelde toetse verkry is, as verwysingwaardes gebruik is, in wit gistende mos (SVP = 14.10 mg/L, R^2 = 0.909, sydigheid = -2.55 mg/L, RVA = 6), maar nie in rooi gistende mos nie. Die Formoltitrasie metode as verwysingsmetode, was geskik vir die ontwikkeling van goeie kalibrasiemodelle in beide rooi- en wit gistende mos (SVP = 16.37 mg/L, R^2 = 0.912, sydigheid = -1.01 mg/L, RVA = 4). ’n Sekondêre, maar baie belangrike bevinding is gemaak met betrekking tot die stoor van mosmonsters wat geneem is van tenke, maar wat nie dadelik met die verwysingsmetodes en FT-IR spektroskopie analiseer kon word nie. Multiveranderlike hoofkomponentanalise op vars en gevriesde sapmonsters het getoon dat gevriesde monsters gebruik kan word om die kalibrasie datastel uit te brei, wanneer benodig. Dus, sapmonsters kan gevries word tot ’n later stadium as onmiddelike analises nie moontlik is nie.

Vir die doel van die tweede navorsingsdoelwit van die studie, naamlik kwalitatiewe af-lyn monitering van alkoholiese fermentasie met FT-IR spektroskopie, is ‘n totaal van 21 industriële-grootte fermentasietenks ge-monitor deur sapmonsters met 8- tot 12-uurlikse intervalle te trek, vanaf die begin van fermentasie, totdat al die druifsuiker gemetaboliseer is. Vir hierdie deel van die werk is die kwantitatiewe data nie gebruik nie; slegs die FT-IR spektra. Multiveranderlike statistiese proseskontrole grafieke is opgestel op grond van die PKK tellings wat bereken is op al die FT-IR spektra wat gemeet is by die verskillende tydsintervalle. Vir hierdie analise is tyd as *y*-veranderlike gebruik. Die vernaamste doel van hierdie ondersoek was om te evalueer of die PKK-gebaseerde modelle kon onderskei tussen normale en slepende gistings. Die modelle wat verkry is, het die variasie oor tyd in die fermentasiepatrone tussen wit- en rooiwyn fermentasies tydens alkoholiese gisting, duidelik uitgewys. Een Colombar tenk wat teen baie lae temperatuur gefermenteer is om ‘n spesifieke wynstyl te verkry, se fermentasiepatroon het aansienlik verskil van die ander Colombar tenks wat gemonitor is, en hierdie atipiese patroon is ook deur die kwalitatiewe modelle identifiseer. ‘n PKK model oor al die Colombar fermentasies kon duidelik tussen normale en slepende gistings onderskei.

Die resultate wat in hierdie studie verkry is, het getoon dat FT-IR spektroskopie baie goeie potensiaal toon vir die aanwending van kwantitatiewe en kwalitatiewe monitering van alkoholiese fermentasie tydens industriële wynproduksie. Die werk wat in hierdie projek gedoen is, het gelei tot die vestiging van ‘n portefeulje van kalibrasiemodelle vir die belangrikste kwaliteitsbepalende parameters in fermenterende mos. Die kwantitatiewe modelle is baie deeglik getoets met onafhanlike toets datastelle, en daarna is die kalibrasiemodelle ge-implementeer vir industriële gebruik by Namaqua Wyne. Multiveranderlike statistiese proseskontrole grafieke wat baseer is op data wat vanaf 21 verskillende fermentasietenks verkry is, het baie goeie potensiaal getoon om probleemfermentasies vroeg te identifiseer. Dié grafieke is ‘n baie nuttige diagnostiese hulpmiddel wat verder ontwikkel kan word om verskillende tipes probleemfermentasies te monitor. Toekomstige navorsing in hierdie konteks, sal toegespits word op die optimisering en uitbreiding van die kwantitatiewe en kwalitatiewe modelle, sowel as toepassing van die tegnieke in die onderskeie kelders van Namaqua Wyne.

This thesis is dedicated to my mother for her continuous support.

Hierdie tesis is opgedra aan my moeder vir haar volgehoue ondersteuning.

Biographical sketch

Cynthia Magerman was born in Pella, South Africa on 31 May 1978. She attended Pella Primary School (Roman Catholic) and matriculated at SA van Wyk Secondary School, Bergsig, Springbok, in 1995. Cynthia obtained a BSc degree in Food Science in 1999 at the University of Stellenbosch.

Cynthia joined Namaqua Wines, Vredendal, South Africa, as laboratory assistant in 2000. In 2003 she was promoted to laboratory manager and in 2007 enrolled for an MSc degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University.

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Preface

This thesis is presented as a compilation of six chapters. Each chapter is introduced separately.

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** Monitoring alcoholic fermentation during industrial wine production
Chapter 3	Univariate and multivariate data analytical tools used in this study
Chapter 4	**Research results** Evaluation of Fourier transform infrared spectroscopy for the quantification of major chemical parameters in fermenting grape must
Chapter 5	**Research results** Qualitative off-line batch monitoring of alcoholic fermentation
Chapter 6	**General discussion and conclusions**
Addendum A	**Enzyme-linked spectrophotometric assays** Reactions and calculations
Addendum B	**List of journals with abbreviation used in this study**

List of abbreviations used in this study

ANOVA	: Analysis of variance
AU	: Absorbance units
B	: Boron
C6	: Hexanoic
C8	: Octanoic
C10	: Decanoic
Ca	: Calcium
CE	: Capillary electrophoresis
CV	: Coefficient of variation
DmodX	: Distance to the model X
DPLS	: Discriminant partial least squares
EC	: Ethyl carbamate
YAN	: Yeast assimilable nitrogen
Fe	: Iron
FT-IR	: Fourier transform infrared
G-G	: Glycosylated compounds
HPLC	: High performance liquid chromatography
H_2S	: Hydrogen sulphide
IR	: Infrared
IWBT	: Institute for Wine Biotechnology
K	: Potassium
Mg	: Magnesium
MIR	: Mid-infrared
MLR	: Multiple linear regression
MSPC	: Multivariate statistical process control
Mn	: Manganese
Na	: Sodium
NIR	: Near infrared
NIRS	: Near infrared spectroscopy
P	: Phosphorus
PCA	: Principal component analysis
PC	: Principal component
PCR	: Principal component regression
PLS	: Partial least squares
PLS-R	: Partial least squares regression
PVPP	: Polyvinyl polypyrrolidone
R^2	: Coefficient of determination
RMSEP	: Root mean square error of prediction
S	: Standard deviation
SA	: South Africa
SDD	: Standard deviation of the difference
SECV	: Standard error of cross validation
SEL	: Standard error of laboratory
SEP	: Standard error of prediction
SIMCA	: Soft independent modelling of class analogy

SSC	: Soluble solids content
SWR	: Stepwise regression
TA	: Titratable acidity
TOS	: Theory of sampling
TSS	: Total soluble solids
VA	: Volatile acidity
Vis-NIRS	: Visible-Near infrared spectroscopy
WL	: Wallerstein laboratories

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Chapter 1

Introduction and project aims

1. INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

In normal batch alcoholic fermentation processes, each tank should have a predictable duration with respect to time of onset of fermentation until a desired endpoint has been reached. In practice however, several factors may affect the fermentation rate and can cause problem fermentations that are stuck or sluggish. These factors include high initial must sugar content, nitrogen limitation, ethanol toxicity, temperature extremes and poor oenological practices (Henschke, 1997; Bisson, 1999).

Fermentation process monitoring during industrial wine production can be much improved, and there is a need for fast and reliable process methods and techniques that can provide real-time information regarding the progress of the process. Increased demands for consistent quality by the consumers, legislators, production cost sensitivity and stiff international competition have been some of the major drivers for the development of new quality-monitoring tools in the wine industry in the last decade. The ideal method for process control should enable direct rapid, precise, and accurate determination of several target compounds, with minimal or no sample preparation and reagent consumption. These requisites are currently fulfilled by spectroscopic methods, most commonly based on infrared spectroscopy (Mazarevica *et al*., 2004). Infrared spectroscopy has numerous advantages over traditional wine analytical methods, including ease of implementation of the technology in wine analytical laboratories, the small sample quantity required for analysis (~ 30 mL), speed (~ 30 seconds analysis time per sample) and the almost complete absence of consumables (Boulet *et al*., 2007).

The use of infrared (IR) spectroscopy for routine analysis of wine began with near infrared spectroscopy (NIRS) being the preferred method in the early 1980’s (Baumgarten, 1984). Since that time, the focus for quantitative analysis of grapes and wine has moved towards Fourier transform infrared (FT-IR) technology in the mid-infrared region, since it offers better accuracy in determination and more constituents and properties can be quantified, than with NIRS (Patz *et al*., 1999; Dubernet & Dubernet, 2000; Soriano *et al*., 2007). Modern infrared spectroscopic instrumentation is fitted with chemometric software packages that facilitate the establishment of calibration models that can be used to quantify many components simultaneously, thereby reducing the analysis time and cost (Eichinger *et al*., 2004). Although the application of FT-IR spectroscopy is well established for quantitative analysis of wine (Patz *et al*., 2004; Cozzolino *et al*., 2007), the application to monitor alcoholic fermentation has been very limited and one pilot study in this regard was done using near infrared spectroscopy (Cozzolino *et al*., 2006).

FT-IR spectroscopy technology and chemometric techniques for analysis of grapes and wine were implemented in South Africa in the early 2000’s (Bauer *et al*., 2007; Paul 2009) and several qualitative and quantitative applications were developed in the last few years. A research program was launched at the Institute for Wine Biotechnology (IWBT), Stellenbosch University, and their industrial partners, to develop quantitative FT-IR spectroscopy based calibrations for all stages of the wine production process, including compounds in bottled wine (Nieuwoudt *et al*., 2004) and grape constituents (Swanepoel *et al*., 2007). These applications of FT-IR spectroscopy in industrial scale wine production did not cover the fermentation processes (both alcoholic- and malolactic fermentation) and the need to develop calibration models for these stages was clear. A summary of the progress of implementation of FT-IR spectroscopy and chemometrics for grape and wine analysis in the South African (SA) wine industry is shown in Figure 1. This MSc project addressed the urgent need to develop quantitative calibrations for

fermenting must in industrial scale fermentations and to explore the possibilities for qualitative monitoring of fermenting must using FT-IR spectroscopy and chemometrics.

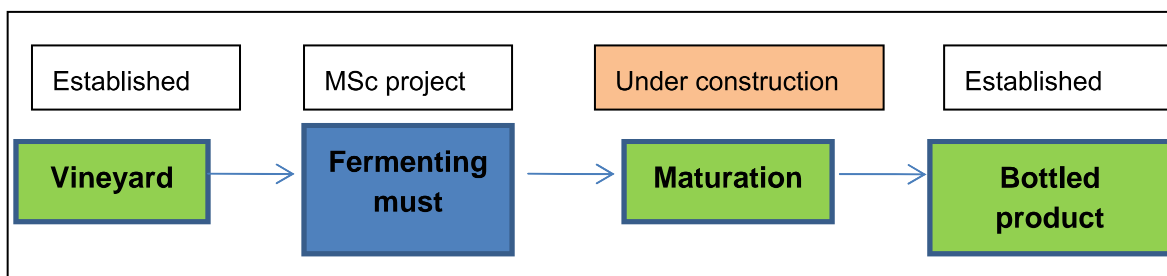

Figure 1 Schematic diagram of the research program of IWBT and industrial partners to develop FT-IR spectroscopy and chemometrics for grape and wine analysis in the South African wine industry, since 2001. This MSc project focused on fermenting must**.**

1.2 PROJECT AIMS

Three clearly defined aims were identified for this project. The first aim was to evaluate the ready-to-use commercial calibrations on the FT-IR spectrometer for quantification of major chemical parameters in fermenting must: ethanol, pH, titratable acidity (TA), total glucose plus fructose and volatile acidity (VA). Ready-to-use calibration models are an advantage for unskilled users and routine analysis, however, different varieties or climatic variations not included in the calibration set may introduce spectral interferences (Moreira and Santos, 2004; Soriano *et al*., 2007) that could affect the accuracy of the results. It is therefore necessary to evaluate if interferences of this nature were present in the SA must samples, and to asses whether the commercial calibrations were indeed suitable for SA samples.

The second aim was to establish and implement new calibration models for glucose, fructose and yeast assimilable nitrogen (YAN). These parameters are important for routine quality control to monitor the alcoholic fermentation process and to be informed regarding the concentrations of residual sugars and the nutrient status of the fermenting must. Since the overall objective with the quantitative stage was to implement the calibration models for use in the industrial cellar, this objective also included development of new calibration models for the classic wine parameters (ethanol, TA, pH, sugars and VA) that were not predicted satisfactorily by the commercial calibration models. It was therefore necessary to build robustness into the calibration models and calibration samples were therefore selected from various vintages, different cultivars, yeast starter cultures, tank volumes, colour intensities, geographic origins and climatic regions.

The third aim was to investigate the use of infrared technology to establish multivariate statistical control charts for qualitative off-line batch monitoring of alcoholic fermentations, by using only FT-IR spectra in combination with chemometric techniques to identify problem fermentations.

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Chapter 2

Literature review

Monitoring alcoholic fermentation during industrial wine production

2. LITERATURE REVIEW

2.1 INTRODUCTION

Alcoholic fermentation is a key process in wine production and entails the critically important stage of yeast-mediated transformation of grape juice into wine. During this process, yeasts utilise the sugars in juice (mainly glucose and fructose) as carbon and energy sources to enumerate and build their biomass (Ribéreau-Gayon *et al*., 2000). Although ethanol and CO_2 are the major end products of alcoholic fermentation, the yeast also produces other metabolic end products such as acetic acid, glycerol and succinic acid. These end products are released into the fermenting juice and contribute to the chemical composition and sensory quality of the wine (Zoecklein, 1995).

In this study the term *must* refers to juice obtained from pressed grapes, and *fermenting must* refers to the stage where the sugar content is being fermented. The duration of alcoholic fermentation in industrial wine production refers to the time required from the onset of fermentation, to an endpoint that is usually determined by the desired style and residual sugar concentration in the resulting wine. In the production of dry table wines, this endpoint (referred to as dryness) is usually less than 5 g/L sugar. It is well known that the time taken to ferment musts to dryness vary considerably in industrial wine production. Factors that have an effect on the conditions of yeast development in fermenting must, such as the nutritional status of the must and the fermentation temperature (Sener *et al*., 2006), are known to have a significant effect on the duration of alcoholic fermentation. The evaluation of the duration of fermentation should therefore be interpreted against the background of continuous temperature recordings (Bisson, 1999; Specht, 2003), initial sugar concentrations in grape juice (Iland *et al*., 2000; Howell and Vallesi, 2004) and the nutritional status of the juice, particularly the ammonia and total nitrogen content (Zoecklein, 1995; 2002).

In modern industrial cellars, one of the most important factors that influence the duration of alcoholic fermentation is temperature. Winemakers typically manipulate and control this factor to obtain different wine styles; for instance a fermentation temperature between 12–15ºC gives more fruity flavours to Colombar wines (D. van der Merwe, winemaker, Namaqua Wines, Vredendal, SA. personal communication, 2009). When available tank space becomes a problem during peak grape harvest periods, the winemaker would typically increase the fermentation temperature to speed up the fermentation process. At Namaqua Wines approximately 105 000 tons of grapes are harvested each year yielding ~ 75 million litres of wine. Tanks sized between 4 000 L and 280 000 L are used for alcoholic fermentation and a total of 200 tanks out of 550 tanks are annually available for fermentation (P. Verwey, winemaker, Namaqua Wines, Vredendal, SA. personal communication, 2009). White wines are fermented at ~15°C, while red wines are fermented at ~25°C for maximum colour extraction and fruit character (Protocol of In-House Fermentation Procedures, Namaqua Wines, SA).

The usual way to obtain qualitative and quantitative information regarding the progress of alcoholic fermentation in a particular tank is to remove a small sample from the tank followed by laboratory analysis. The types of sampling most wineries use include tank sampling at the sample valve (Figure 1), or from the top lid using a collection flask (plunger) and barrel sampling (Payette, 2006). With large industrial tanks (e.g. 1 million litres) it is expected that such a sampling system will not accurately represent the whole tank; however the option of pumping over the fermentation tank each time a sample is collected is not practical both from time- and cost implications. This aspect contributes to the challenges associated with monitoring industrial

size fermentation tanks, since the uncertainty associated with the sampling methods used remains unquantified (Paakkunainen *et al*., 2006).

Figure 1 Typical fermentation tanks (280 000 L capacities) at Namaqua Wines, Vredendal, SA with sample valves indicated with black arrows.

Under controlled conditions, alcoholic fermentation progresses until the wine is dry (<5 g/L sugar) or a specific wine style is achieved. A fermentation that progresses very slowly is referred to as a sluggish fermentation, whilst a fermentation that stops prematurely, leaving the resulting wine with undesired natural sweetness, is referred to as a stuck fermentation (Bisson, 1999). Problem fermentations will be discussed in more detail in section 2.3.

Problem fermentations have been a huge reality in winemaking for centuries, and are still today a serious problem for many winemakers. Although problems are more likely to occur with the formation of high alcohol content during alcoholic fermentation, there are several other factors that can contribute to this situation. For instance, the risks of grape sugars in must not being consumed to dryness increase with high initial sugar content, low yeast available nitrogen in the juice and late fungicidal treatments of vineyards. In addition, some grape varieties (Chardonnay, Merlot and Shiraz) are known to be difficult to ferment due to low yeast available nitrogen or their high fructose content. Overall, a lack of control over the winemaking process also increases the risks of problem fermentations. The logistic implications of sluggish fermentations include the requirement for extended fermentation time which could consequently consume tank space for an uncertain time period.

The evolution of fermentation end products over the duration of alcoholic fermentation reveals a significant amount of information about the progress of the process, and the viability and metabolic acitivity of the yeast (Fleet and Heard, 1993; Zoecklein, 1995). Analytical monitoring of fermentation components therefore forms the basis of quantitative monitoring of alcoholic fermentation. To date, infrared spectroscopy has established itself as an analytical tool used for indirect quantitation of organic compounds in wine as discussed in detail in section 2.4.2. However the application towards fermentation monitoring using infrared spectroscopy during winemaking has been limited. One approach included quantitation of important alcoholic

fermentation components with mid-infrared spectroscopy in samples taken from fermentation tanks (Dubernet and Dubernet, 2000), although this was done on only a few samples. Initial work on qualitative off-line batch process monitoring using near infrared spectra was also investigated (Urtubia *et al*., 2004; Cozzolino *et al*., 2006). Blanco *et al*., 2004 used near infrared spectroscopy for on-line monitoring of small-scale laboratory fermentations in synthetic wine. Recently there were several developments in the application of sensor technology for monitoring fermentation processes during winemaking. Esti *et al*., 2004 used amperometric biosensors consisting of platinum-based probes coupled with appropriate enzymes to monitor malolactic fermentation, and Xiu-Ling *et al*, 2008, developed electrochemical biosensors for quantitation of alcohol, glucose, glycerol and lactic acid in wine.

This review highlights some important aspects related to the alcoholic fermentation process and biological, physicochemical and biotechnological factors that influence its progress. Problem fermentations are also briefly discussed. The different strategies for analytical monitoring of alcoholic fermentation are presented and a short section on sampling issues in the industrial cellar concludes the review.

2.2 ALCOHOLIC FERMENTATION

2.2.1 THE ALCOHOLIC FERMENTATION PROCESS

Alcoholic fermentation is a complex biochemical process that is conducted principally by the facultative anaerobic wine yeast *Saccharomyces cerevisiae* (Pretorius, 2000; Bell and Henschke, 2005). Results obtained by Blanco *et al.* 2004 in controlled small-scale laboratory fermentations in synthetic medium, show some typical trends in the changes in the concentrations of some major fermentation components during yeast-mediated alcoholic fermentation (Figure 2).

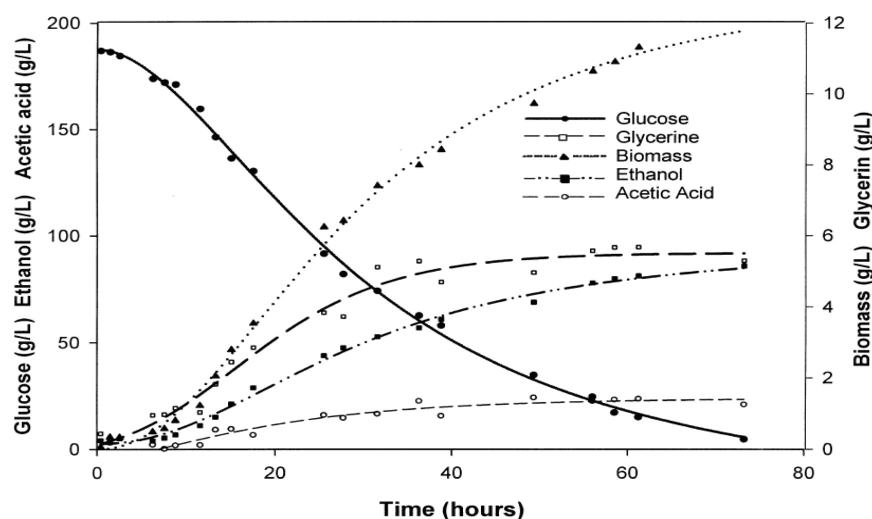

Figure 2 Changes over time in the concentrations of the major analytes involved during yeast-mediated alcoholic fermentation of synthetic medium (Adapted from Blanco *et al*., 2004).

Although ethanol and CO_2 are the major end products of alcoholic fermentation, the yeast also produces glycerol and pyruvic acid through the glyceropyruvic metabolic pathway (Pronk *et al*., 1996). Glycerol is released into the fermenting juice under fermentative conditions, while pyruvic acid evolves through several biochemical steps to amongst other, acetic acid, succinic acid and 2,3 butanediol. Theoretical information regarding the yeast growth cycle, metabolism and

physiology, and the factors that affect yeast development has been provided in fundamental publications on this topic (Pronk *et al*., 1996; Fleet, 1998; Ribéreau-Gayon *et al*., 2000).

2.2.2 FACTORS INFLUENCING ALCOHOLIC FERMENTATION

The majority of industrial wine fermentations are conducted by inoculating grape juice with an adequate dosage (usually >10^6 cells / mL) of commercial wine yeast, although uninoculated fermentations that rely on the proliferation of indigenous yeast populations in must, are sometimes preferred in order to achieve a specific wine style. Based on the crucially important role that the wine yeast plays in the alcoholic fermentation process, it is clear that prevailing must conditions that affect yeast development will also have a large influence on the kinetics of alcoholic fermentation. The successful completion of fermentation depends on many factors and primarily requires undisrupted growth and metabolism of the yeast (Sener *et al*., 2006). In this section selected biological, physicochemical and process technological practices that affect fermentation progress in connection with practical and industrial wine production are discussed.

2.2.2.1 MICROBIAL FLORA

The microbial flora on grape berries typically consists of bacteria, moulds and yeasts. The bacterial species on the surface of grapes are members of the genera *Bacillus, Pseudomonas* and *Micrococcus* (Fleet, 1998; Pretorius, 2000). Acetic acid bacteria, principally *Acetobacter* and lactic acid bacteria are frequently isolated from grape berries, while the four most commonly isolated moulds are members of the genera *Aspergillus, Penicillium, Rhizopus* and *Mucor* (Zahavi *et al*., 2000). Four yeast genera, *Hanseniaspora, Metschnikowia, Hansenula* and *Candida* are found on the surface of the grape berry (Fleet, 1998; Pretorius, 2000). The wine yeast *S. cerevisiae* is hardly ever isolated in significant numbers from the surface of berries, and therefore the practice of inoculating grape juice with *S. cerevisiae* to induce a rapid onset of alcoholic fermentation, is used. Fermentation difficulties can lead to proliferation of undesired microbes that are antagonistic to the wine yeast, and winemakers must be aware of the risks associated with the native flora on grape berries.

Spontaneous fermentations are conducted by vineyard and winery flora. Native flora fermentations are not necessarily problematic, but it is the responsibility of the winemaker to monitor these closely and to take appropriate action should a problem arise during the fermentation. The primary benefit of native flora fermentation is the perceived increased complexity in the flavour of the resulting wine, also, referred to as so-called “microbial characters” by some authors (Romano *et al*., 1997; Soden *et al*., 2000). However, native flora can produce undesirable flavour characteristics in wine that may affect the wine quality negatively. On the other hand, yeast-inoculated fermentations are more predictable in terms of the onset, duration and maximal rate of fermentation, as opposed to native flora fermentations. The advantages and disadvantages of spontaneous and inoculated fermentations have been discussed in several publications (Kunkee, 1984; Heard, 1999; Pretorius, 2000).

Mould growth on fruit has been reported to cause fermentation problems due to the production of metabolites antagonistic to the wine yeast and the depletion of nitrogen by these microbes (Doneche, 1993). Inhibitory metabolites such as acetic acid, medium chain fatty acids, killer toxins produced by native yeast or bacteria growing on fruit or proliferating during the early stages of fermentation, may have a significant effect on the fermentative performance of the *Saccharomyces* species. Acetic acid-, lactic acid bacteria and native yeast can produce potent wine yeast inhibitors and decrease must nitrogen and vitamin levels. Acetic acid is a strong

inhibitor of *Saccharomyces* species especially when combined with other antagonistic factors like ethanol toxicity (Drysdale and Fleet, 1988). Inhibitory peptides produced by some strains of *Saccharomyces* affect other strains of the same yeast. Certain non-*Saccharomyces* yeasts produce broader spectrum killer factors. Their presence in fermentation can be inhibitory to other yeasts (Tredoux *et al*., 1986). Bacteria produce bacteriocins that are inhibitory to other bacteria (Fimland *et al.,* 1996).

Killer yeasts are also known to occur in wineries. These yeasts secrete a proteinaceous killer toxin lethal to susceptible or sensitive strains of the same species (van Vuuren and Wingfield, 1986). Toxins are most commonly considered to derive from other microbes and impact the biological activities of *Saccharomyces* (Tredoux *et al*., 1986). Some *Saccharomyces* species and strains, and some non-*Saccharomyces* yeasts, can produce killer toxins that inhibit other sensitive strains and may play a role in stuck fermentations (van Vuuren and Wingfield, 1986; Radler and Schmitt, 1987). The killer toxin can change the nitrogen metabolism of the yeast 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 Peňa *et al*., 1981).

The mould *Botrytis cinerea* produces a group of heteropolysaccharides collectively referred to as "Botryticine" (Doneche, 1993). These mycotoxins stimulate *Saccharomyces* species to produce high and inhibitory levels of acetic acid at the onset and during the last stages of alcoholic fermentation (Doneche, 1993). Moulds, while not present in the fermentation, may produce mycotoxins on the surface of the berry, to which *Saccharomyces* is susceptible (Bisson, 1999; Sage *et al*., 2002).

2.2.2.2 NUTRITIONAL STATUS OF MUST

Deficiencies in the supply of essential nutrients in fermenting must remain the most common causes of poor performance of the yeast and stuck or sluggish fermentations (Bisson, 1991; Bisson, 1999). Grape juices with nitrogen levels below 150 mg/L have a high probability of becoming problem fermentations due to insufficient yeast growth and poor fermentative activity (Pretorius, 2001). It should be noted that the concentration of nitrogen levels below 150 mg/L is a broad-based generalisation and it might differ in different sources of the literature. A nitrogen deficiency during fermentation can prevent the formation of the essential yeast sugar transport proteins. Transportation of sugar across the yeast cell membrane is slowed down and might stop completely, resulting in a stuck fermentation. In fermentations of botrytis-infected grapes, the musts can often have insufficient vitamins and minerals to support yeast growth. These microelements are co-factors in cellular enzymatic reactions and a shortage can slow down fermentation. Too much nitrogen in fermenting must can also lead to problems that include excessively fast fermentation, fermentation above 32°C, increased yeast biomass and reduced fruity aromas in the wine. Rapid fermentation can increase aroma compound loss due to their increased volatility, resulting in the loss of complexity in the flavour characteristics of the wine (Zoecklein, 2002). Residual nitrogen in the form of arginine can also feed potential spoilage microbes (Franson, 2005).

A phosphate deficiency in grape juice may also have a direct impact on yeast cell growth and fermentative performance (Boulton *et al*., 1996). Inorganic phosphate is required for synthesis of ATP, ADP and nucleic acids by the wine yeast. Juice and fermenting must can be vitamin deficient when there is a high population of microorganisms (mould, yeast and/or bacteria). Growth of *Kloeckera apiculata* has been reported to rapidly reduce thiamine levels below those required by *Saccharomyces* species (Bataillon and Rico, 1996). The addition of

SO_2 as discussed later, may lead to additional reduction in levels of thiamine necessary for yeast growth (Lafon-Lafourcade and Ribereau-Gayon, 1984; Alexandre and Charpentier, 1998). Acetic acid has been reported to reduce the ability of *Saccharomyces* to transport and retain thiamine (Iwashima *et al*., 1973). Biotin is the only vitamin that the yeast cannot synthesise and at least a precursor of this vitamin must be present in the grape juice. While other vitamins can be synthesised, yeast growth and fermentation are accelerated in the presence of these compounds in juice or fermenting must.

Potassium is needed for phosphate uptake by the yeast, while magnesium is required for yeast growth and also acts as an enzyme activator and stabiliser of the cell membranes (Franson, 2005). Small amounts of zinc, manganese, calcium and copper are also needed as growth factors by the yeast (Franson, 2005). Limitation of zinc and magnesium directly affects yeast sugar catabolism and hence also fermentative activity (Dombeck and Ingram, 1986; Monk, 1994). A calcium limitation increases ethanol sensitivity of the yeast (Nabais *et al*., 1988).

Oxygen should be considered an essential yeast nutrient (Zoecklein, 2002). Although fermentation is an anaerobic process, oxygen has a stimulating influence on yeast growth and fermentation kinetics, largely as a result of inducing ethanol tolerance in the yeast (Zoecklein, 2002). Limited aeration of the fermentation tanks during active fermentation promotes the formation of survival factors, particularly fatty acids and sterols, by the wine yeast and enables the yeast to build large and healthy populations (Dharmadhikari, 1999; Blateyron *et al*., 2003). Oxygen helps the yeast to produce its own lipids (Specht, 2003). It is recommended that the addition of small amount of O_2 to a fermentation in the form of macro-oxygenation is done in order to obtain increased fermentation speed and alcohol tolerance (Lourens and Reid, 2003).

2.2.2.3 PHYSICOCHEMICAL FACTORS

Increased osmotic pressure associated with high sugar concentrations can inhibit yeast growth (Dharmadhikari, 1999). Yeasts differ in their tolerance of initial must sugar levels, as well as in tolerance to the resulting final alcohol levels. Higher initial juice sugar concentrations, particularly higher than 30ºBrix, have a retarding effect on the progress of fermentation; and the process can stop before all the sugar is utilised. High sugar musts can place the yeast cell membrane under severe osmotic stress and thus weaken it. Towards the end of fermentation the cell membrane can become unable to tolerate the high alcohol concentration. Sugar transport across the membrane can also shut down. When high sugar content musts are fermented, the choice of yeast strain for inoculation is absolutely critical and it is recommended by commercial wine yeasts manufacturers, that winemakers ensure that the strains they select are able to ferment high sugar content musts (Bisson, 1999; Zoecklein, 2002).

Alcohol also has an inhibitory effect on yeast growth and the toxic effect is enhanced with increasing temperature (Dharmadhikari, 1999). Ethyl alcohol is the major desired metabolic product of grape juice fermentation, but it is also a potent chemical stress factor that is often the underlying cause of sluggish or stuck fermentation. The production of excessive amounts of ethanol, resulting from harvesting of over-ripe grapes, is known to inhibit the uptake of solutes such as sugars and amino acids by the yeast and to inhibit its growth rate, viability and fermentation capacity (Pretorius, 2001). Several intrinsic and environmental factors are known to enhance the inhibitory effects of ethanol. These factors include high fermentation temperatures, nutrient limitation, for example, oxygen, nitrogen, lipids and magnesium ions, and fermentation metabolic by-products such as higher alcohols, aldehydes, esters, organic acids, certain fatty acids, carbonyl and phenolic compounds (Pretorius, 2001). Ethanol tolerance can

be reduced if the yeast cells do not have sufficient resources of sterols and unsaturated fatty acids that are needed by the yeast to generate ethanol resistant cell membranes.

Acids with longer hydrocarbon chains are generally known as fatty acids. Yeasts produce medium fatty acids during fermentation (Pretorius, 2001) and these fatty acids can have an inhibitory affect on fermentation. Three of them, hexanoic- (C6), octanoic- (C8) and decanoic acid (C10) have been implicated in the inhibition of sugar transport across the yeast membrane. Yeast hulls, which are by-products of commercial manufacturing of yeast extract, have been described to lower the concentration of inhibitory C_{8-10} fatty acids in fermenting must and addition of hulls during industrial winemaking is recommended by the manufacturers in order to rectify problematic fermentations (Lafon-Lafourcade *et al.*, 1984; Lourens and Reid, 2003).

Carbon dioxide in concentrations of up to 0.2 atmosphere (atm) stimulates yeast growth. The release of carbon dioxide helps to decrease the lag phase of yeast growth (Zoecklein, 2002). Above this level, carbon dioxide becomes inhibitory to yeast growth and reduces the yeast's uptake of amino acids.

Fruit from diseased vines may also contain inhibitory levels of phytoalexins that are produced by the plant in response to the parasite (Smith and Banks, 1986). These may be inhibitory towards *Saccharomyces* species. Pesticides and fungicides applied to the vineyard can influence the fermentation kinetics by producing stress metabolites such as mycotoxins that inhibit and/or prevent fermentation (Zoecklein, 2002). Anti-fungal pesticides are used in the vineyards to protect grape vines against botrytis and other mould infection. Some pesticides such as dichlofluanide can increase the length of the lag phase, thus delaying the start of fermentation. High concentrations of pesticide residues may remain on the grapes at the time of harvest, resulting in higher incidences of stuck and sluggish fermentations. The style of vinification can influence the concentration of pesticide residue in fermenting must. For instance, pre-fermentation clarification and the utilisation of bentonite, for fining purposes and protein stability, can lower the final concentrations of contact fungicides in white wine production (Specht, 2003).

2.2.2.4 PROCESS TECHNOLOGICAL PRACTICES

In this section, the effects of some process technological practices used in industrial wine production on alcoholic fermentation kinetics are discussed. These practices are must clarification, selection of fermentation temperature, sulphiting of juice and inoculation practices.

Must clarification refers to the removal of grape solids before inoculation with selected wine yeast. Highly clarified musts are more difficult to ferment successfully to dryness, because extensive settling, which refers to the precipitation of solids in the juice before the onset of alcoholic fermentation, removes most of the sterols and long chain fatty acids from the musts (Alexandre & Charpentier, 1998). These survival factors are responsible for alcohol tolerance in yeasts, as discussed in earlier sections. Extensive clarification also removes a large percentage of wild yeasts and thus spontaneous natural fermentations will be difficult to achieve. Yeasts are also known to produce more acetic acid in very clear musts because of increased stress, due to the lack of important sterols and long chain fatty acids. Very turbid musts can, however, lead to off-flavours in the resulting wine.

Most industrial fermentations are conducted in the temperature range of 10 - 30°C. At the higher end of the range, fermentations become sluggish and above 32°C, they can stop prematurely. The effect of high temperature is enhanced at higher ethanol concentrations, such as the levels formed towards the end of alcoholic fermentation, and the higher temperatures also lead to greater loss of volatile components (Bisson 1999). Fermentation temperature

affects the rate of spontaneous chemical reactions in the ferment and processes such as volatilisation of chemical compounds. Wines produced at low fermentation temperatures (10 - 15°C) tend to have higher alcohol content and fresh and fruitier aroma. Lower temperatures also result in slow rates of metabolism, allowing other non-*Saccharomyces* organisms to persist in the ferment. There is also better retention of volatile characters at lower fermentation temperatures (Bisson, 1999; Pretorius, 2001). Red wines are fermented at slightly higher temperatures (22-30°C) to facilitate the extraction of colour and other skin constituents (Dharmadhikari, 1999; Pretorius, 2001). Like ethanol, temperature directly affects membrane fluidity and therefore nutrient transport. Temperature also has an influence on the yeast's capacity to assimilate amino acids during alcoholic fermentation (Urtubia *et al*., 2007).

Sudden or extreme changes in fermentation temperature can cause the yeast to undergo thermal shock with resulting loss in viability (Specht, 2003). Temperature shock refers to a dramatic (greater than 5°C) change in the mean temperature of the tank (Zoecklein, 2002). This may arise due to super cooling that occurs as the fermentation rate slows down and the heat released as a result of yeast metabolism, decreases. Temperature swings during fermentation can also inhibit sugar catabolism. It is therefore very important that fermentation temperature is carefully monitored in industrial wine production.

Sulphur dioxide is widely used in wineries to suppress the growth of unwanted microbes, such as bacteria and some strains of indigenous yeast other than the wine yeast. The wine yeast also produces SO_2 during alcoholic fermentation, and the amount formed is yeast strain dependent (Pretorius, 2001). Although *S. cerevisiae* tolerates higher levels of sulphite than most unwanted yeasts and bacteria, excessive SO_2 dosages may cause sluggish or stuck fermentation (Pretorius, 2001). SO_2 inhibits the enzyme polyphenyloxidase and in the complete absence of SO_2, this common plant enzyme system conducts the chemical reaction using large concentration of available oxygen (Zoecklein, 2002). This enzyme is responsible for the browning reaction which occurs after bruising of the grapes or during the ripening process.

Grape juice is inoculated with commercial yeast starter cultures in the wine industry, when desired. The yeast cultures are obtained as active dried preparations and these are rehydrated prior to inoculation into juice. Rehydration protocols should strictly adhere to the supplier's recommendations to ensure maximum yeast viability and vigour (Boulton *et al*., 1996). Some yeast manufacturers recommend rehydration in a nutrient mix, consisting of sugar, water and nutrient supplements. After rehydration, the yeast starter culture should be added to the juice or must within 20 - 30 minutes. If this is not done, yeasts undergo a premature decline phase resulting in an inoculum of low viable cell density. Significant yeast cell death occurs when temperature differences between the starter culture and juice are more than 5 - 7°C (Monk, 1986). Liquid starter cultures can be prepared in either juice or a defined medium, usually a mixture of sugar and water, and used to inoculate juice or must.

Yeast populations of about 10^6 cells / mL should be large enough to dominate unwanted microflora and should ideally enumerate to 2 to 5 x 10^6 yeast cells / mL juice (Zoecklein, 2002). These concentrations apply when the °Brix is below 24; the juice pH is above 3.1 and the fermentation temperature above 13°C. Increases in the inoculum volume should be made when parameters are outside these values. Survival factors are important for the maintenance of cell viability by providing the nutrients needed to repair cellular damage and support the limited synthesis of needed proteins and other cellular components (Zoecklein, 2002).

2.3 PROBLEM FERMENTATIONS

Slow or sluggish fermentations are defined as those that are progressing very slowly, requiring a period of several weeks to complete and stuck fermentations are defined as a fermentation containing a high or undesired level of residual sugar (Bisson, 1999). Figure 3 shows different types of problem fermentations (Bisson, 2005). Several factors might affect yeast growth during alcoholic fermentation, including clarification of grape juice, addition of sulphur dioxide, temperature of fermentation, composition of grape juice, inoculation with selected yeasts and interactions with other organisms as discussed in section 2.2. Glucose and fructose are the main fermentable sugars in grape juice and at the ripening stage, glucose and fructose are usually present in equal amounts (Fleet, 1998). In overripe grapes, the concentration of fructose might exceed the concentration of glucose (Fleet, 1998; Snyman, 2006). It is known that *Saccharomyces cerevisiae* is glucophilic (Fleet, 1998) and in the later stages of alcoholic fermentation, fructose becomes the main sugar present. Therefore the yeast has to ferment this sugar under conditions of high ethanol concentration and nitrogen limitation, which may lead to stuck or sluggish fermentations (Alexandre and Carpentier, 1998). Unfortunately problem fermentations need tank space for unlimited periods of time and therefore limit the flexibility of tank usage during harvest season, causing major logistical problems in the cellar.

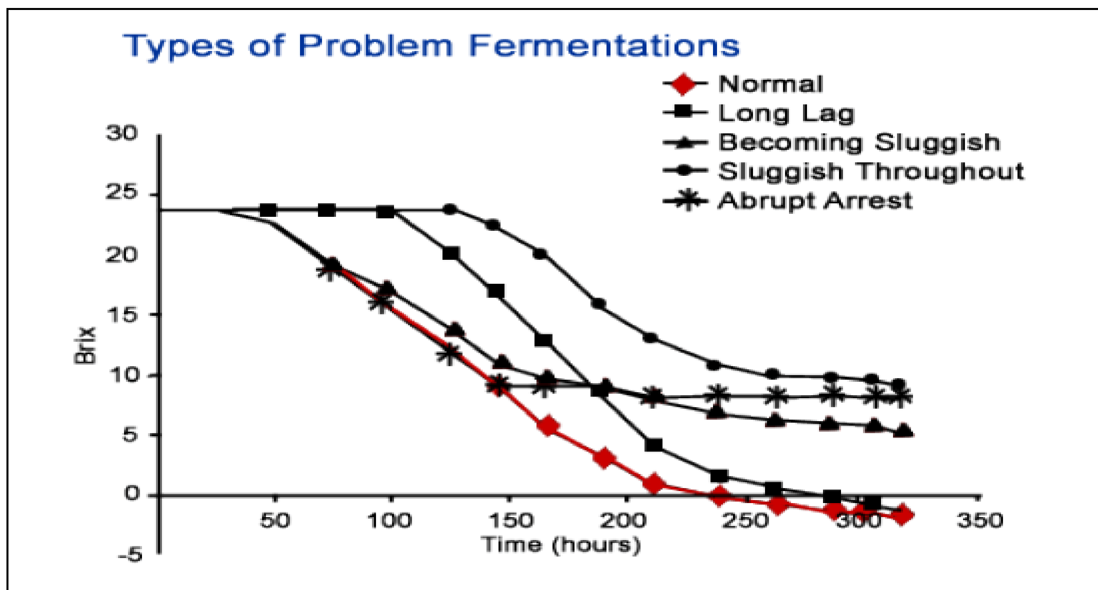

Figure 3 Illustration of different types of problem fermentations (Adapted from Bisson, 2005).

During wine production, one of the first concerns for a winemaker is to ensure steady and complete alcoholic fermentation so that all the sugars in the must are metabolised. This should be done in order to avoid problems and risks arising due to stuck fermentations or problems related to the aroma and taste of the wine (Garcia *et al*., 2006). The completion of fermentation may prevent problems, by preventing proliferation of acetic acid bacteria and lactic acid bacteria that could metabolise residual sugars and result in increased volatile acidity (O'Connor-Cox and Ingledew, 1991). As discussed before, many factors such as vitamin, magnesium, nitrogen and oxygen deficiencies or toxic fatty acids, and acetic acids may be responsible for stuck or sluggish fermentations.

2.3.1 OFF-CHARACTERS RESULTING FROM PROBLEM FERMENTATIONS

"Off-characters" are defined as unpleasant flavour characteristic of a wine often resulting from a lack of experience or, carelessness on the part of the winemaker. Frequently however, off-characters can also originate in the wine due to factors beyond the winemaker's control. Although not directly related to alcoholic fermentation kinetics, the negative effects of problem fermentations on wine quality are of a very serious nature. Conditions leading to slow or incomplete fermentations also result in the production of undesirable yeast metabolites such as sulphur volatiles.

The appearance of hydrogen sulphide (H_2S) in wine as a consequence of yeast metabolism is considered to be a serious sensory defect (Linderholm *et al.*, 2008). The majority of H_2S produced during alcoholic fermentation occurs during the synthesis of sulphur-containing amino acids by *Saccharomyces* (Linderholm and Bisson, 2005). There mechanisms by which H_2S is produced by *S. cerevisiae* include the degradation of sulphur-containing amino acids, the reduction of elemental sulphur and the reduction of sulphite or sulphate (Linderholm *et al*, 2008). However, H_2S produced early in fermentation can be driven off by the carbon dioxide produced during fermentation. H_2S may arise from the degradation of sulphur containing amino acids or from the reduction of organic sulphur used as fungicide in the vineyard. If sulphur is applied in the vineyard close to harvest, the reductive conditions that are created during fermentation can lead to chemical conversion of this sulphur to H_2S (Linderholm and Bisson, 2005). Deficiencies in vitamins and micronutrients that are essential for the synthesis of sulphur containing amino acids may contribute to H_2S production (Linderholm *et al.*, 2008). A nitrogen shortage is also accompanied by the production of higher levels of H_2S. Fermentation temperature (Rankine, 1963), juice turbidity (Karagiannis and Panos, 1999), the levels of soluble solids and titratable acidity (Vos and Gray, 1979) have been shown to significantly affect the final H_2S levels.

Higher alcohols are produced by yeast metabolism of sugars and amino acids during fermentation (Singh and Kunkee, 1976). These higher alcohols may also be considered as off-characters depending upon the amount produced and the style of wine desired. Higher alcohols such as propanol, butanol, isobutanol and isoamyl alcohol as well as phenolic alcohols are usually responsible for unpleasant flavours. Phenethyl alcohol has been described as having a floral aroma that, if present in high concentration, may be too intense for some wines. Higher alcohol production during fermentation is influenced by yeast strain, temperature, oxygen levels, nutrition levels and acidity (Singh and Kunkee, 1976).

High levels (>1.3 g/L) of acetic acid are often associated with stuck or sluggish fermentations. The heterofermentative lactic acid bacteria (Fleet and Heard, 1993), commercial wine yeasts and acetic acid bacteria (Drysdale and Fleet, 1985), all have the ability to produce high levels of acetic acid that directly increases volatile acidity (Malherbe *et al.*, 2007). An increase in acetic acid concentrations can inhibit yeast growth, enhance ethanol toxicity and prevent the completion of fermentation. High levels of acetic acid may also be produced by contaminating organisms or wine spoilage yeasts, especially under a deficiency of oxygen (Specht, 2003). The production of acetic acid is affected by the yeast strain, the must composition, vitamin content, initial sugar concentrations and fermentation conditions such as variations in temperature (Bely *et al.*, 2003).

Saccharomyces produces many esters as a result of fatty acid degradation during fermentation (Iland *et al.*, 2007). The most common ester in wine is ethyl acetate and it is formed by chemical interaction between ethanol and acetic acid. High levels (>200 mg/L) of ethyl acetate are a common microbial fault associated with wine spoilage yeasts, particularly

Pichia and *Hanseniaspora,* but ethyl acetate is also produced by lactic acid bacteria and acetic acid bacteria (Iland *et al*., 2007).

Acetaldehyde and higher aldehydes can be considered as off-characters if present in high concentration (>125 mg/L). These compounds are desired in some styles, such as sherry production, and are associated with wine age. Acetaldehyde is the primary aldehyde found in wine. It is released when ethanol formation is blocked due to absence of alcohol dehydrogenase. It is also released as the detoxification mechanism for sulphites and the oxidation of alcohol by acetic acid bacteria (MilláN and Ortega, 1988; Saucier *et al*., 1997).

Vinyl phenols have very distinctive medicinal aromas and are responsible for the barnyard characters found in wines. Decarboxylated phenols are reduced to vinyl phenols by yeast enzymatic activity. The principle yeast producing vinyl phenols is *Brettanomyces*. Vinyl phenol formation is dependent upon the phenolic composition of the fruit and compounds that can be reduced. The main constituents are 4-ethylphenol (>140 µg/L), 4-ethylguaiacol (>600 µg/L) and isovaleric acid (Couto *et al*., 2006; Larcher *et al*., 2007).

2.4 TECHNIQUES USED TO MONITOR ALCOHOLIC FERMENTATION

There is a need for fast and reliable analytical techniques for monitoring and screening throughout the whole wine production chain: from the start of grape ripening in the vineyard to harvest, at grape reception, for the purposes of fermentation control and finally for quantification of important parameters in the final wine product. The requirements for suitable monitoring techniques are speed, a high degree of automation, good reproducibility, precision and accuracy, cost effectiveness, and good comparability to results obtained with the reference methods (Patz *et al*., 2004). While standard instruments such as temperature and pressure gauges are useful for tracking basic must conditions, advanced analytical instrumentation is needed to detect changes in nutrient levels as fermentation progresses (Urtubia *et al*., 2007).

Fermentation monitoring may be as simple as measuring Brix or sugar level, or may involve analysis of many other parameters including organic acids and nitrogen content. It is important to have a good understanding of how what is being measured, relates to the information desired. It is equally important to know the reproducibility, precision and accuracy of the method used for monitoring and what types of factors will interfere in the measurements. One of the important aims in monitoring strategies is to shorten the time required for a given measurement and subsequently, to make the information available in a short time period. This can be done through the development of quantitative and screening methodologies, combinations of the different methods, and the application of chemometric techniques for data analysis, as discussed in the following sections.

2.4.1 CHEMICAL AND MICROBIOLOGICAL ANALYSES

Must sugar levels can be monitored in one of several different ways. The most common is to use the Brix scale or a similar means to assess the specific gravity or density of the ferment. The amount of carbon dioxide liberated can be used to determine the amount of sugar consumed (Iland *et al*., 2000; Howell and Vallesi, 2004). The levels of glucose and fructose can be evaluated, either using enzyme-linked spectrophotometric assays that can be automated, or by high performance liquid chromatography (HPLC). The latter method is more accurate and precise, but requires sophisticated analytical equipment and expertise (Holler *et* al., 2007; Howell and Vallesi, 2004). Ethanol evolution can also be monitored as a means to determine

the amount of sugar consumed. Eubillometry is the most common method used for ethanol quantitation, but gas chromatography (GC), capillary electrophoresis (CE), HPLC, near infrared (NIR) and mid-infrared (MIR) spectroscopy are also applied (Iland *et al.*, 2000; Vallesi and Howell, 2002).

The nitrogen content of the must or juice should be measured to provide the winemaker with information on the amount of supplementation required. Amino acid analysis can be performed using HPLC or measured as YAN using enzymatic assays. Other chemical assays have been developed that serve to measure the compounds possessing a certain type of nitrogen moiety. The three most common is the free amino nitrogen analysis (FAN), yeast assimilable nitrogen (YAN) analysis using the Formol method, and the o-phthaldialdehyde/N-acetyl-L-cysteine (NOPA) spectrophotometric assay (Zoecklein, 1995; Iland *et al.*, 2000).

Yeast and bacteria can be distinguished from each other under the microscope so the relative numbers of these organisms can easily be determined in a counting chamber. However, it is not possible to distinguish between different yeast genera or even different bacteria using microscopic observation. Qualitative estimates of relative numbers of microbial populations can also be made, but are less reliable. Viable organisms can be monitored directly by plating (i.e. spreading of inoculae over the surface of an agar medium contained in a petri dish) a sample of the must or juice. General media (Wallerstein Laboratories (WL) nutrient medium) can be used to support the growth of a broad spectrum of organisms including bacteria, mould and yeast, or more selective media such as acetic acid bacteria agar, or apple juice agar for lactic acid bacteria can be employed that support the growth of a subset of microbes. In this context it is also important to emphasise the importance of statistically valid sampling of the tank for microbial profiling, because flora are not uniformly present in tanks. High concentrations of bacteria will appear at the surface of the tank and the yeast at the bottom of the fermentation tank. Analysis of one sample taken from the racking valve might not provide an accurate picture of the distribution of the flora throughout the tank. For organisms present in low numbers, it may be necessary to collect the microbial flora from a large sample by sterile filtration, using a 0.2 micron membrane filter, or plating indiluted or concentrated samples (Iland *et al.*, 2007).

The amount of a specific end product could be an indication of the metabolic activity of the microbes present, and not just their presence or absence (Iland *et al.*, 2000). Volatile acidity (VA) analysis can be used to measure acetic acid content. This can be an indication of the presence of *Acetobacter* or lactic acid bacteria. The level of vinyl phenols in fermenting must could be an index of the presence and metabolic activity of *Brettanomyces*. Hydrogen sulphide is associated with *Saccharomyces*. One of the most important analytical tools available to the winemaker is their own sense of smell. Off-characters can be detected by nose and it is important that ferments are sniffed on a regular basis in order to detect problematic compounds and undesirable metabolic activities.

Another factor that is very important to be monitored during fermentation is changes in acidity. The acidity of grape juice and wine plays an important role in many aspects of winemaking and wine quality, including the sensory quality of the wine and its physical, biochemical and microbial stability (Pretorius, 2001). This can be done by monitoring titratable acidity and measurement of pH. Levels of malate and lactate are typically directly measured during industrial wine production as these are correlated with the presence of the lactic acid bacteria. These acids can be measured by HPLC, paper chromatography, NIR- and MIR spectroscopic analysis (Iland *et al.*, 2000).

2.4.2 INFRARED SPECTROSCOPIC TECHNIQUES

Infrared (IR) spectroscopy was first used to analyse biological samples in 1911 by W.W. Coblentz (Holler *et al*., 2007). The basis of the technology has been described in detail in several textbooks (Willard *et al*., 1988; Holler *et al*., 2007) and in this review, only some theoretical aspects of the application of the technology to viticulture and oenology are discussed. IR spectroscopy utilises the measurement of infrared light intensity, as for example absorbance, transmission or reflection, at specific instrument-defined wavelength (nm) or wavenumber (cm^{-1}) regions (Willard *et al*., 1988). Wavelength refers to the distance between points in an electromagnetic wave and wavenumber refers to the number of waves in a unit distance. The cm^{-1} is obtained when the frequency is expressed in Hertz and the speed of light is expressed in cm/s, while nm is a measure of wavelength that is one thousand-millionth of a meter. Mid-infrared waves are referred to as wavenumbers and near infrared waves are referred to as wavelengths. Upon interaction with infrared radiation, portions of the incident radiation are absorbed at specific wavelengths by covalent bonds, resulting in vibrational motions such as twisting, bending, or stretching of the bonds. The multiplicity of vibrations occurring simultaneously produces a highly complex absorption spectrum that is uniquely characteristic of the functional groups that make up the molecules, as well as of the overall configuration of the molecule (Willard *et al*., 1988). The infrared region of the electromagnetic spectrum extends from 14000 cm^{-1} to 10 cm^{-1}. The applications of IR spectrometry fall into three major categories based on the three infrared spectral regions (Holler *et al*., 2007). The region of most interest for chemical analysis is the mid-infrared region (4000 cm^{-1} to 400 cm^{-1}) which corresponds to changes in vibrational energies within molecules (Holler *et* al., 2007; Urtubia *et al*., 2007). Absorption, reflection, and emission spectra are employed for both qualitative and quantitative analysis. The near infrared region (4000 – 14000 cm^{-1}) also finds considerable use for the routine quantitative determination of certain species, such as water, carbon dioxide, sulphur, and other compounds of interest in agriculture and in the wine industry. The far infrared region (400 cm^{-1} to 10 cm^{-1}) is useful for molecules containing heavy atoms such as inorganic and metal-organic compounds based on absorption measurements, but requires rather specialised experimental techniques (Holler *et al*., 2007). Both near and far infrared regions correspond to changes in vibrational energies within molecules.

The near infrared (NIR) spectroscopic method of analysis is an instrumental method for rapid and reproducible measurement of the chemical composition of samples, requiring little or no sample preparation (Manley *et al*., 2001). NIR instruments are manufactured to measure in the region (4000 cm^{-1} to 14000 cm^{-1}) and provide more information related to the vibration behaviour of combinations of fundamental absorptions (Willard *et* al., 1988; Cen and He, 2006; Holler *et al*., 2007). A NIR spectrum is composed of combination and overtone bands of molecular bonds such as O–H, N–H and C–H, that can be related to fundamental absorption frequencies in the mid-infrared region. In this way a characteristic spectrum is built that can be considered a "fingerprint" of the sample (Downey, 1994, 1996; Cozzolino *et al*., 2003). The first overtones of the O–H and N–H stretching vibrations are near 7140 cm^{-1} and 6667 cm^{-1} (Willard *et al*., 1988).

The IR spectral properties of both grapes and wines have been used as a means of determining their chemical composition (e.g. pH, titratable acidity, alcohol and colour) by the Australian wine industry (Dambergs *et al*., 2002, 2003). This approach provided a means of characterising complex features of wine quality including aroma as well as possibly assisting in determining the relationship between the chemical composition and sensory characteristics (Cozzolino *et al*., 2003). Absorption bands at 6897 cm^{-1}, 5587 cm^{-1} and 4413 cm^{-1} were reported

to be associated with sucrose, fructose, and glucose in fruit juices, respectively (Lanza and Li, 1984; Cozzolino *et al*., 2003; Cen and Hi, 2006). Similarly, the regions 4926 – 4694 cm^{-1} and 6757 – 6329 cm^{-1} were associated with the glucose concentration; for the determination of concentrations, calibrations are necessary (Blanco *et al*., 2004). In white wine absorption bands at 6897 cm^{-1} were related to the O–H second overtone of water and ethanol (Cozzolino *et al*., 2003; Cen and Hu, 2006). The spectral region from 5128 cm^{-1} to 4926 cm^{-1}, which corresponds to the spectral range where the COOH group absorbs, was reported to be associated with acetic acid content in synthetic wine (Blanco *et al*., 2004). The calibrations developed for total soluble solids use wavenumbers that are related to O–H and C–H bonds, around 10204 cm^{-1}, 7142 cm^{-1}, 5263 cm^{-1} and 4608 cm^{-1} respectively (Cozzolino *et al*., 2006). Absorptions at 4413 cm^{-1} and 4344 cm^{-1} are most likely C–H combination bands of methanol (Cozzolino *et al*., 2003).

A NIR spectrometer instrument (Figure 4) mainly consists of light source, beam splitter system, sample detector, optical detector, and data processing analysed system (Cen and He, 2006). The NIR beam penetrates deeper than MIR into a sample in reflectance techniques, giving a more representative analysis. Furthermore, minor impurities are less troublesome in both reflectance and transmission methods (Willard *et al*., 1988). Applications of NIR spectroscopy in viticulture and oenology are described in Table 1.

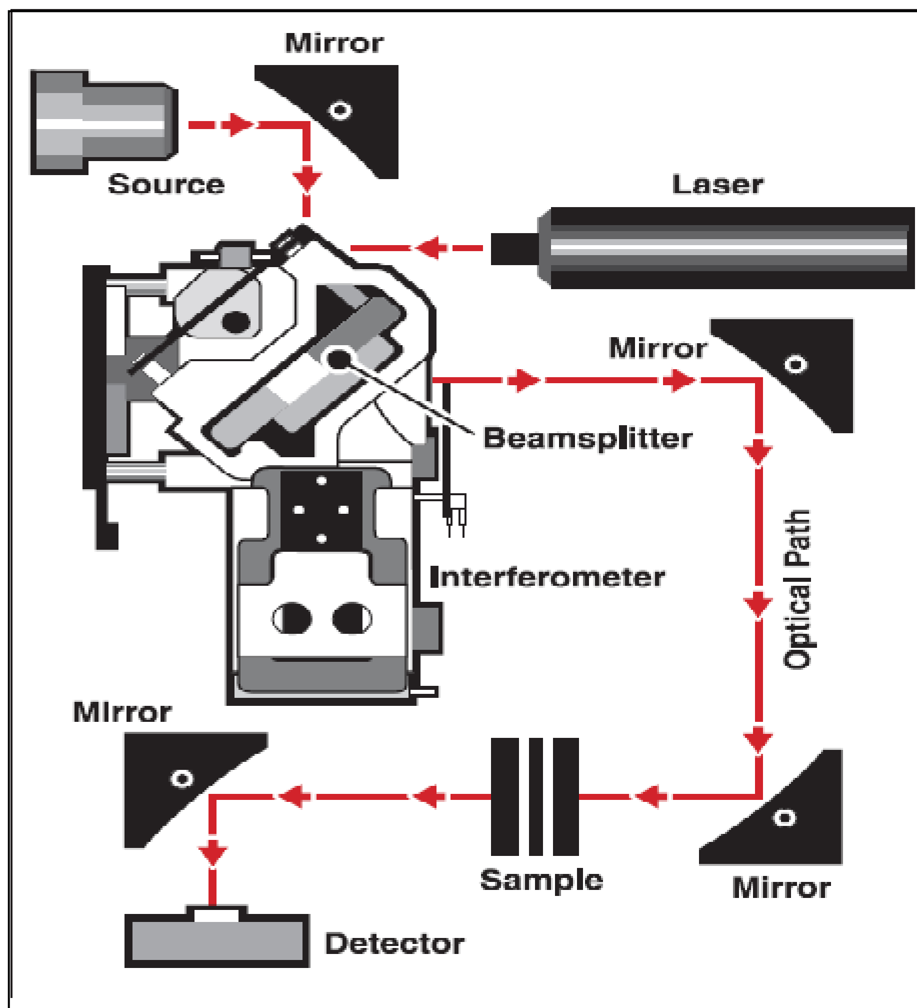

Figure 4 A typical layout of an NIR spectrometer (Adapted from Thermo Electron Corporation, www.thermo.com)

Table 1 Applications of near infrared spectroscopy in viticulture and oenology

Applications	References
Determination of ethanol, glycerol, fructose, glucose and residual sugars in botrytised sweet white wines	Garcia-Jones and Medina, 1997
Determination of total soluble solids (TSS) in different grape varieties	Jaren *et al*., 2001 Herrera *et al*., 2003
Measurement of YAN, sugar, malic- and lactic acids and ethyl carbamate in must and wines	Manley *et al*., 2001
Determination of sodium, potassium, magnesium and calcium in white wine	Sauvage *et al*., 2002
Determination of methanol in grape spirits	Dambergs *et al*., 2002
Discrimination between Chardonnay and Riesling wines	Cozzolino *et al*., 2003
Determination of total anthocyanins, pH and TSS in different grape varieties	Dambergs *et al*., 2003
Measurement of malic and tartaric acid in different grape varieties	Chauchard *et al*., 2004
Determination of total anthocyanins and pH in grape varieties	Cozzolino *et al*., 2004
Determination of 15 parameters in different types of wine	Urbano-Cuadrado *et al*., 2004
Measurement of malvidin 3 glucoside, pigmented polymers and tannins in red varieties (must and wine)	Cozzolino *et al*., 2004
Analytical monitoring of alcoholic fermentation in synthetic medium	Blanco *et al*., 2004
Monitoring of red wine fermentation in a pilot scale	Cozzolino *et al*., 2006
Determination of soluble solids content (SSC) and pH of rice wine	Liu *et al*., 2007
Measure concentration of calcium, potassium, magnesium, phosphorus, sodium, sulphur, iron, boron and manganese in Australian wines	Cozzolino *et al*., 2008
Measurement of glycosylated compounds (G-G) in white grape juice	Cynkar *et al*., 2007
Measurement of volatile aroma compounds in Riesling wine	Smyth *et al*, 2007
Prediction of wine quality ratings in Australian red wines	Cozzolino *et al*., 2008
Prediction of SSC and pH and varieties discrimination of grapes	Cao *et al*., 2009
Determination of fermentative volatile compounds in aged red wines	Lorenzo *et al*., 2009

The use of vibrational spectroscopy for routine quantitative analysis of wine began with NIR spectroscopy being the preferred method in the early years. Recently however, the focus has moved towards Fourier transform infrared (FT-IR) technology in the mid-infrared region, since it offers a more accurate determination of more compounds than NIR spectroscopy (Patz *et al*., 1999; Soriano *et al*., 2006). The first purpose-built wine analyser of this type, the WineScan FT120 instrument (Foss Analytical, Denmark, http://www.foss.dk) was released on the market in 1998 (Soriano *et al*., 2006). Since its introduction to wine applications, FT-IR spectroscopy has increasingly been used for quantitative and qualitative analyses in many diverse applications (Table 2). This technology is based on the measurement of the absorbance of radiation in the mid-infrared region by molecules that contain covalent bonds such as C–C, C–H, O–H and N–H (Willard *et al*., 1988; Holler *et al*., 2007). The radiation from the infrared light source passes through an interferometer before reaching the sample and the detector (Figure 5). The signal is digitised by an analog-to-digital converter and transferred to the computer for the Fourier transformation (Burgula *et al*., 2007). FT-IR is an indirect method, which means that absorbance data generated by the instrument must first be converted by a mathematical equation or calibration model that is applied to predict quantitative or qualitative results (Kupina and Shrikhande, 2003). The WineScan uses FT-IR spectroscopy together with multivariate statistical procedures to correlate the spectral response of a sample with compositional data as determined by reference laboratory methods.

IR spectra of wine samples show that water and ethanol absorption peaks dominate the spectrum, with the C–O stretch for primary alcohols at 1050 cm^{-1} prominent. The contribution of the C–H stretch from 2850 – 2960 cm^{-1} from ethanol is also significant. The region from 1690 –

1760 cm^{-1} contains information relating to C=O stretching for aldehydes, carboxylic acids, and esters (Bevin *et al*., 2006; 2008). Peaks between 1157 cm^{-1} and 1504 cm^{-1} are related to –CH groups in the alcohol and acid components, while the peaks at 1003 – 1099 cm^{-1} are related to –COH groups (Bevin *et al*., 2006; 2008). The region associated with sugars (C–O stretch for fructose at 1060 cm^{-1} and glucose at 1030 cm^{-1}) contributes to the separation among wines. Phenolic compounds have a major contribution for both red- and white wines and are associated with the aromatic–OH stretches at 1520 cm^{-1}, 1280 cm^{-1}, and 1200 cm^{-1} (Bevin *et al*., 2008).

The use of a FT-IR instrument with ready-to-use calibration models for different products provides a useful starting point for unskilled users and for routine analysis. In the case of the WineScan commercial calibrations, several studies have shown that the calibration models had to be evaluated and in most cases redeveloped when applied to samples originating from geographic origins not included in the original calibration models. This was true for glycerol quantification in South African wine samples (Nieuwoudt e*t al*., 2004), ºBrix, TA and pH in South African grape juice samples (Swanepoel *et al*., 2007) and for several routine parameters in German wine samples (Patz *et al*., 2004). Extending the database of calibration samples to include representative real life samples originating from the geographic area that future unknown samples will originate from, and to encompass the widest possible range and scale of concentrations for the parameter of interest, is crucial for each laboratory using this technology. Extensive evaluation, and, where necessary, modifications of the commercial calibrations are necessary to develop robust local models. However, the gain in robustness of a calibration model can result in a weakening of analytical precision; therefore, a compromise must be found between robustness and analytical accuracy in order to be able to deal with maximum number of wine types using the same calibration, while at the same time, being sufficiently accurate for the requests of enological analysis. When grape, must or wine is analysed for payment or quality control, analysis time, accuracy and precision are key parameters.

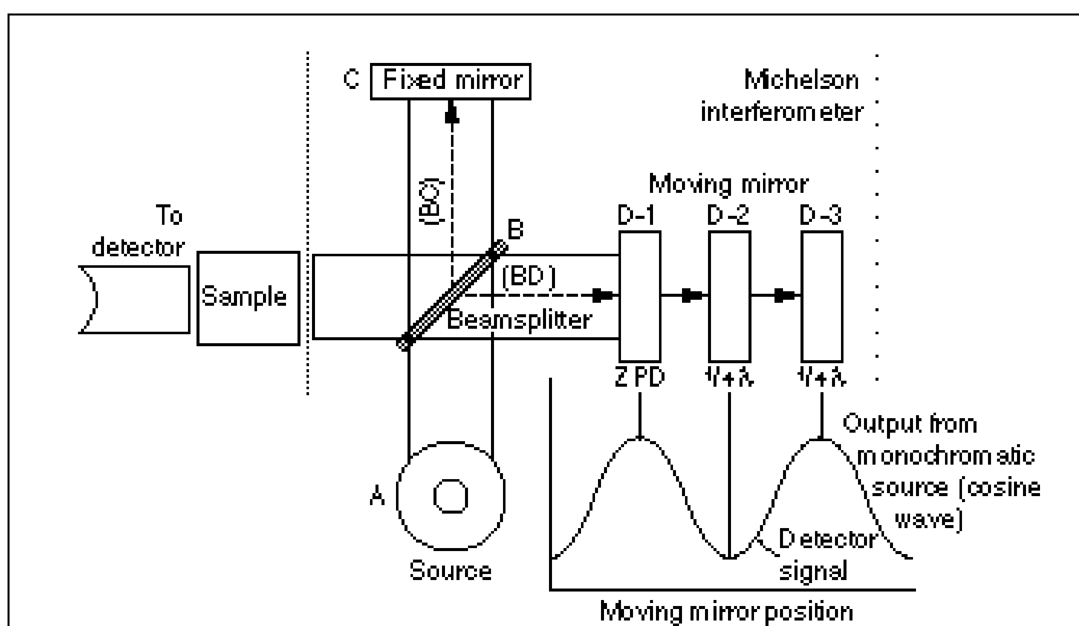

Figure 5 Simplified optical layout of a typical FT-IR spectrometer (adapted from Nicolet Instrument Corporation; www.thermonicolet.com).

Table 2 Applications of Fourier transformation infrared spectroscopy in viticulture and oenology.

Applications	References
Determination of carbohydrates, alcohols and organic acids	Vonach *et al*.,1998
The use of FT-IR for routine wine analysis	Dubernet and Dubernet, 2000; Patz *et al*., 2004
Characterisation and classification of wines, brandies and other distilled drinks during their ageing process.	Palma and Barroso, 2002
The determination of glycerol in South African table wines	Nieuwoudt *et al*., 2004
Develop calibrations for monitoring glucose, fructose, glycerol, ethanol and organic acids during large scale wine fermentations in Cabernet Sauvignon.	Urtubia *et al*., 2004
Rapid analysis of grape aroma glycoconjugates	Schneider *et al*., 2004
Determination of anthocyanins in red wine	Soriano *et al*., 2006
Development of a rapid (fingerprinting) system for wine authenticity	Bevin *et al*., 2006
Determination of wine polysaccharides	Boulet *et al*., 2007
Quantification of total soluble solids (TSS), pH and TA in South African grape must	Swanepoel *et al*., 2007
Determination of grape quality at harvest	Versari *et al*., 2008
Discrimination between different red and white wine varieties of Australian wine	Bevin *et al*.,2008
Characterisation of selected South African young wines	Louw *et al*., 2009

2.5 SAMPLING ISSUES IN THE INDUSTRIAL WINE CELLAR

Data analysis is often regarded as separate from chemical analysis and separate from the process by which the sample is produced, while in reality, both chemical analysis and data analysis depend on the process. Sampling representativity will always be strongly coupled to process and product types, because each process or product type poses a unique heterogeneity characteristic (Esbensen *et al*., 2006). Sampling is the evaluation of a portion of a population for the purpose of obtaining useful information about it and very important decisions could be made for an entire lot based upon the results of a sample. Taking a representative sample of the tank to be monitored is the first and most important step in ensuring meaningful analysis in an industrial cellar. However, there are constraints on what can be achieved in this respect, and it is not always possible and practical, especially in large wineries that operate hundreds of tanks with different tank volumes, to pump fermentations over for the sake of obtaining a representative sample. The topic: *Theory of Sampling* has been described in detail by different sources (Gy, 1992, 1998; Esbensen *et al*., 2006). Detailed discussion of this topic is beyond the scope of this dissertation.

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Chapter 3

Univariate and multivariate data analytical tools used in this study

3. UNIVARIATE AND MUTIVARIATE DATA ANALYTICAL TOOLS USED IN THIS STUDY

3.1 INTRODUCTION

Scientists in the food and beverage industries are faced with many different quality control tasks. To name a few, these include verification that products meet the required standards, identification of changes in process parameters that might lead to a change in quality, detection of product adulteration, and verification of product authenticity (Cozzolino *et al*., 2009). Many of the research in the field of grape and wine analysis has been conducted in a manner that can be described as “univariate”, since the investigations examined the effect of a single variable at a time on the overall data matrix. These methods include calculation of the average, standard deviation, standard error of laboratory and coefficient of variation.

Analysing the effect of one variable at a time by analysis of variance (ANOVA) can give useful descriptive information, but this will not give specific information about relationships in the background matrix (Esbensen, 2002; Manly, 2005; Cozzolino *et al*., 2009). Multivariate data analytical methods of both quantitative and qualitative nature are increasingly being used by research scientists in combination with traditional data analytical methods (Balabin *et al*., 2007). Compared to traditional wet chemistry methods for routine wine analysis, multivariate analysis combined with modern instrumental techniques (e.g. near infrared and mid-infrared spectrometers) often give new and better insight into complex problems by measuring several chemical compounds simultaneously (Dubernet and Dubernet, 2000; Patz *et al.,* 2004). For example, due to the complexity of the information contained in the FT-IR spectra, an extensive calibration process that involves multivariate statistical procedures such as principal component analysis (PCA) and partial least squares (PLS) regression are required to extract the maximum amount of information (Eriksson *et al*., 1999; Esbensen, 2000; Næs *et al*., 2004). The mathematical tools in chemometrics provide the means to convert raw data into information, information into knowledge, and ultimately knowledge into understanding (Willard *et al*., 1988). Both quantitative and qualitative applications of multivariate data analysis have been reported in the literature for the analysis of grape juice and wine (Dubernet and Dubernet, 2000; Patz *et al*., 2004; Urtubia *et al*., 2004). Possibly the most commonly used multivariate data analysis techniques applied to grape and wine analysis are PCA and PLS regression (Manly *et al*., 2001; Cozzolino *et al*., 2003; Urbano-Cuadrado *et al*., 2004; Soriano *et al*., 2007).

The technique of PCA was first described by Karl Pearson in 1901 (Manly, 2005). It is used as a tool for extracting, compressing, and screening multivariate data (Cozzolino *et al*., 2009). Large data tables usually contain a large amount of information that is partly hidden, because the data sets are too complex to be easily interpreted. PCA is a projection method that aims to make a graphic visualisation of all the information contained in a data set. PCA models the maximum directions of variation in a data set and provides an overview of the data structure by revealing relationships (differences and similarities) between the samples (Eriksson *et al*., 1999; Esbensen, 2002). Principal components (PC) are constructed to capture, in decreasing order, the maximum variation in the data set and the first few PCs often describe the largest proportion of variation in the data. PCs are calculated to be orthogonal to one another, therefore they can be interpreted independently (Esbensen, 2000).

PLS regression is a technique that combines features from PCA and multiple regression. It is particularly useful when a set of dependent variables must be predicted from a very large set of independent variables (Esbensen, 2002; Abdi, 2003). PLS regression is a bilinear modelling

method whereby the original **X** data matrix is projected onto a small number of underlying variables, called PLS components. The computation of PLS components actively uses the **Y** data matrix to ensure that the first PLS components are most relevant for predicting the *y* variables (Næs *et al*., 2002).

Classification using the Soft Independent Modelling of Class Analogy (SIMCA) approach, aims to identify local models for possible groups and to use these models to predict a probable class membership for new observations. At first, this approach runs a PCA or PLS (according to the available data structure) on the whole data set in order to identify groups of observations. Local models are then estimated for each class. Finally, new observations are classified to one of the established class models on the basis of their best fit to the respective model (Esbensen, 2000).

The univariate and multivariate statistics that are discussed in this chapter have been described in standard statistical textbooks and were used in this study (Martens & Martens, 2001; Esbensen, 2002; Manly, 2005). Where possible, the different techniques have been illustrated with examples based on own results generated in this study.

3.2 UNIVARIATE ANALYSIS

Univariate statistics are used when one variable at a time is dealt with. It provides useful information on the properties of the data set and relationships between samples in terms of a single variable (Kaufmann, 1997, Esbensen, 2002). Univariate models do not consider the contributions of more than one variable source and can result in models that oversimplify the system under analysis.

3.2.1 MEAN

The mean is the average of the observed values, i.e. the sum of the values, divided by the number of samples in the group. The mean gives an indication of the central location of the samples, i.e. a value around which the most typical samples are located.

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

where:
x_i is item *i* in the set (measurement for i^{th} sample)
bar *x* is the mean of the number set
n is the number of samples

3.2.2 STANDARD DEVIATION

The standard deviation (s) is the root mean square of deviation from the mean of the set of *n* numbers; it is denoted by *s* and is defined by

$$s = \sqrt{\frac{\sum_{i=1}^{n} \left(x_i - \bar{x}\right)^2}{n-1}}$$

where:

x_i is item i in the set (measurement for i^{th} sample)
bar x is the mean of the number set
n is the number of samples

3.2.3 STANDARD ERROR OF LABORATORY

The standard error of laboratory (SEL) is used to determine the measuring error of the analytical method based on two measurements of the same samples (Nieuwoudt *et al*., 2004; Urbano-Cuadrado *et al*., 2004). SEL is based on the sum of the difference between two measurements in terms of the size of the sample set.

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

where:
y_1 and y_2 are duplicate measurements of a sample
n is the number of samples

3.2.4 STANDARD DEVIATION OF THE DIFFERENCE

The standard deviation of the difference (SDD) can also be used to evaluate the accuracy of the measurements. SDD is the difference between two repeat measurements of a sample in terms of the average difference between measurements (Esbensen, 2002).

$$\text{SDD} = \sqrt{\frac{\sum (d_1 - d_m)^2}{(n-1)}}$$

where:
d_1 is the difference between duplicate measurements of a sample
d_m is the average difference between duplicate measurements
n is the number of samples

3.2.5 COEFFICIENT OF VARIATION

The coefficient of variation (CV) can be used to express the variation between replicate measurements of the same sample, thereby giving an indication of the precision of the measurements. CV can be described as the ratio of the standard deviation and the sample average and is expressed as a percentage. CV was calculated as:

$$\text{CV (\%)} = \frac{s}{\bar{x}} \times 100$$

where:
s is the standard deviation and
bar x is the sample mean

3.3 MULTIVARIATE DATA ANALYSIS

One of the important reasons for collecting data is to develop a sufficient understanding of that data to be able to use the information in characterisation of future similar data sets. The goal of **exploratory data analysis** (e.g. PCA) is to provide a quality check on the data; for example to expose key measurements in the data, to expose possible outliers, to indicate whether there are

patterns or trends in the data, and to indicate whether there is sufficient modelling power in the data collected to do further investigations (Cozzolino *et al*., 2009). Ultimately, the purpose of most multivariate analyses is to develop a model to predict a property of interest. The property may be categorical or a continuous property that cannot be measured directly. When the property of interest has a discrete category assignment, then **batch data analysis** is the appropriate approach. Continuous properties are most often modelled and predicted by **regression analysis** (e.g. PLS) methods.

3.3.1 EXPLORATORY DATA ANALYSIS

Exploratory data analysis can be described as the graphical display of patterns of association in multivariate data sets. The algorithms for this exploratory work are designed to reduce large and complex data sets into a set of *best views* of the data that provide insight into the structure and correlation that exist among the samples and variables in the data set (Esbensen, 2000; Næs *et al*., 2002).

3.3.1.1 Principal component analysis (PCA)

PCA is a commonly used statistical tool for reducing the dimensionality of a data set while retaining the relevant patterns hidden in it. PCA achieves this by mapping the original data set onto a reduced orthogonal space assigned in such a manner as to account for most of the original data set's variability (Martens & Næs, 1989; Urtubia *et al*., 2007). PCA can be considered as the workhorse in exploratory data analysis and is frequently the first technique employed. PCA consists of score and loading plots, where scores are estimated in bilinear modelling methods and the information carried by several variables is concentrated onto a few underlying variables. This means the scores show the locations of the samples along each model component and can therefore be used to detect sample patterns, groupings, similarities or differences. Loadings on the other hand, are estimated in bilinear methods where the information carried by several variables is concentrated onto a few components. The loadings show how well a variable is taken into account by the model; therefore, you can use them to understand how much each variable contributes to the useful variation in the data. The objective of this exercise was thus to see if FT-IR spectroscopy can distinguish between red- and white fermenting must (Figures 1 and 2).

PCA is designed to reduce the number of variables that need to be considered, to a smaller number of indices (called the principal components), that are linear combinations of the original variables. The objective of the analysis is to take p variables X_1, X_2, ..., X_P uncorrelated in order of their importance, and the ordering is such that $Var\ (Z_1) \geq Var\ (Z_2) \geq ... \geq Var\ (p_1)$, where $Var\ (Z_i)$ denotes the variance of Z_i. The Z indices are then the principal components (Manly, 2005). The first linear combination of p variables is the first principal component (PC). PC1 explains the largest possible variance in the sample set. Similarly, the other principal components explain the remainder of the variation in decreasing order. All PC's are calculated to be uncorrelated to one another. For further analysis, only the first few principal components were used, providing that the sum of their variances is a high percentage of the sum of their variances for all p components.

Simply explained, PCA helps to identify in what way one sample is different from other samples, which variables contribute most to this difference, and whether those variables contribute in the same way or independently from each other. In this way sample patterns can be detected. PCA also quantifies the amount of useful information by excluding noise or meaningless variation, in the data. PCA transforms complex data into ways in which the most important or relevant information is made more obvious and simple for interpretation purposes.

This is accomplished by constructing a new set of variables that are linear combinations of the original variables in the data set (Esbensen, 2002). PCA forms the basis for several classification (SIMCA) and regression (PLS) methods.

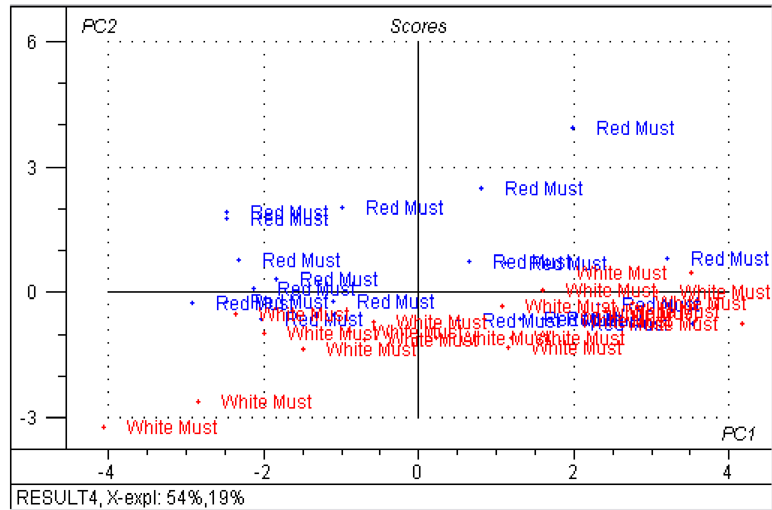

Figure 1 PCA scores plot, PC1 versus PC2, of FT-IR spectra of fermenting red- and white must samples. PC1 explains 54% of the variance in the sample set and PC2 19%. Red- and white fermenting must separate on PC2 (Own data, analysed with Unscrambler 9.2, Camo ASA, Trondheim, Norway, www.camo.com).

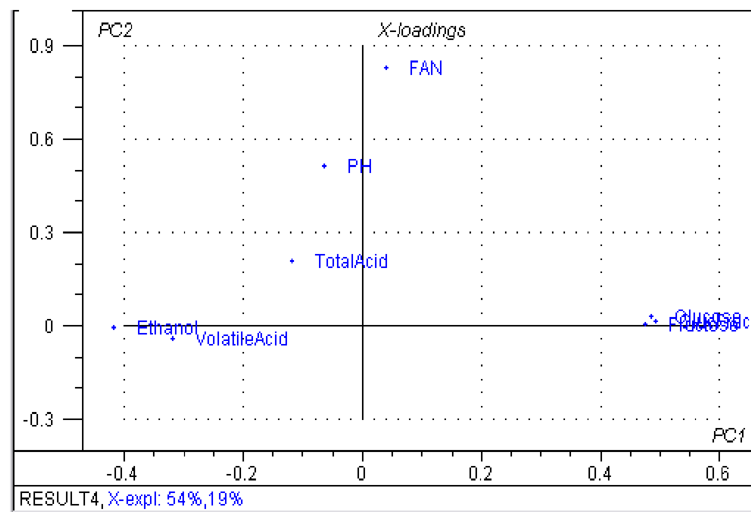

Figure 2 PCA loadings plot of fermenting red- and white must samples. Each marker represents the PCA loadings of a specific variable on PC1 and PC2. According to the loadings plot, glucose, fructose, ethanol and volatile acid contribute the most to the meaningful variation on PC1, while the FAN content in the samples contributed most towards the separation of samples of PC2. (Own data, analysed with Unscrambler 9.2, Camo ASA, Trondheim, Norway, www.camo.com).

3.3.2 BATCH DATA ANALYSIS

An investigation into monitoring of alcoholic fermentation is based on measurements, in order to generate data from which pertinent information can be extracted. Nowadays, analytical instrumentation has large numbers of sensors that generate huge amounts of data, frequently at very short time intervals, from all parts of the process. Typical variables related to winemaking, include input variables like the raw material, in this case grape must; controlled process

variables; such as fermentation temperature or nitrogen content of must; and result variables, also referred to as response values (User Guide, SimcaP+, Umetrics AB, Umeå, Sweden. www.umetrics.com). Response values can be chemical components such as the amount of ethanol or sugar concentration, but also an instrumental measurement such as FT-IR spectra. Based on the large amounts of variables (1056 wavenumbers) generated in spectra, the useful information in the spectra can be condensed by extracting PCA or PLS scores from the data, and the scores used as input response values, as described below. In wine production, it is useful to compare the fermentation progress of one specific tank, with patterns established over time, from similar related fermentations, in order to predict the duration of the fermentation and to foresee problems arising during the process.

The manner in which data of this nature is analysed, is very important, since useful information can easily be lost if a univariate approach is used. Multivariate batch data analysis is an ideal tool for this task and software packages such as SimcaP+ version 11.5 (Umetrics AB, Umeå, Sweden. www.umetrics.com) are often used for this purpose. In essence, batch processes require three-way analysis of the data, where K variables are measured on N batches at regular time intervals, J. This gives a three-way data matrix for each batch (N x J x K) as shown in Figure 3. Multi-way analysis and the methods have been discussed in detail in several publications (Kiers, 2000; Lorho *et al.*, 2006).

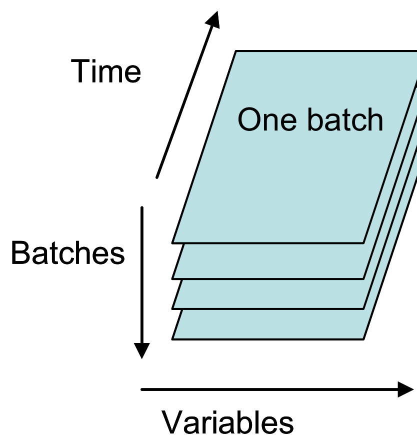

Figure 3 Diagrammatic illustration of three-way analysis of batch process data. Typically, N batches are measured at J time points for K number of variables. In wine fermentations, the batches usually have different lengths of time, not indicated in this figure.

Multivariate batch data analysis uses PCA or partial least squares regression (PLS) to calculate the respective score vectors; t_1, t_2, t_3,…t_j for the **X**-data and u_1, u_2, u_3,…u_j score vectors for the **Y**-data respectively, where 1,2,3..j refers to the number of vectors extracted from the data matrices. Score vectors are also referred to as principal components (Kiers, 2000). It is customary to extract two to three score vectors and plot them against each other, since these first components usually describe the most important variation in a data set. The scores are then used to construct **tt**, **uu** or **tu** graphical plots. The **tt** plots show how the **X**-variables and response values are situated with respect to each other, while and **uu** plots show how the **Y**-variables and response values are situated with respect to each other. The **tu** plots display the relation between **X** and **Y**. In this way the PCA – or PLS score plots provide a simple and visual way of interpreting the progress of the alcoholic fermentation process, as well as the status of the fermentations at a given point in time (User Guide, SimcaP+, Umetrics AB, Umeå, Sweden. www.umetrics.com). An example of a $\mathbf{t_1 t_2}$ plot that was constructed from PCA analysis of FT-IR spectra of fermenting must is shown in Figure 4. The plot serves to illustrate the relationships between the different fermentations, from the onset to complete consumption of the sugars, of

the respective white cultivars and wine styles shown in the figure. The extreme location of the Extra Light (ethanol content <10.5 %v/v) towards the left of the plot shows that the process behaviour of this fermentation deviated significantly from the behaviour of the other fermentations. The tanks shown in this example were white must fermentations that were sampled at 8-hourly or 12-hourly intervals at Vredendal Cellar, SA.

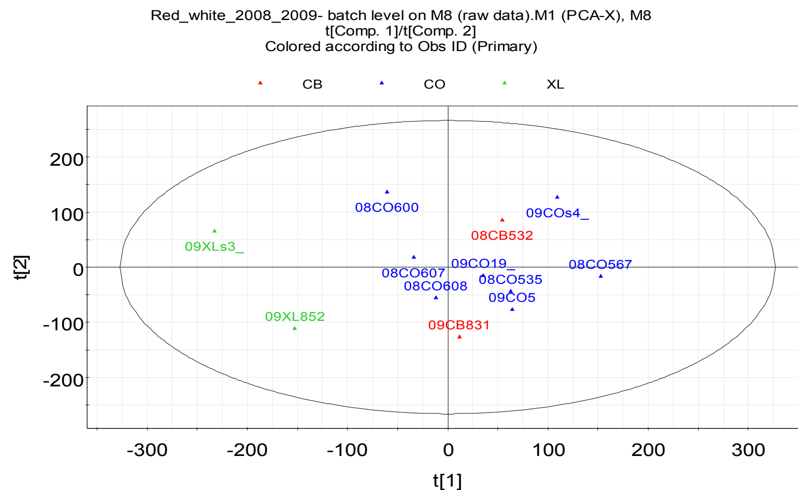

Figure 4 PCA score plot, t_1t_2 based on FT-IR spectra at the batch level of all white wines monitored during 2008 and 2009. Colombar (blue markers), Chenin blanc (red markers) and Extra Light (green markers). Each marker represents a complete fermentation, from onset to completion, sampled at 8-hourly or 12-hourly intervals. Duplicate fermentations were conducted for each wine style. The ellipse indicates a 95% confidence interval. None of the fermentations were identified as outliers. (Own data, analysed with SimcaP+ version 11.5. Umetrics AB, Umeå, Sweden. www.umetrics.com).

3.3.3 REGRESSION ANALYSIS

The goal of a regression analysis is to develop a model, also referred to as a calibration model that correlates information in a set of measurements to some desired property. This is particularly useful when a component or parameter is difficult to measure directly, since regression methods can be used to predict this value once the calibration model has been established. To fully test a model created in the calibration stage, a validation procedure is required. In general, validation entails the application of a model to test samples for which the components or parameters have been determined by the reference method. Thus, by comparison of the values predicted by the calibration model to that obtained with the reference values, a measure of reliability of the calibration model can be established (Esbensen, 2000)

3.3.3.1 Partial least squares regression (PLS)

PLS is built on PCA technology and is used for regression analysis (Martens and Næs, 1989; Esbensen, 2002). The goal of PLS regression is to predict the independent variable matrix **Y** from the dependent variable matrix **X** and to describe their common structure. The information extracted from the **X** matrix is passed to the dependent variable vector and vice versa and the mathematics for these calculations has been described in chemometric textbooks (Martens and Næs, 1989; Martens and Martens, 2001; Esbensen, 2002). PLS2 is a predictive two-block (in the case of one set of *x* variables and one set of *y* variables) regression method, while PLS3 uses multi-blocks (more than one set of *x* variables and one or more sets of *y* variables). The result from PLS is also a regression vector, but one in which correlations between the **X** block and the **Y** block are included. The regression analysis is applied simultaneously to the various blocks of data, for example, data set FT-IR spectra and physical and/or chemical data of the

same wine samples. The main purpose of PLS regression in this study was to build linear calibration models that enable prediction of desired characteristics, such as alcohol or sugar content of fermenting must.

Figure 5 shows a PLS1 regression plot of reference ethanol values versus WineScan predicted ethanol values. The statistical indicators used to evaluate the model are discussed in the following sections, and include bias, standard of cross validation (SECV), root mean square error of prediction (RMSEP), the coefficient of determination and the residual predictive deviation.

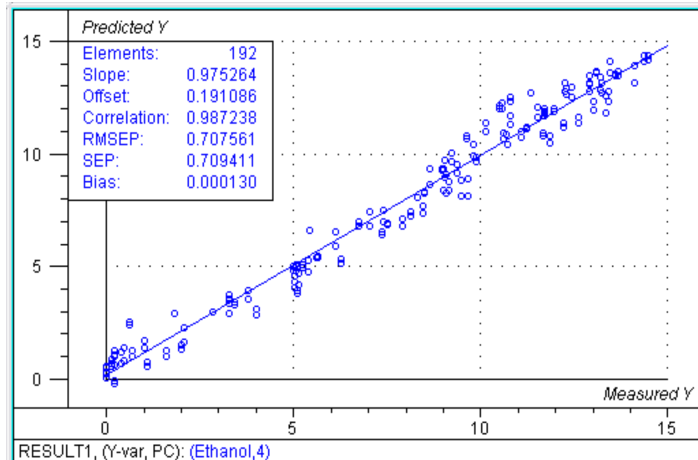

Figure 5 PLS regression plot of 192 measured ethanol values obtained by distillation analysis of fermenting must samples, versus WineScan predicted ethanol values, obtained with a PLS1 calibration model established in this study, for the prediction of ethanol. The different statistical indicators shown in the plot are discussed in the followig sections. (Own data, analysed with Unscrambler 9.2, Camo ASA, Trondheim, Norway, www.camo.com).

3.3.4 BIAS

Bias is one of the statistical indicators that are used to evaluate multivariate calibration data. Bias refers to the mean difference between the predicted and the measured reference values for all the samples in a validation set. It is a measure of the overall accuracy of a prediction model and is expressed in the same unit as the original reference data, in this case %v/v.

$$\text{Bias} = \frac{1}{n}\sum_{i=1}^{n}\left(y_i - \hat{y}_i\right)$$

where:

$\hat{y}_i$ is the predicted value for item i in the set

y_i is the measured reference value for item i in the set

n is the number of samples in the set

The bias (0.000130) for the ethanol calibration (Figure 5) indicated a negligible systematic error in the predicted data.

3.3.5 STANDARD ERROR OF CROSS VALIDATION

The standard error of cross validation (SECV) decribes the predictive accuracy of the calibration model in relation to the reference data and the equation used for the calculation of SECV was:

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

where:
y_i is the reference value for the ith sample
$\hat{y}_i$ is the predicted value for item i^{th} sample
n is the number of samples in the set

3.3.6 STANDARD ERROR OF PREDICTION

The standard error of prediction (SEP) gives an indication of the average prediction error obtained for several samples. Terminology used on the WineScan calibration software, uses SEP to denote the prediction error (WineScan Reference Manual, Foss Analytical Denmark), while in Unscrambler software this same property is referred to as root mean square error of prediction (RMSEP). SEP (or RMSEP) is expressed in the same unit as the original reference data, in this case %v/v. Data obtained with the ethanol calibration, showed a relatively small prediction error (SEP = 0.707561 %v/v, Figure 5) for the ethanol content in fermenting must samples. SEP can also be described as the scatter around the regression line and is expressed when corrected for bias.

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

where:
$\hat{y}_i$ is the predicted value for item i in the set
y_i is the measured reference value for item i in the set
n is the number of samples in the set

3.3.7 COEFFICIENT OF DETERMINATION

The coefficient of determination (R^2) is the ratio of the explained variation to the total variation. If there is no explained variation the ratio is 0 and if all the variation is explained the ratio is 1. In all other cases the ratio is between 0 and 1.

$$R^2 = 1 - \frac{s_{y.x}^2}{s_y^2}$$

where:
s^2_y is the standard deviation
$s^2_{y.x}$ is the root mean square error of prediction

3.3.8 RESIDUAL PREDICTIVE DEVIATION

The residual predictive deviation (RPD) is an important criterion that can be used to interpret the calibration error of the calibration models. RPD is defined as the ratio of the standard deviation of the reference values to the standard error of the predicted values when using independent test set validation (Williams, 1995; Esbensen, 2002). An important drawback of the RPD

criterion is that standard deviation, which forms part of the calculation, is influenced by the concentration range of the sample set.

3.3.9 DETECTION AND CLASSIFICATION OF OUTLIER SAMPLES

Outlier detection is one of the most important tasks in practical multivariate calibration (Nieuwoudt *et al*., 2004). Outliers describe extreme deviating samples that show an abnormal pattern in variability when compared with other samples in a particular sample set (Esbensen, 2002). An outlier could be different from other points with respect to the value of one variable or, it could only be detected when several variables are considered simultaneously. The latter example can therefore be considered as a true multivariate outlier.

There are different types of outliers and these include calibration and validation outliers, and **X**- and *y*-outliers (Naes, 2002). Calibration outliers are present in calibration sets and will influence the calculation of the calibration algorithms; these outliers should be investigated carefully and removed from the data set if necessary, since the prediction of all future samples will be affected by them. This could lead to mistaken conclusions, inaccurate predictions and a lack in quality control. A prediction outlier is present after the calibration stage and will not have an effect on the calibration model, although represent an incorrect value. An indicator of calibration outliers is SECV, while SEP indicates prediction outliers. These values are given in the same units as the original reference values, and can thus easily be interpreted. Outliers can be visualised in 2-dimensional **X**-**Y** relation plots (Figure 6) where the **t** scores are shown as the horizontal axis and the **u** scores as the vertical axis. **X**-**Y** relation outliers can be constructed with the Unscrambler software (version 9.2, Camo ASA, Trondheim, Norway, www.camo.com). The plots show the relation between samples in the **Y**-space and the variables in the **X**-space. It is wise to remove only one or two outliers at a time, starting with the most extreme ones in the first component (Esbensen, 2002).

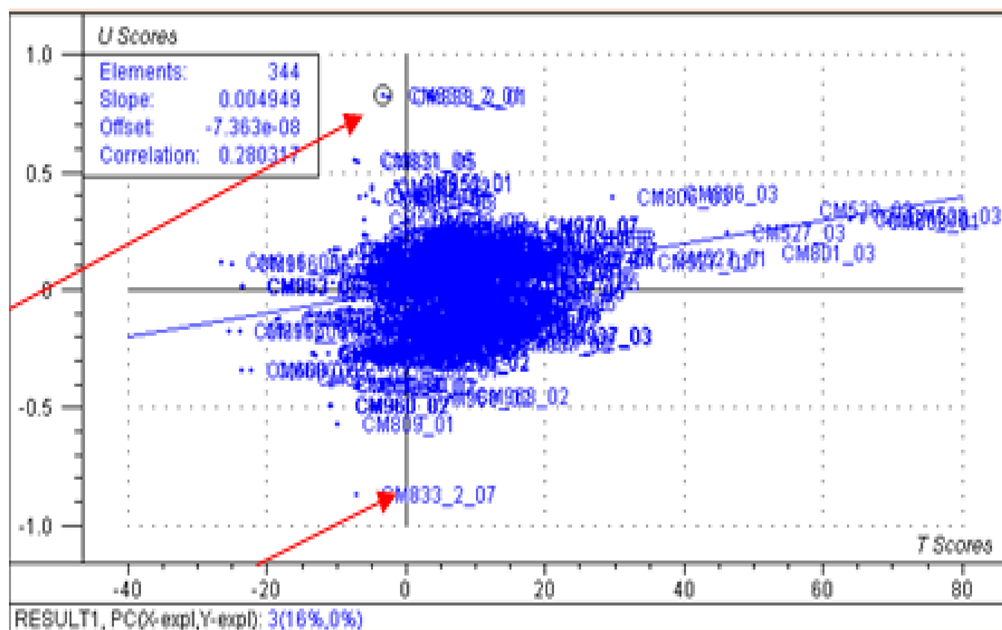

Figure 6 **X**-**Y** relation plot showing two outlier samples (flagged with red arrows) that are projected far away from the model component PC3. **T** scores show the abscissa, and **u** scores show the ordinate. The result was obtained during the development of a glucose calibration in fermenting must using own data. (Data analysed with Unscrambler 9.2, Camo ASA, Trondheim, Norway, www.camo.com).

For the glucose calibration implied in Figure 6, the SECV was 2.46 g/L with outlier samples included in the analysis. Cross validation, with 10% of the samples excluded from the PLS1 calculation at time, was used to evaluate the calibration model. A much improved SECV value of 1.79 g/L was obtained with the outlier samples removed from the calibration sample set. These results serve to illustrate the effect that outlier samples in the calibration set can have on the regression statistics.

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Chapter 4

Research results

Evaluation of Fourier transform infrared spectroscopy for quantitative monitoring of alcoholic fermentation

4. RESEARCH RESULTS

4.1 ABSTRACT

Fourier transform infrared (FT-IR) spectroscopy plays an important role in quality control in wine production by providing a rapid and cost effective method to determine a range of important wine compounds. To date, the technology has not been implemented to any large extent to quantify components in fermenting must. In this study, samples from 284 industrial-scale white- and red actively fermenting tanks were collected at regular time intervals, during the 2007, 2008 and 2009 harvests, and analysed with FT-IR spectroscopy and appropriate reference methods. The data were used to establish new calibration models for the determination of glucose (n = 465), fructose (n = 465) and yeast assimilable nitrogen (n = 471). The models were evaluated in terms of the coefficient of determination (R^2), bias and prediction error (SEP). The performances of the commercial (also referred to as global) FT-IR calibrations for ethanol (n = 322), pH (n = 394), titratable acidity (n = 351), volatile acidity (n = 282) and total glucose plus fructose content (n = 465) in fermenting must were also evaluated. The global ethanol calibration model gave very good prediction (SEP = 0.15% v/v, R^2 = 0.999, bias = 0.04% v/v) and with a residual predictive deviation (RPD) of 30, rendered an excellent model for quantitative purposes in fermenting must. However, concentration ranges of the global model did not span the ethanol concentration ranges found in South African (SA) wines, and a new model, based on SA samples was developed. The new models for pH (SEP = 0.04, R^2 = 0.923, bias = -0.01, RPD = 4) and VA (SEP = 0.07 g/L, R^2 = 0.894, bias = -0.01 g/L, RPD = 3) showed that the models were suitable for screening purposes. The calibration model for TA (SEP = 0.35 g/L, R^2 = 0.797, bias = -0.004 g/L, RPD = 2), proved unsatisfactory for quantification purposes, but useful for screening purposes. The calibration model for the total content of glucose plus fructose (SEP = 0.6.19 g/L, R^2 = 0.993, bias = 0.02 g/L, RPD = 13), showed very good prediction and can be used to quantify total glucose + fructose content in fermenting must. The newly developed calibration models for glucose (SEP = 4.88 g/L, R^2 = 0.985, bias = -0.31 g/L, RPD = 8) and fructose (SEP = 4.14 g/L, R^2 = 0.989, bias = 0.64 g/L, RPD = 10) also proved fit for quantification of these important parameters. Two reference methods were compared for measuring yeast assimilable nitrogen (YAN), namely the enzyme-linked spectrophotometric assay and the Formol titration method. The results showed that the YAN reference values obtained with the enzyme-linked assays, resulted in a good calibration model for white fermenting must (SEP = 14.10 mg/L, R^2 = 0.909, bias = -2.55 mg/L, RPD = 6), but the regression statistics for predicting YAN in red fermenting must, were less satisfactory (data not shown). Reference data obtained with the Formol titration method could be used successfully in both red- and white fermenting must (SEP = 16.37 mg/L, R^2 = 0.912, bias = -1.01 mg/L, RPD = 4). The results confirmed that FT-IR spectroscopy is a useful technique for the quantification of major chemical parameters in fermenting must and is a very useful tool for off-line monitoring of alcoholic fermentation.

4.2 INTRODUCTION

Alcoholic wine fermentation is a biotechnological process characterised by yeast-mediated transformation of grape juice into wine. During the process yeast biomass is formed, and sugars, mainly glucose and fructose, are converted into ethanol, carbon dioxide and other minor

fermentation products that all contribute to the final composition of wine (Ribéreau-Gayon *et al*., 2000). Major chemical changes take place such as an increase in ethanol levels, the consumption of nitrogen substrates, acidity changes and decrease in sugar levels. The endpoint of fermentation is dictated by the style of wine; for example dry table wine (<5 g/L reducing sugar), off-dry table wine (5-10 g/L reducing sugar) or semi-sweet table wine (15-20 g/L reducing sugar). In industrial situations the duration from the start to the endpoint of a fermentation varies significantly from tank to tank, due to the inherit variation in juice as well as unpredictable external factors. Large wineries such as Namaqua Wines, Vredendal, South Africa operate several fermentation tanks (usually 100–200 tanks at once during peak harvest time) simultaneously and need quantitative chemical analysis that is rapid and accurate for quality control. The successful outcome of a fermentation process is only possible if real time measurements of decisive parameters are available (Urtubia *et al*., 2008).

Mid-infrared spectroscopy, and in particular the Winescan FT 120 mid-infrared spectrometer (Foss Analytical, http://www.foss.dk) is well established in wine analytical laboratories worldwide for quantitation of components in finished wine (Bauer *et al*., 2008), however only a few applications of this technology for monitoring alcoholic fermentation have been reported in the literature. Dubernet and Dubernet, 2000 developed some preliminary quantitative calibrations on the WineScan instrument for fermenting must samples originating from France. Urtubia *et al*., 2004 used a quantitative approach to monitor industrial scale Cabernet Sauvignon wine fermentations including malolactic fermentation. Cozzolino *et al*., 2006 reported the use of visible-near infrared spectroscopy to monitor spectral changes through the time course of pilot scale Cabernet Sauvignon and Shiraz wine fermentations, while Blanco *et al*., 2004 monitored alcoholic fermentation in a synthetic wine medium using near infrared (NIR) spectroscopy. Critically important parameters such as fructose and nitrogen content were not included in the mentioned studies. The South African (SA) wine industry has a large Winescan instrument user group and from these limited applications reported in the literature, the need to develop and expand the quantitative FT-IR calibrations in fermenting must is clear.

FT-IR spectroscopy is based on the measurement of the frequencies of fundamental vibrations of chemical bonds in functional groups such as C-C, C–H, O–H, C=O and N-H, upon absorption of radiation in the mid-infrared region. The mid-infrared (MIR) region is usually defined as ranging from 4000 to 400 cm^{-1}, or in terms of nanometers, from 2500 to 2.5×10^4 nm (Willard *et al*., 1988; Nieuwoudt *et al*., 2004; Holler *et al*., 2007). Recent developments in design and performance of FT-IR spectrometers combined with advances in chemometrics have provided an analytical tool that is suitable for rapid product screening and process control. This technology can replace available methods of analysis which require sample preparation, costly equipment, are frequently time consuming due to several steps of purification in sample preparation, sometimes unreliable and the information limited to a few parameters (Cozzolino *et al*., 2006). These factors all delay the time taken to deliver results and are not optimal for the purposes of effective real time fermentation monitoring.

Monitoring of the alcoholic fermentation process should include tracking the increase in ethanol concentration during the course of the process, since it provides a sensitive indicator of wine yeast metabolism (Ribereau-Gayon *et al*., 2000; Bowyer, 2006). The final ethanol concentration in wine is also of high importance in terms of international labeling laws. Payments made by wholesalers to the producers are often based on the ethanol content and there are legal specifications for the ethanol content in different types of wine (Baumgarten, 1984). Ethanol is responsible for several flavour characteristics of wine, such as warmth and body and, at higher concentrations; it also contributes a small amount of perceptible sweetness (Bowyer, 2006). In wineries, acidity measurements are usually split into three components by

most winemakers. These are titratable acidity (TA), a measurement of the amount of acidity in the solution, pH and finally the volatile acidity (VA) (Goode, 2005). TA and pH are important for sensory properties and microbial stability of the wine (Iland *et al*., 2000). Wine acidity also balances ethanol and residual sugar in wine. VA is present in all wines, generally at very low levels (0.2 – 0.4 g/L), but it becomes a problem when the levels exceed 0.8 g/L. High amounts of VA can be formed by bacterial or oxidative yeast activity during or after fermentation (Iland *et al.*, 2000; Goode, 2005). VA influences the quality of a wine and large amounts of this parameter can indicate microbial spoilage. The determination of VA is routinely used as an indicator of wine spoilage (Iland *et al*., 2000). There are different types of sugars in grape must, but glucose and fructose are most abundant and therefore also most important to oenologist and winemakers (Howell and Vallesi, 2004). Monitoring sugar levels in fermenting must is of critical importance to evaluate yeast metabolic acitivity, as well as to check that residual levels after alcoholic fermentation satisfy the requirements of the intended wine style (Roig & Thomas, 2003). It is also important at the final quality control and regulatory level before approval for production or bottling to know the content of the individual sugars in the final product (Garcia-Jares & Medina, 1997). The referral to nitrogen in must refers to the combination of ammonia nitrogen and yeast assimilable nitrogen, and can be considered as one of the most important nutritional elements required by a healthy yeast population to successfully complete alcoholic fermentation. Insufficient nitrogen levels in musts (<150 mg/L) are mostly responsible for stuck fermentation and development of H_2S odors (Falchek, 2000). The measurement and interpretation of must and wine parameters are therefore far more important to winemakers than often realised.

Several factors can lead to fermentation problems under enological conditions and these can be the direct cause of stuck fermentations. Factors can be of biological, microbial or physicochemical nature, or interactions of these (Ingledew and Kunkee, 1985; Henschke, 1997; Bisson, 1999). The critical quality control factors that provide early indications of problematic wine fermentations, include the sugars (glucose and fructose), nitrogen substrates (free available nitrogen), total titratable acids, alcohol levels and volatile acidity, and these must be monitored at regular and intervals for effective quality control. Currently residual reducing sugar concentrations are measured using hydrometers, or the progress of the fermentation process from the amount of CO_2 released. Many instruments are useful for tracking basic must conditions, but advanced instrumentation is needed to detect change in nutrient levels and glucose/fructose ratio as a fermentation progresses (Urtubia *et al*., 2008).

This study reports on the establishment of a portfolio of partial least squares regression (PLS1) calibration models, based on FTIR spectra of fermenting must samples, suitable for quantitative off-line monitoring of alcoholic fermentation during industrial wine production. The specific focus was on the WineScan FT 120 spectrometer and the components of interest were ethanol, pH, volatile acidity (VA), titratable acidity (TA), glucose, fructose and yeast assimilable nitrogen (YAN). The ultimate objective was to achieve the best possible accuracy and precision, and at the same time build robustness in the models, so they could be implemented for the maximum number of different sample types, including different grape varieties, geographic origin, climatic conditions, vintages, tank volumes, colour intensities for red grape varieties, different yeast starter cultures and at all different stages of the alcoholic fermentation process.

4.3 MATERIALS AND METHODS

4.3.1.1 FERMENTING MUST SAMPLES

For the course of this paper “fermenting must” refers to fermenting grape juice and spans the period immediately after settling, which involves the precipitation of grape skin solids in the freshly pressed grape juice for 24 hours, followed by inoculation with yeast starter cultures, until an endpoint determined by the style of wine is reached. A total of 284 actively fermenting tanks were sampled from 2007 to 2009. These included the white grape cultivars Chardonnay (28), Chenin blanc (23), Colombar (59), Sauvignon blanc (24), Muscat d’ Alexandrie (Hanepoot) (1), and Nouvelle (1) that were sampled at Vredendal Winery, Vredendal, South Africa. In addition, different wines styles were included in the project and these were White blend (12), Extra Light (2) and Rosé (19). The red grape cultivars Merlot (22), Shiraz (23), Pinotage (25), Cabernet Sauvignon (31), Cabernet Franc (3), Ruby Cabernet (8), Petit Verdot (2), and Malbec (1) were sampled at Spruitdrift Winery, Vredendal, South Africa. Samples from different wine styles were also included in the project. These include a white blend that refers to different white varieties fermented together in one tank, while Extra Light refers to a wine style with a low final ethanol level smaller than 10.5 %v/v. The Rosé is produced from different types of red cultivars with very little skin contact. The vineyards from which grapes are harvested by Namaqua Wines are located in different areas, namely Lutzville, Vredendal and Spruitdrift within the Olifants River Valley region which is located about 250 km from Cape Town, South Africa. The vineyards in the Lutzville area are situated on the banks of the Olifants River while the Vredendal and Spruitdrift vineyards are not. A total of 105 000 tons of grapes are harvested annually from these regions and taken in at Vredendal- and Spruitdrift wineries. Aliquots of 350 mL were collected in clean sample vials from the sample valves of the respective fermentation tanks that ranged in size from 4 000 L to 280 000 L. Samples were transported in a cooler bag with ice packs to the laboratory. Tanks were sampled at regular intervals during the active phase of fermentation in order to span the complete range of the components of interest.

4.3.1.2 SAMPLING PLAN

A total of 284 different fermenting tanks were sampled at different stages of fermentation to include the widest possible concentration range in the calibration sample set and the major changes in the fermenting matrix profiles. The strategy was to obtain FT-IR spectra throughout the fermentation process and as many different tanks as possible.

4.3.2.1 SAMPLE PREPARATION AND STORAGE

Upon arrival at the laboratory, the fermenting must samples were immediately prepared for further analysis. Aliquots of 50 mL were centrifuged (2 minutes; 5000 rpm) using a Hermle 200A centrifuge (LASEC, Cape Town, SA) and these samples were specifically used for the enzyme-linked spectophotometric assays and FT-IR spectroscopy. The samples were degassed for 5 minutes in an ultrasonic bath (UMC 5, Krugersdorp, SA). The remainder of the original 350 mL sample volume was degassed to remove excess CO_2 by manual shaking for 5 minutes, followed by 5 minutes in an ultrasonic bath (In-House chemical laboratory Standard Operating Procedure, Namaqua Wines, 2007). The degassed samples were used for alcohol, pH, TA and VA analyses using appropriate reference methods as described below. In some instances, samples were frozen directly after collection and stored at -20ºC until analysis could be done. Frozen samples were brought to ambient laboratory temperature (±20ºC), thoroughly mixed for

at least 5 minutes before they were degassed as described before. The thawed samples were thereafter used for both FT-IR spectroscopy and the appropriate reference analysis. The same pretreatment procedure was used for all samples and the spectra were used for the development of calibration models and evaluation of commercial calibrations.

4.3.2.2 CO_2 REMOVAL

The objective was to remove as much CO_2 as possible to obtain accurate results for all analysis. Certain analysis such as VA, TA and pH can be influenced by the presence of CO_2 and therefore samples need to be properly degassed. All samples were degassed for 5 minutes in an ultrasonic bath, manual shaking for another 5 minutes, and followed by another 5 minutes in the ultrasonic bath. The target concentration of CO_2 levels after degassing, were below 1000 mg/mL.

4.3.3 REFERENCE METHODS

4.3.3.1 Enzyme-linked spectrophotometric assays

4.3.3.1.1 Glucose and Fructose

Glucose and fructose concentrations were determined with an enzyme-linked assay (D-Fructose and D-Glucose, K-FRUGL 11/05, www.megazyme.com) in 1 mL plastic disposable cuvettes (LASEC, Cape Town, SA) and measured spectrophotometrically (Cecil, CE 1011, England, LASEC, Cape Town, SA) at 340 nm. The complete procedure for the enzymatic assays is given in Addendum A of this dissertation. Red fermenting must samples were first decolorised by the addition of 0.2 g of polyvinyl polypyrrolidone (PVPP) (Wintrust, Cape Town, SA), per 10 mL sample. After addition of PVPP, samples were shaken vigorously by hand for 5 minutes and thereafter filtered through Whatman No. 1 filter paper (Merck, Cape Town, SA). Dilutions were made where necessary, as described in Addendum A and each kit could perform 110 assays.

4.3.3.1.2 Ammonia and Primary Amino Nitrogen

The measurement of yeast assimilable nitrogen (YAN) was determined using both ammonia (Ammonia (Rapid), K-AMIAR 11/05, www.megazyme.com) and primary amino nitrogen assays (Primary Amino Nitrogen (PAN), K-PANOPA 03/06, www.megazyme.com). The results of each assay (ammonia + PAN) per sample were combined to determine the YAN content of each sample. Both assays were determined in 1 mL plastic disposable cuvettes and measured spectrophotometrically at 340 nm. The complete procedure for the enzymatic assays is given in Addendum A of this dissertation. No dilutions were necessary for the measurements and each kit performed 96 and 100 assays respectively. A second procedure for determination of the YAN content in fermenting must, the formal titration method, was also used, as describe in 4.3.3.2.5.

For all the enzymatic assays, two absorbance readings were taken for the enzymatic analysis, A_1 and A_2, at 340nm, and the absolute difference in absorbance $|A_1 - A_2|$ between the two readings must be least 0.1 AU and not exceeding 1.0 AU. The total volume of each assay, D-Glucose and D-Fructose, Ammonia and Primary Amino Nitrogen, recommended by the manufacturer was reduced in half in order to increase the number of measurements performed per kit. The accuracy of the down-scaling was tested with a normal volume sample as well as the assay controls provided with each kit. The standard error of laboratory (SEL) and the coefficient of variation (CV) were 0.25 g/L and 2.2% for glucose respectively and 0.23 g/L and 3.2% for fructose respectively. The standard error of laboratory (SEL) and the coefficient of variance were 0.53 mg/L and 8.6% respectively for YAN. The SEL is used to determine the

measuring error of the method based on two measurements of the same sample. The CV is used to express the variation between replicate measurements of the same sample, thus giving an indication of the precision of the measurements.

4.3.3.2 Wet chemistry

The wet chemistry methods used to generate reference values for routine wine parameters described below are those recommended by the Office International de la Vigne et du Vin (http://www.oiv.com). These methods are fully described in standard laboratory method textbooks (Zoecklein *et al*., 1999; Iland *et al*., 2000). All tests were done in the chemical laboratory, Vredendal Winery.

4.3.3.2.1 Alcohol

Alcohol was measured by distillation using a distillation unit (Glasschem, Cape Town, SA) and expressed as %v/v.

4.3.3.2.2 pH

pH was determined using an automatic titrator (Crison Compact Titrator D, SN 01714, Spain, LASEC, Cape Town, SA) with a combination electrode and a temperature probe. Certified buffers (Crison pH 7.00 and pH 4.00, LASEC, Cape Town, SA) were used to calibrate the electrode.

4.3.3.2.3 Volatile acidity (VA)

Volatile acidity was expressed as g/L acetic acid and measured by steam distillation using a Cash Still unit (Glasschem, Cape Town, SA). A freshly prepared standard acetic acid working solution of 0.60 g/L from 99% glacial acetic acid (Merck, Cape Town, SA) was measured daily to validate the accuracy and performance of the Cash Still unit.

4.3.3.2.4 Titratable acidity (TA)

TA was expressed as g/L tartaric acid and measured by potentiometric titration (Crison Compact Titrator D, SN 01714, Spain, LASEC, Cape Town, SA) using standardised 0.33 N sodium hydroxide (Merck, Cape Town, SA) to the endpoint of pH 7.00. A standard TA solution of 7.5 g/L was measured daily from L (+) tartaric acid (Merck, Cape Town, SA) to validate the accuracy and performance of the titrator. The solution was stable for one week at 4°C.

4.3.3.2.5 Yeast assimilable nitrogen

The Formol titration method was used to determine the yeast assimilable nitrogen concentration in the fermenting must samples. The samples (50 mL) were neutralised with 1 N NaOH to pH 8.5. An excess of neutralised formaldehyde (pH 8.5) was added followed by the re-titration of the solution after 10 minutes to the endpoint of pH 8.5.

4.3.4 EVALUATION OF REFERENCE MEASUREMENT ERRORS

Several parameters can be used to evaluate the precision of an analytical method and to describe variation in a data range. In this study the accuracy of the reference methods was expressed as the standard error of laboratory (SEL), the standard deviation of the difference (SDD) and the coefficient of variation (CV) as described below. SEL was calculated as:

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

Where y_1 and y_2 are the values from duplicate determinations and n is the number of samples. This method was used to determine the measuring error of duplicate measurements of the same sample (Nieuwoudt *et* al., 2004; Urbano-Cuadrado *et al*., 2004).

The SDD refers to the difference between two repeat measurements of a sample in terms of the average difference between measurements and calculated as:

SDD = $\sqrt{\frac{\sum(d_1 - d_m)^2}{(n-1)}}$

Where d_1 is the difference between duplicate measurements of a sample, d_m is the average difference between duplicate measurements of all samples tested and n is the number of samples (Esbensen, 2002).

Coefficient of variation (CV) can be described as the ratio of the standard deviation and the sample average and is expressed as a percentage. The CV can be used to express the variation between replicate measurements of the same sample, thus giving an indication of the precision of the measurements. CV was calculated as:

$$\text{CV (\%)} = \frac{s}{\bar{x}} \times 100$$

Where s is the standard deviation and bar x is the sample mean.

4.3.5 MULTIVARIATE DATA ANALYSIS

4.3.5.1 Principal component analysis (PCA)

The PCA technique is one of the simplest and most used methods of multivariate analysis since it provides a visual presentation of the variation in a sample set that is easy to interpret (Esbensen, 2002; Naes, 2002). It is a very important technique used to explore the data structure, in particular to detect possible outliers or deviating objects and to identify clusters based on relationships between the samples. PCA reduces the number of original variables, by computation of a new set of variables called principal components (PC's) that each consists of a linear combination of the original variables. PC's describe the variation in the sample set and each PC extracts the variation amongst the objects in decreasing order, with PC1 capturing the most variation, PC2 the most variation in the residual unexplained variation not explained by PC1 and so on, until the maximum variation in the sample set has been explained. PC's are calculated orthogonally to one another and can therefore be interpreted independently.

In calculating the PC's, the original data matrix, defined by $\mathbf{X}(n,m)$, is decomposed into the object space, the variable space and the error matrix. The latter represents the variation not explained by the extracted PC's and is dependent on the problem definition. The algorithm describing this decomposition is presented as:

$$\mathbf{X}(n,m) = \mathbf{T}(n,k)\mathbf{P}(k,m)^{T} + \mathbf{E}(n,m)$$

where **X** is the independent variable matrix, **T** the scores matrix, **P** the loadings matrix, **E** the error matrix, *n* the number of objects, *m* the number of variables and *k* the number of PC's used (Esbensen, 2000). The new data matrices calculated by PCA, are graphically presented as scores plots and loading plots that can be interpreted to establish the relationships between samples, variables and between samples and variables.

FT-IR spectra were exported to the Unscrambler Software (version 9.2, Camo ASA, Trondheim, Norway, www.camo.com) and spectra were averaged and autoscaled (mean centered and standardised). The complete data set consisted of the spectra of the fermenting must samples, defined by the variables (1056 wavenumbers) in the columns and the samples in the rows. The data matrix was centered by column. In this study, PCA was used to investigate the variation in the fermenting must sample sets due to cultivar, vintage and sample preparation procedures.

4.3.5.2 Outlier detection

X- outliers refer to the **x**-vectors that are abnormally positioned to the majority of **x**-data, while *y*-outliers are defined as those samples where the relation between **x**-data and *y* variables are different in comparison to the majority of the samples (Esbensen, 2002). Outliers were identified in X-Y relation outlier plots in the Unscrambler software (version 9.2, Camo ASA, Trondheim, Norway, www.camo.com).

4.3.6 FT-IR SPECTROSCOPY

4.3.6.1 FT-IR spectral measurements

FT-IR spectral measurements were done with the mid-infrared Winescan FT 120 spectrometer (Foss Analytical, http://www.foss.dk) that is an application instrument designed especially for quantification in grape- and wine-derived matrices. Certain instrument settings cannot be changed by the user and these include the temperature at which samples are scanned (40°C), the scanning interval (930 – 5011 cm^{-1} at 4 cm^{-1} intervals) and conditions of spectral collection. The WineScan is equipped with a Michelson interferometer and pyroelectric detector. A HeNe laser (632 nm) tracks the position of the moving mirror in the interferometer The CaF_2-lined cuvette has a sample volume of 3.276 µL with a path length of 37 µm. A total number of 10 scans is taken for each sample and then averaged. The final interferogram is then processed through a series of mathematical treatments that include Fourier transformation, and a single beam spectrum is created. Duplicate scans were obtained of each fermenting must sample immediately after degassing and sample preparation as described in section 4.3.2.1, with the WineScan FT 120. The instrument was cleaned and zeroed every 60 minutes using the cleaning and zero WineScan solutions supplied by Foss. No further spectral preprocessing was done.

4.3.6.2 Evaluation of commercial calibration models

The WineScan FT 120 instrument is supplied with commercial calibration models for the quantification of major wine components. These ready-to-use calibration models are referred to as "global" calibrations (Foss Analytical, http://www.foss.dk) and were developed in the late 1990's on samples of mostly French origin. The descriptive statistics of the chemical composition of the calibration samples used for the global WineScan calibrations are given in Table 1.

As part of the applications of the WineScan, the Advanced Performance Software Module with partial least squares regression (PLS1) and multiple-linear regression (MLR) are available

as multivariate regression techniques. For the purpose of this study, PLS1 was used to develop and evaluate the prediction performance of each calibration model. PLS is a bilinear regression modelling method where the original **x** variables are projected onto a smaller number of PLS components, also referred to as “factors”. The ***Y***- data matrix is incorporated into these calculations. After their generation, the FT-IR spectra and corresponding reference values were organised into calibration and validation samples sets as described in further sections.

FT-IR spectra of fermenting must samples collected for the purpose of this study were used as independent test sets to evaluate the global calibrations. The indicators used to evaluate the goodness of fit of the SA fermenting must samples on the global calibrations were the coefficient of determination (R^2), bias and standard error of prediction (SEP). These indicators were calculated as:

$$R^2 = 1 - \frac{s_{y.x}^2}{s_y^2}$$

where s^2_y is the standard deviation and $s^2_{y.x}$ is the root mean square error of prediction

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

where y_i is the reference value for the ith sample; $\hat{y}_i$ is the predicted value for the ith sample; n is the number of samples.

Bias gives an indication of a systematic error in the predictive values and it was calculated as the average of the difference between the reference values and the predicted values, also referred to as residuals (Esbensen, 2002; Naes, 2002). SEP describes the bias-corrected prediction error of the calibration model in relation to an independent validation set. The calculations of these indicators are standard statistical procedures and have been described by several authors (Esbensen, 2002; Naes, 2002).

Table 1: Descriptive statistics of the global calibration for fermenting must provided with the WineScan FT120

Model	PLS factors	n[a]	Mean (Min[b]-Max[c])	Reference method	Reference[d]
Ethanol %v/v	12	2813	11.50 (0.0-14.14)	Distillation, electronic density meter	Application note 157 P/N 1025295
Glucose + Fructose g/L	14	1494	5.66 (0.0-175.0)	Enzymatic	Application note 158 P/N 1025296
pH	14	2999	3.49 (2.73-4.10)	Potentiometer	Application note 160 P/N 1025298
Titratable acidity g/L	8	3142	4.43 (1.60-18.55)	Automatic titration with NaOH	Application note 162 P/N 1025301
Volatile acidity g/L	14	2589	0.38 (0.0-1.64)	Distillation, titration	Application note 163 P/N 1025302

[a]Number of samples; [b]Minimum; [c]Maximum; [d]Application notes for WineScan FT 120 P/N 1000823, Issue 2GB, Foss Analytical, Denmark

4.3.6.3 Development of new calibration models and wavenumber selection

The PLS1 (PLS method with only 1 *y*-variable) function on the WineScan Advanced Performance Software Module (Foss Analytical, Denmark, 2001; http://www.foss.dk) was used to establish new calibrations. Cross validation of calibration models was automatically done by

the software and involved keeping out successive groups of 10% of the total number of calibration samples at a time, and using these subsets for calculating the calibration error, standard error of cross validation (SECV), until all samples have been kept out of the calculation once (Foss Analytical, Denmark; Nieuwoudt *et al.*, 2004).

By default, 15 filters (wavenumbers or small groups of adjacent wavenumbers) were automatically selected by the software (Table 2). These wavenumbers are selected to collectively capture the maximum variation in the concentrations of the analyte, or *y*-variable, under investigation (WineScan Reference Manual, Foss Analytical Denmark). The Advanced Performance software only allows the following regions to be available for wavenumber selection: 964 - 1562 cm^{-1}, 1716 - 1813 cm^{-1} and 2700 - 2970 cm^{-1}. Absorbance in the regions 1543 – 1716 cm^{-1} and 2970 – 3626 cm^{-1} is due to water (Patz *et al.*, 2004) and contributes to significant noise in the spectra. The software allows slope and/or intercept adjustments of the commercial calibrations, as one strategy to improve their prediction performance.

Table 2: An example of the selection of filters by the WineSan Advanced Performance Software Module (Foss Analytical, Denmark, 2001; http://www.foss.dk) (Own data).

Number of filter	From wavenumber	To wavenumber	% variance
1	1168	1172	93.29
2	1003	1006	95.21
3	1728	1728	95.41
4	1527	1527	95.64
5	1199	1199	95.87
6	1226	1226	96.40
7	1381	1385	96.55
8	1724	1724	96.62
9	1288	1300	96.65
10	1130	1130	96.77
11	2897	2901	96.84
12	1477	1481	96.91
13	1539	1539	97.09
14	995	995	97.20
15	1161	1161	97.24

4.3.6.4 Evaluation of the performance of the calibration models

For the evaluation of the new calibration models, the reference sample set for each compound was divided into a calibration and validation set containing 70% and 30% of the samples respectively. The maximum and minimum values in each range were always in the calibration sets. The statistical indicators for evaluating the accuracy of the predictive abilities of the new calibration models included bias, R^2, and the prediction errors, SECV when based on the calibration sample sets, and SEP when based on independent validation sample sets. SECV describes the predictive accuracy of the calibration model in relation to the reference data and the equation used for the calculation of SECV was:

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

where y_i is the reference value for the ith sample; $\hat{y}_i$ is the predicted value for the ith sample; *n* is the number of samples.

The indicators R^2, SECV*SEL, SEP: SDD and residual predictive deviation (RPD) were used as a means to interpret the prediction error of the calibration models. RPD is defined as the ratio of the standard deviation of the reference values to the standard error of the predicted values when using independent test set validation (Williams, 1995; Esbensen, 2002). It has been proposed that an RPD value of less than three is an indication that the calibration model is unsuitable for accurate quantification, a value between three and five indicates that the model is suitable for screening and a value greater than five indicates that the model is suitable for excellent quantification (Williams, 1995). The disadvantage of the RPD criterion is that the standard deviation, which is part of the calculation, is influenced by the concentration range of the sample set. Therefore, alternative criteria were also used in this study to interpret the prediction errors of the calibration models. One such a criterion is the value of SECV*SEL and it has been proposed that a SECV value lower than 1.5 times the laboratory error, SEL, indicates excellent precision, while a value between 2 to 3 times SEL indicates good precision (Shenk and Westerhaus, 1996). Another criterion of evaluation was the ratio of SEP and SDD (standard deviation of difference), and SEP smaller than 2 times SDD proved it fit for quantification purposes (Esbensen, 2002). A summary of the interpretation of the performance indicators is given in Table 3.

Table 3: Different criteria used to evaluate the performance of the calibration models.

Parameter	Excellent quantification	Good quantification	Good for screening	Unsuitable for quantification
R^{2a}	>0.9	0.7 – 0.9	0.5 – 0.7	<0.5
SECV*SEL[b]	<1.5	2 – 3	n/a	n/a
SEP:SDD[c]	<2	<2	n/a	n/a
RPD[d]	>5	>5	3 - 5	<3

[a]Coefficient of determination; [b]Standard error of cross validation*standard error of laboratory; [c]Standard error of prediction:standard deviation of difference; [d]Residual predictive deviation

4.3.6.5 Validation of the reference methods

The standard error of laboratory (SEL) and an in-house validation procedure for each parameter were used to validate the performance of the appropriate reference methods, as well as to set a internal laboratory target for precision of the laboratory measurements. This target was also used to evaluate the Winescan calibrations. Glucose, fructose, glucose plus fructose and YAN were not included in the normal reference analysis performed by the laboratory, therefore no internal targets were established for these parameters, to date.

Table 4 : Summary of the standard error of laboratory (SEL) and internal laboratory target of each parameter to validate the performance of the reference methods.

Parameters	SEL	Internal target
Ethano %v/vl	0.04	0.20
pH	0.01	0.10
VA g/L	0.04	0.05
TA g/L	0.14	0.30
Glucose g/L	0.25	n/a
Fructose g/L	0.23	n/a
Glucose + fructose g/L	0.20	n/a
YAN (enzymatic) mg/L	0.53	n/a
YAN (Formol) mg/L	0.45	n/a

4.4 RESULTS AND DISCUSSION

4.4.1 FERMENTING MUST SAMPLES

In this study, red- and white fermenting must samples were selected to be representative of the major red cultivars (Shiraz, Merlot, Pinotage, and Cabernet Sauvignon) and white cultivars (Chardonnay, Chenin blanc, Colombar, and Sauvignon blanc) produced in the Vredendal area. The number of fermenting must samples from Cabernet Franc, Malbec, Petit Verdot, Muscat d' Alexandrie (Hanepoot), Nouvelle and Ruby Cabernet each made out ~5% of the total number and reflect the typical annual intake of these cultivars at Vredendal- and Spruitdrift Wineries. White blends, Rosé and Extra Light products, all different wine styles produced at the wineries, were also included in the sample set to try and achieve maximum representivity. Fermenting must samples were collected at different stages of the fermentation process to include the widest possible concentration range in the calibration sample set and the major changes in the fermenting matrix profiles. Fermenting must samples were selected from cultivars originating from regions with different climatic conditions, since distinct wine flavour characteristics are associated with the respective regions. For example, in Sauvignon blanc wine produced from grapes originating from the Lutzville area, more grassy flavours are typically perceived, while more fruity flavours are characteristic of white wine produced from grapes originating from the inland areas. The Lutzville area is situated 30 - 50 km from the wineries, and ~5 km from the cold Atlantic Ocean. Day temperatures can be as high as 45ºC and night temperatures as low as 15ºC during harvest season. The inland Olifants River Valley producing areas are on average warmer than the Lutzville area. The selection of samples were important, in order to build robust calibration models.

The descriptive statistics (minimum, maximum, mean and standard deviation) for alcohol, pH, VA, TA, glucose plus fructose, glucose, fructose and YAN are shown in Table 5. The number of samples in Table 5 represents the amount of samples that were analysed during the study. The samples that were used to develop the calibration models exclude the outlier samples.

Table 5: Descriptive statistics of fermenting must samples collected during vintages 2007 to 2009 used to evaluate the global WineScan calibrations and to establish new calibration models.

Parameters	No. of samples (white;red)[a]	Min.[b]-Max[c]	Mean	SD[d]	SDD[e]	SEL[f]
Ethanol %v/v	322(205;117)	0.00-15.11	8.90	4.45	0.06	0.04
pH	394(243;151)	3.16-3.99	3.55	0.15	0.03	0.01
VA g/L[g]	282(191;91)	0.04-1.74	0.32	0.24	0.05	0.04
TA g/L[h]	351(200;151)	3.48-9.59	6.87	0.79	0.54	0.14
Glucose g/L	465(352;113)	0.06-141.30	34.99	40.32	2.79	0.25
Fructose g/L	465(352,113)	2.89-154.56	57.17	41.39	1.94	0.23
Gluc.+fruct. g/L[i]	465(352;113)	3.00-295.86	92.16	80.66	3.36	0.20
YAN[j] mg/L	471(471;0)	16.88-352.52	90.47	77.69	3.08	0.53
YAN (Formol)[k] mg/L	168(127;41)	49.89-381.64	138.14	60.93	n/a	0.45

[a]Sample number (white fermenting must; red fermenting must); [b]Minimum; [c]Maximum; [d]Standard deviation; [e]Standard deviation of difference; [f]Standard error of laboratory; [g]VA expressed as acetic acid; [h]TA expressed as tartaric acid; [i]Total glucose + fructose; [j]Yeast assimilable nitrogen (enzymatic); [k]Yeast assimilable nitrogen (Formol titration method)

4.4.2 FT-IR SPECTRA

Figure 1 shows an example of mid-infrared spectra of fermenting must at different stages of alcoholic fermentation from grape juice (Day 0) to dry wine (Day 8). A normal red wine alcoholic fermentation at Spruitdrift Winery usually takes between 5 - 7 days for completion, depending on the influence of external factors such as fermentation temperature. The duration of a normal white wine fermentation at Vredendal Winery, is usually between 10 - 14 days. Distinct variation between the FT-IR spectra of the fermenting must at different stages of fermentation was observed. Given that grape juice, fermenting must and finished wine are complex mixtures of components sharing the same functional groups (e.g. C-O, C-H); it is difficult to assign spectra peaks to specific compounds. It is reasonable however to presume that the largest peaks represent chemical bond vibrations of the most abundant components in the respective mixture (Urtubia *et al*., 2008). The C-O stretch of primary alcohols is found at 1050 cm^{-1} while the contribution of the C-H stretch of ethanol occurs in the region 2850 – 2960 cm^{-1} (Bevin *et al*., 2006; 2008). Peaks associated with sugars are the C-O stretch for fructose at 1060 cm^{-1} and glucose at 1030 cm^{-1} respectively (Bevin *et al* ., 2006; 2008). The peaks at 900 – 1100 cm^{-1} include absorption bands characteristic of carbohydrates and alcohols. As the fermentation progresses the strong initial carbohydrate peaks decrease and the ethanol peak increase in the 900 – 1100 cm^{-1} region and, at the end of the fermentation, the ethanol absorption band dominates in the region 900 – 1100 cm^{-1} (Figure 1).

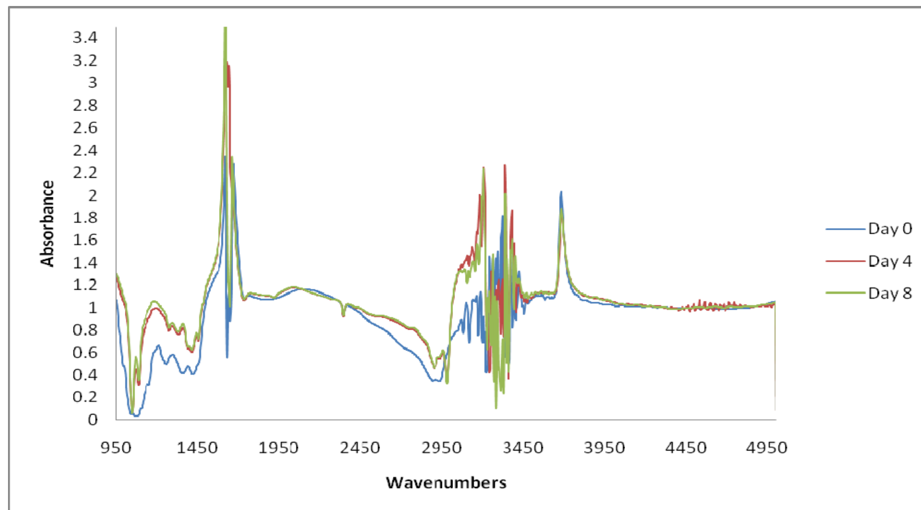

Figure 1 An example of mid-infrared spectra of fermenting grape must of Merlot at different stages of alcoholic fermentation. Distinct variation between the FT-IR spectra of the fermenting must at different stages of fermentation was observed. Grape juice (Day 0), Mid fermentation (Day 4) and Finished wine (Day 8).

Although the whole spectral range (5012 - 926 cm^{-1}) was stored for each sample, only the following areas were used for wavenumber selection: 1543 - 956 cm^{-1}, 2280 - 1717 cm^{-1}, and 2971 - 2435 cm^{-1}. The other ranges of frequencies were eliminated to prevent noise in the calculation. The two regions 1717 - 1543 cm^{-1} and 3627 - 2791 cm^{-1} are strong water absorption bands that prevent any energy from passing through the cuvette, whereas the region 5012 - 3627 cm^{-1} contains very little useful information as mentioned before (Patz *et al*., 2004).

4.4.3 PRINCIPAL COMPONENT ANALYSIS (PCA)

4.4.3.1 Discrimination within red- and white cultivars

PCA was performed on the FT-IR spectra of grape juice sampled prior to alcoholic fermentation, to get an overview of the data structure and to investigate possible effects of fermentation stage, cultivar or vintage. These factors are all important considerations in the design of calibration sets. All red- and white grape juice samples were included for the purpose of the analysis. The red cultivars (Pinotage, Merlot, Petit Verdot, Cabernet Sauvignon, and Malbec), white cultivars and wine styles (Chardonnay, Colombar and Chenin blanc, and Extra Light) were included for the purpose of the analysis. The grapes were harvested at Namaqua Wines at cultivar-specific ripeness levels. Some separation between the white- and red cultivars could be observed (Figure 2). Within the red grape juice, no clear separation was seen between the various cultivars. PC1 explained 92% of the variance in the sample set and clearly described the sugar content of the samples. The white cultivars tended to locate towards the negative end of PC1 and the red cultivars towards the positive side of PC1. Sugar levels of white grape juice were between 22 - 24ºBrix, with the exceptions of the Extra Light samples (21ºBrix) and Chardonnay samples (27ºBrix). Sugar levels of red grape juice were between 26 - 30ºBrix. Most white cultivars were harvested at sugar levels between 21 - 23ºBrix and red cultivars were harvested at sugar levels between 25 - 28ºBrix. PC2 could be interpreted in terms of the TA content of the samples. The Pinotage, Extra Light and Chenin Blanc grape juices had very high TA contents (7.5 - 8.5 g/L).

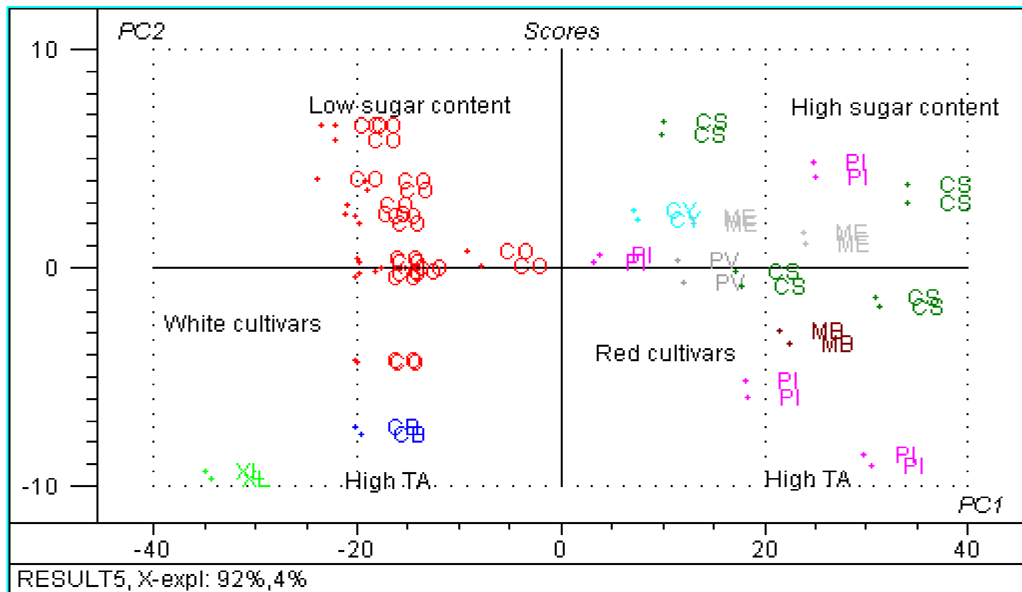

Figure 2 PCA scores plot (PC1 vs. PC2) of FT-IR spectra of grape juice samples. White grape juice: XL=Extra Light; CY=Chardonnay; CB=Chenin blanc; CO=Colombar. Red grape juice: ME=Merlot; CS=Cabernet Sauvignon; PI=Pinotage; MB=Malbec; PV=Petit Verdot. PC1 explained 92% of the variance in the sample set, and PC2 4%. Some clear cultivar groupings could be observed. The samples used were taken before the fermentation process.

Within the white grape juices some clustering was seen in Colombar and Chardonnay (Figure 3). As before, separation of samples along PC1 could be interpreted on the basis of the sugar content of the grape juices. Samples locating towards the far right end of PC1 had sugar levels of 26 - 27ºBrix, and those to the far left of PC1, had sugar levels of 16 - 21ºBrix. One Colombar sample located at the far positive side of PC2 and it could be interpreted on the basis of the high TA content (8.5 g/L).

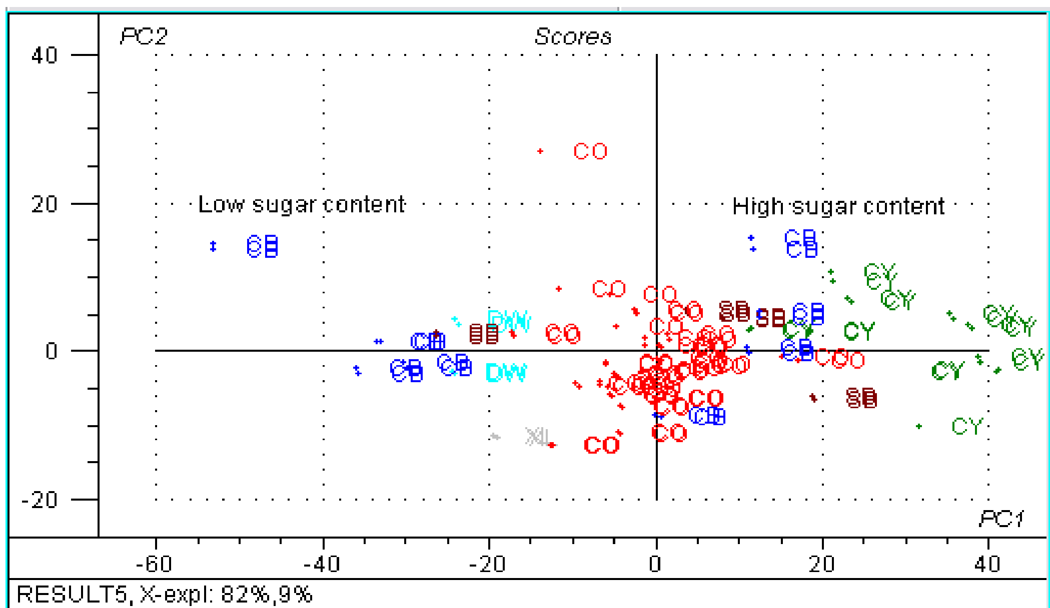

Figure 3 PCA score plot (PC1 vs. PC2) of white grape juice samples (CY=Chardonnay; DW=Dry White blends; CB=Chenin blanc; CO=Colombar; XL=Extra Light; SB=Sauvignon blanc). PC1 explained 82% of the variance between the samples and PC2 9%. The samples used were taken before the fermentation process.

No clear separation according to vintage was observed as shown for the white cultivars (Figure 4). Colombar and Chardonnay cultivars are annually harvested at more or less the same sugar levels. The Chenin blanc of 2007 was harvested at a higher sugar level and the Chenin blanc of 2008 was harvested at very low sugar levels to obtain a different wine style for each vintage.

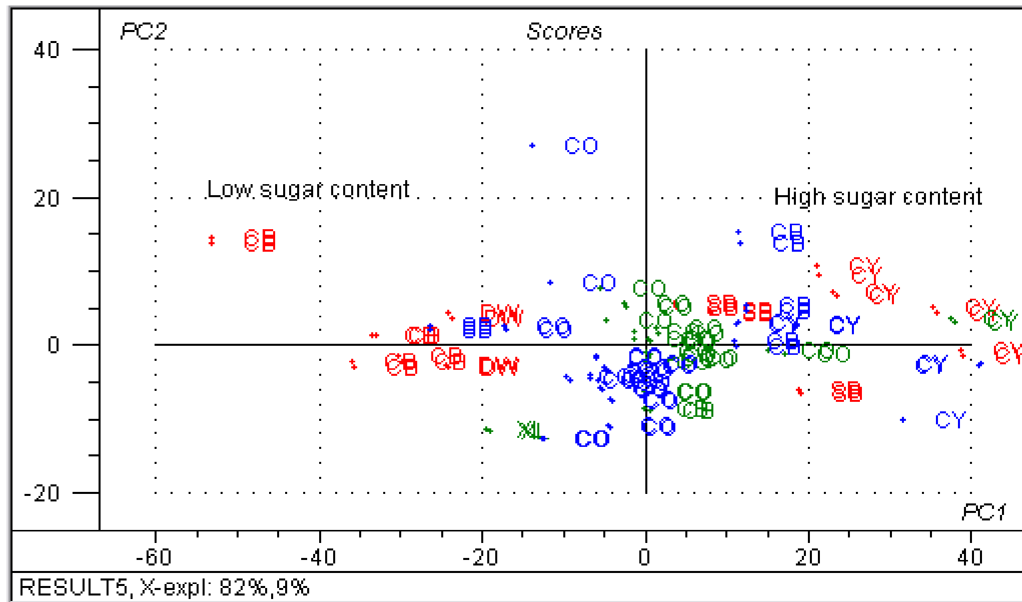

Figure 4 PCA score plot (PC1 vs. PC2) of white grape juice samples of the 2007, 2008 and 2009 vintages. PC1 explained 82% of the variance between the samples and PC2 9%. The samples used were taken before the fermentation process.

4.4.4 EFFECTS OF SAMPLE FREEZING ON FT-IR SPECTRA

During 2007, 81 fermenting must samples were collected at different stages of alcoholic fermentation that could not be analysed immediately after collection, and these samples were stored frozen at -20°C until they were analysed 3 months later. The aim was to use these samples to extend and optimise the calibration models for ethanol, pH, TA, VA, glucose, fructose and glucose plus fructose where possible. PCA was used to compare the FT-IR spectra of corresponding fresh and frozen sample pairs (Figure 5). A shift in position on the resulting scores plots, between paired normal (fresh) and frozen samples was seen for many of the observations, although the distances of the shifts were mostly small (indicated by the length of the lines linking the members of the pair), except for two samples where the lines linking the sample pairs were notably longer than the other pairs (Figure 5). These samples were poorly predicted when included in calibration sample sets for all mentioned parameters and were considered as outlier samples. Their outlier status was confirmed by the X-Y relation outlier plots that were used to detect outlier samples for each parameter. It is possible that the two outlier samples were negatively affected by freezing. The PCA results confirmed that there were no major differences between the normal and frozen samples and that fermenting must samples can definitely be frozen for at least up to 3 months to expand the calibration models for parameters mentioned above. These results were in accordance to those reported for total soluble solids and pH determination in frozen Riesling and Chardonnay grape samples that were analysed by NIR spectroscopy (Cynkar *et al.*, 2009).

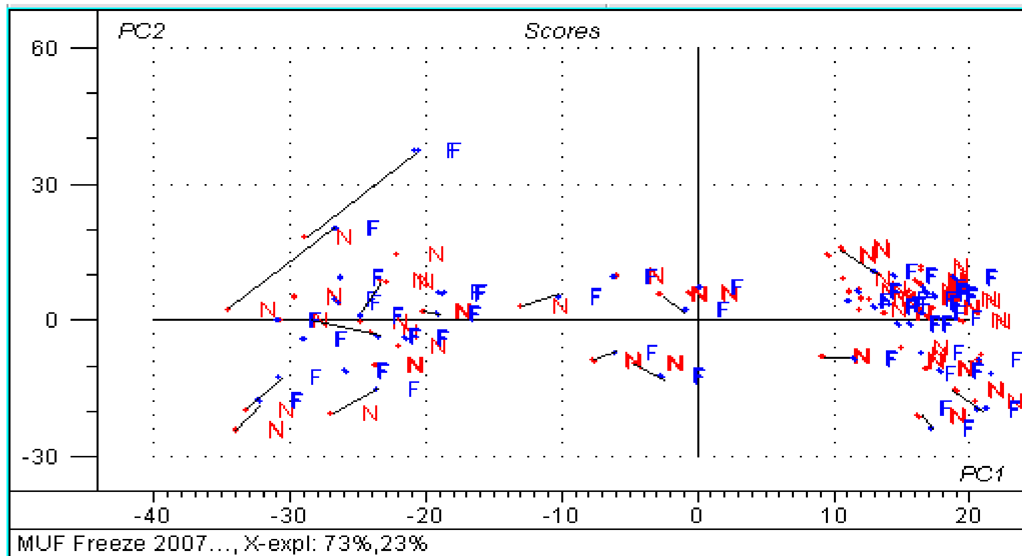

Figure 5 PCA score plot of duplicate FT-IR spectra of freshly analysed (referred to as nomal) and frozen fermenting must samples. The black lines linking samples refer to the shift in position on the scores plot of the same samples, frozen (F) and normal (N). PC1 explained 73% of variation in the sample set and PC2 23%.

4.4.5 EVALUATION OF QUANTITATIVE CALIBRATION MODELS

4.4.5.1 Evaluation of global and new ethanol calibration models.

For the validation of the global ethanol calibration model 322 red- and white fermenting must samples were used (Table 5). The value range of the sample set was between 0-15.11 %v/v. The bias of the validation was -0.21 %v/v and the coefficient of determination (R^2) 0.9980 (Table 6). The standard error of prediction (SEP) for ethanol was 0.199 %v/v, indicating a high accuracy in relation to the independent set (Figure 6A). A total of 4.3% of the samples had a prediction error larger than 0.5 %v/v and 1% of the samples had a prediction error larger than 1.0 %v/v. A total of 2.8% of the samples that were predicted larger than 0.5 %v/v were between 0-10 %v/v. Part of the reference method is to remove all the CO_2 in the fermenting must during sample preparation for accurate results and it is not always possible to remove it all, especially if the fermenting must is still in the active phase of fermentation (ethanol <10 %v/v), therefore CO_2 in the fermenting must might lead to inaccurate results which could be the reason for the large prediction error.

The parameter range (0 – 15.11 %v/v) for ethanol for the fermenting must samples fell outside the calibration range (0 – 14.14 %v/v) of the global WineScan FT 120 calibration model. Therefore it was necessary to develop a new calibration model for ethanol to include the samples that fell outside the global calibration range. For the new ethanol calibration model, the sample set (n = 316) was divided in a calibration set (n = 211) and an independent validation set (n = 105). Wavenumbers 1153 – 1157cm^{-1} were selected as the first filters that explained 93.2% of the variance between the samples for ethanol content. Peaks near 1157 cm^{-1} are largely due to the –CH groups in the alcohol component. The contribution to the C-H stretch from 2850 – 2960 cm^{-1} from ethanol can also be observed (Bevin *et al*., 2006; 2008). The new calibration model performed very well and a smaller SEP (0.15 %v/v) and bias (0.04 %v/v) were obtained (Figure 6B; Table 7). A total of 10 factors had shown the lowest SEC (0.19 %v/v), indicating the accuracy of the predictive ability of the calibration model. A total of 99.8%

variance between samples were explained by 15 PLS filters. The RPD value was 30 and R^2 was 0.999 which indicate an excellent calibration model for quantification purposes.

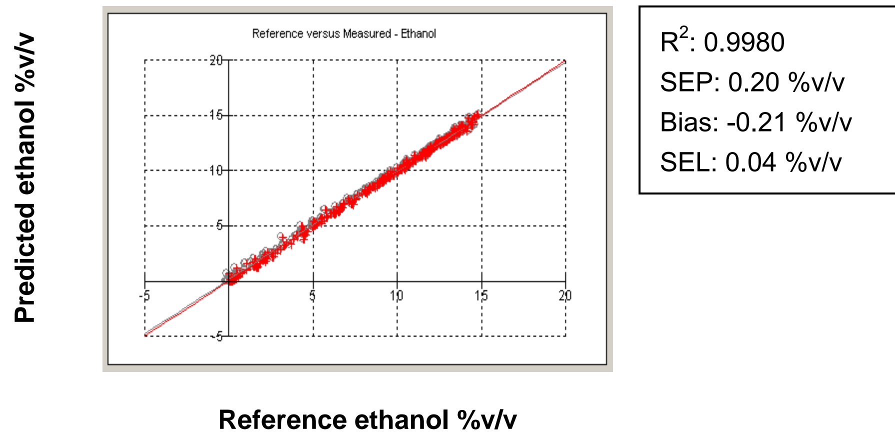

Figure 6A Plot of FT-IR predicted values for alcohol using global WineScan calibration vs. reference alcohol values.

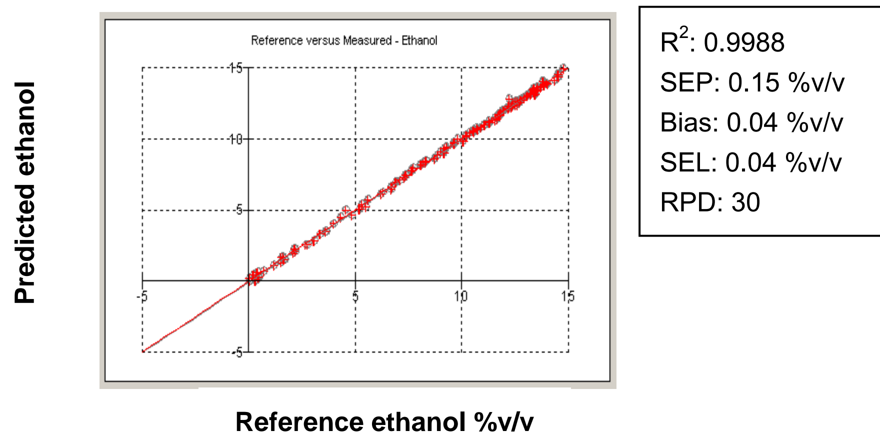

Figure 6B Plot of FT-IR predicted values for new ethanol calibration vs. reference ethanol values.

4.4.5.2 Evaluation of global and new pH calibration models.

A total of 394 red- and white fermenting must samples were used for the validation of the global pH parameter (Table 5). Validation statistics of the data generated with the unadjusted global calibration showed a small bias and SEP, but also a low R^2 for pH (bias = 0.03, SEP = 0.09, R^2 = 0.6676); (Table 6). The R^2 value of 0.6676 indicates that the global calibration was not suitable to quantify pH in fermenting must, but for screening purposes, although the bias of 0.03 and SEP of 0.09 were very small. The regression plot for reference pH values vs. predicted values showed a very poor fit between the true regression line (red) and the target regression line (grey) (Figure 7A). To improve the prediction of pH and the accuracy of the model a new calibration model was developed.

For the new pH calibration model, the sample set (n = 383) was divided in a calibration set (n = 256) and an independent validation set (n = 127). A total of 12 factors obtained the lowest SEC of 0.043. The bias of the new calibration model was much smaller (-0.004), the R^2 equal to 0.9225 and the SEP was 0.042 (Figure 7B; Table 7). A total of 3.3% of the samples were

predicted larger than 0.1. Wavenumber 1450 cm^{-1}, representing the first filter, explained 93% of the variance for pH between samples. The peaks near 1504 cm^{-1} are due to the –CH groups associated with the acid components (Bevin *et al.*, 2006; 2008). The absorption zone between 1200 cm^{-1} and 1500 cm^{-1} contains vibrations of several groups found in organic acids (Bevin *et al.*, 2006). The prediction error was large relative to the standard deviation in the sample set, resulting in a RPD value of 4. The value of 4 indicates that the model is suitable for screening purposes. The standard deviation which is part of the RPD calculation is influenced by the concentration range. The sample set covers a relatively small range of pH values and it could be considered the reason for the poor performance. Nevertheless, the model can be considered fit for the quantification of pH in fermenting must, if the R^2 of 0.9225 is used as criterion to evaluate the performance of the model. The SEP is also smaller than 2 times SDD which proved it fit for quantification purposes.

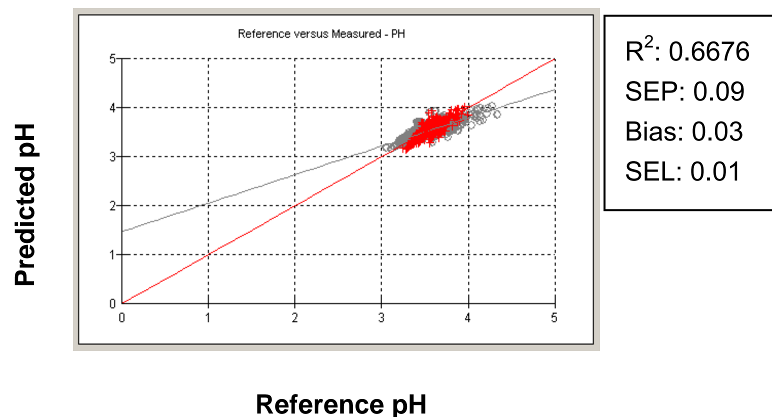

Figure 7A Plot of FT-IR predicted values for pH using the global Winescan calibrations vs. reference pH values. The grey line (circles) represents the target line of the global calibration and the red line (crosses) the proposed line for the adjusted calibration.

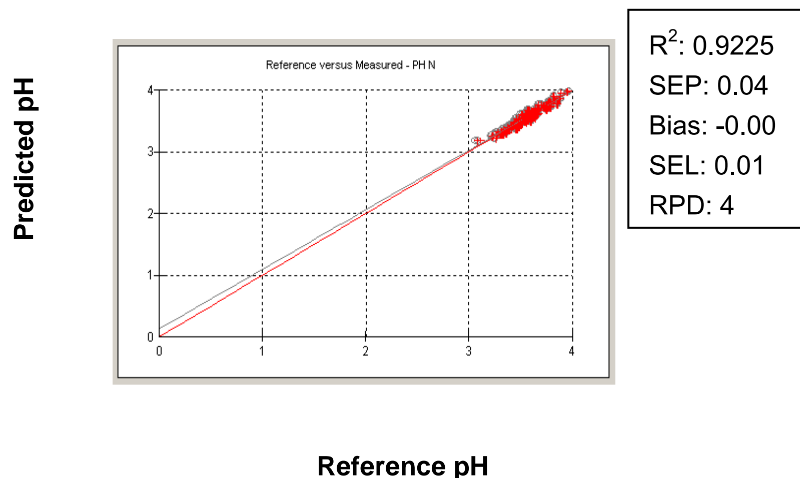

Figure 7B Plot of FT-IR predicted values for new pH calibration vs. reference pH values.

4.4.5.3 Evaluation of global and new VA calibration models.

For the validation of the global VA model 282 red- and white fermenting must samples were used (Table 5). Validation statistics of the data generated with the global calibration showed a small bias and SEP, but also a high R^2 for VA (bias = -0.10 g/L, SEP = 0.09 g/L, R^2 = 0.8909); (Table 6). The high R^2 value indicates that the global calibration was suitable to quantify VA in fermenting must. In addition, the bias was also very low. The regression plot for reference VA

values vs. predicted values showed a slight deviation between the true regression line (red) and the target regression line (grey) (Figure 8A). The parameter range (0.04 – 1.74 g/L) for VA for the fermenting must samples fell outside the calibration range (0 – 1.64 g/L) of the global WineScan calibration model and a new calibration was develop to include the samples that fell outside the calibration range.

For the new VA calibration model, the sample set (n = 282) was divided in a calibration set (n = 181) and a validation set (n = 90). A total of 95% variance between samples were explained by 15 PLS filters. The lowest SEC (0.061 g/L) was obtained by 10 factors. The bias of the validation of the new calibration model was -0.006 g/L, the R^2 0.8937 and the SEP was 0.074 g/L (Figure 8B; Table 7). A total of 92% of the samples had a prediction error smaller than 0.1 g/L. Samples with reference VA values smaller than 0.1 g/L had very large prediction errors and it could be the result of excessive CO_2 in the fermenting must that was not removed properly and can lead to higher reference values (Payette, 2006). A better SEP and bias could be achieved with the new prediction model, however, a RPD value of 3 was obtained and it indicates that the model is only suitable for screening purposes, but it could also be considered suitable for quantification purposes, if the R^2 of 0.8937, bias of -0.01 g/L and SEP of 0.074 g/L are used as criteria to evaluate the performance of the model. The SEC value is 1.5 times SEL which indicates excellent precision and the SEP is smaller than 2 times SDD which also proved it fit for quantification.

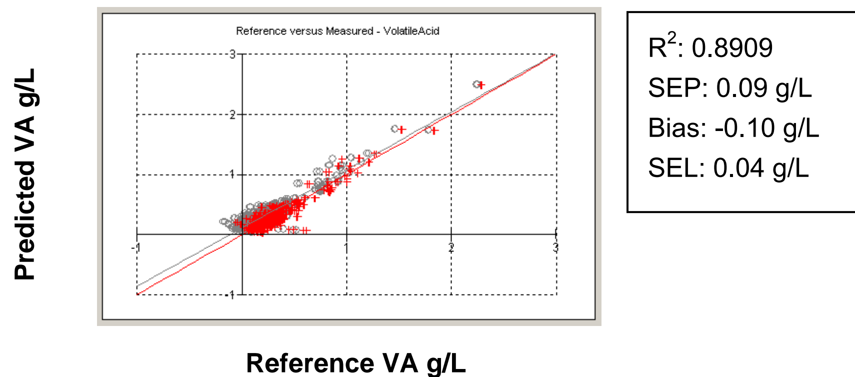

Figure 8A Plot of FT-IR predicted values for VA using the global WineScan calibrations before slope and/or intercept adjustments vs. reference VA values. The grey line (circles) represents the target line of the unadjusted global calibration and the red line (crosses) the proposed line for the adjusted calibration.

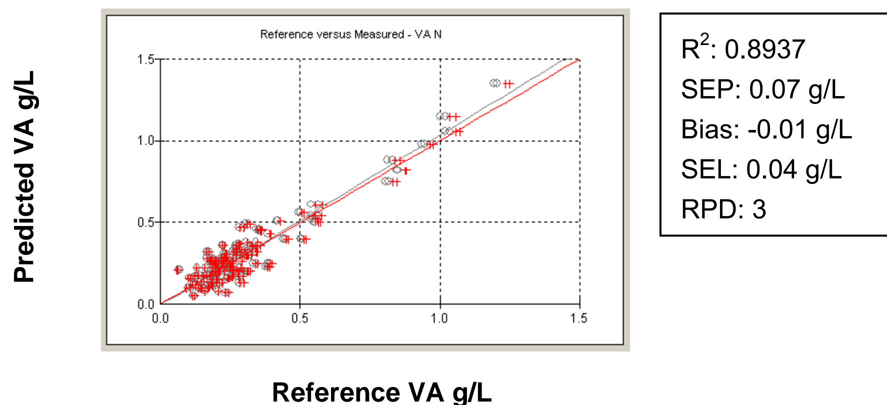

Figure 8B Plot of FT-IR predicted values for new VA calibration vs. reference VA values.

4.4.5.4 Evaluation of global and new TA calibration models.

A total of 351 red- and white fermenting must samples were used for the validation of the global TA parameter (Table 5). Validation statistics of the data generated with the unadjusted global calibration showed substantial systematic errors for TA (bias = -2.85 g/L, SEP = 0.68 g/L, R^2 = 0.4563); (Table 6). The bias (-2.85 g/L), indicating that the averages of the residuals (difference between reference and predicted values) of the samples were very high. It means that the regression statistics will give a high systematic error as evident by the bias. The low R^2 value (0.4563) also indicates that the model was not suitable for quantification of TA in fermenting must. The regression plot for reference TA values vs. predicted values showed a significant lack of fit between the target regression line (grey) and the true regression line (red) (Figure 9A). There was no correlation between the TA values determined with the reference method and the values predicted by the global calibration model. The model could not be used for either screening or quantification of TA in fermenting must due to the high prediction error (SEP), high bias and low R^2.

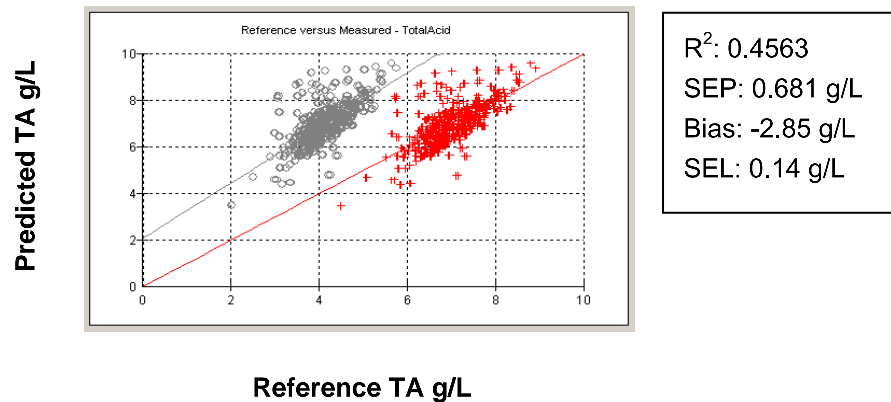

Figure 9A Plot of FT-IR predicted values for TA using the global WineScan calibrations before slope and/or intercept adjustments vs. reference TA values. The grey line (circles) represents the target line of the unadjusted global calibration and the red line (crosses) the proposed line for the adjusted calibration.

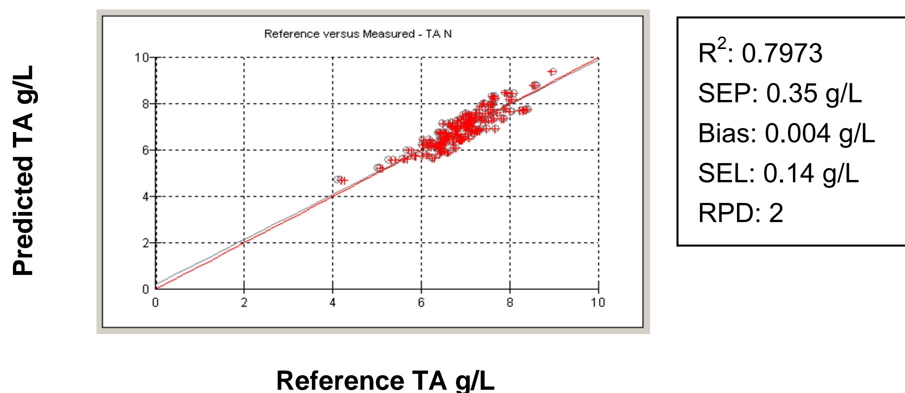

Figure 9B Plot of FT-IR predicted values for new TA calibration vs. reference TA values

A new calibration model for TA was therefore developed to improve the prediction capabilities of the model. For the new TA calibration model, the sample set (n = 351) was divided in a calibration set (n = 210) and a validation set (n = 105). A total of 82% variance between samples was explained by 15 PLS filters. Peaks between 1157 cm^{-1} and 1504 cm^{-1} are due to

the –CH groups in the acid components, while peaks at 1003 – 1099 cm^{-1} are due to the –COH groups (Bevin *et al*., 2006; 2008). The absorption zone between 1200 and 1500 cm^{-1} also contains groups found in organic acids (Bevin *et al*., 2006). The lowest SEC (0.38 g/L) for the calibration model (15 filters and 12 factors) was achieved. The bias of the validation of the new calibration model was -0.004 g/L, the R^2, 0.7973 and the SEP was 0.35 g/L (Figure 9B; Table 7). Samples with reference TA values larger than 8.0 g/L had very large prediction errors. A total of 84% of the samples had a prediction error smaller than 0.5 g/L and 93% of the samples had a prediction error smaller than 1.0 g/L. Unfortunately, a RPD value of 2 was obtained and it indicates that the model is unsuitable for quantification or screening purposes. Although the bias was small and the R^2 high, the model was characterised by a high prediction error (0.35 g/L) relative to the standard deviation (0.79 g/L) in the sample set, resulting in a RPD value much lower than 3. The SEC value is almost 3 times SEL which indicates good precision and with the low bias, high R^2 and a SEP that is smaller than 2 times SDD, the model can be considered fit for the quantification of TA in fermenting must.

4.4.5.5 Evaluation of global and new glucose plus fructose calibration models

A total of 465 red- and white fermenting must samples were used for the validation of the global glucose plus fructose model (Table 5). Validation statistics (Table 6) of the data generated with the unadjusted global calibration showed also substantial systematic errors for glucose plus fructose (bias = -7.1661 g/L, SEP = 17.041 g/L, R^2 = 0.9560). Despite the high R^2, the prediction error had to be decreased to ensure accurate quantitative determination of glucose plus fructose in fermenting must. The bias of the global calibration was also very high. These factors indicated that the calibration model was unsuitable for screening or quantification of glucose plus fructose in fermenting must. The regression plot for reference glucose plus fructose values vs. predicted values showed a slight deviation between the true regression line (red) and the target regression line (grey) (Figure 10A). The parameter range (3.0 – 295.9 g/L) for glucose plus fructose for the fermenting must samples also fell outside the calibration range (0 – 175.0 g/L) of the global WineScan FT 120 calibration model.

A new calibration model for glucose plus fructose was developed to include the samples not part of the global calibration. For the new glucose plus fructose calibration model, the sample set (n = 465) was divided in a calibration set (n = 256) and a validation set (n = 127). Wavenumber 1168 cm^{-1} was selected as the first filter that explained 96% of the variance between the samples for glucose plus fructose. The region associated with sugars is the C-O stretch for fructose at 1060 cm^{-1} and glucose at 1030 cm^{-1} (Pistorius, 1995; Bevin *et al*., 2008). According to literature (Foyolle *et al*., 1996; Sivakesava *et al*., 2001) the bands 1036 cm^{-1}, 1065 cm^{-1}, 1080 cm^{-1}, 1104 cm^{-1} and 1152 cm^{-1} correspond to glucose and fructose in fruit juice, fermenting must and wine. A total of 10 factors had shown the lowest SEC (5.617 g/L). The bias of the validation of the new calibration model was 0.018 g/L, the R^2 0.9928 and the SEP was 6.19 g/L (Figure 10B; Table 7). A total of 86% of the samples had a prediction error smaller than 10.0 g/L. Samples with reference glucose plus fructose values larger than 200.0 g/L had very large prediction errors (>5 g/L) and it could be the result of a possible error in the dilution series that were used to obtain the results. A better SEP, R^2 and bias could be achieved with the new prediction model. A RPD value of 13 was obtained and it indicates that the model is suitable for quantification purposes. The bias, SEP and R^2 all indicated that the new calibration model was suitable to quantify glucose plus fructose in fermenting must.

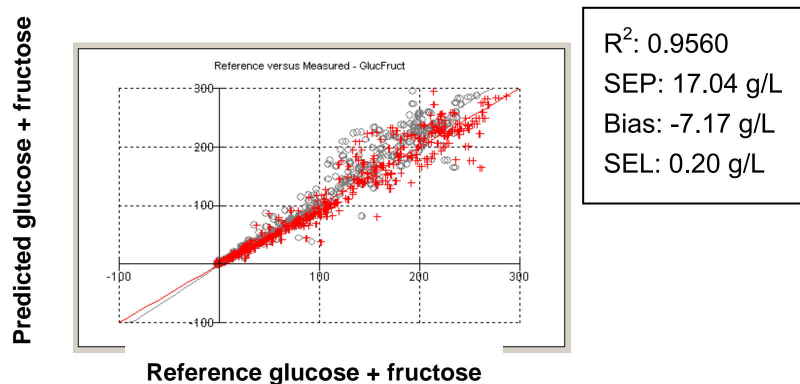

Figure 10A. Plot of FT-IR predicted values for glucose + fructose using the global WineScan calibrations before slope and/or intercept adjustments vs. reference glucose + fructose values. The grey line (circles) represents the target line of the unadjusted global calibration and the red line (crosses) the proposed line for the adjusted calibration

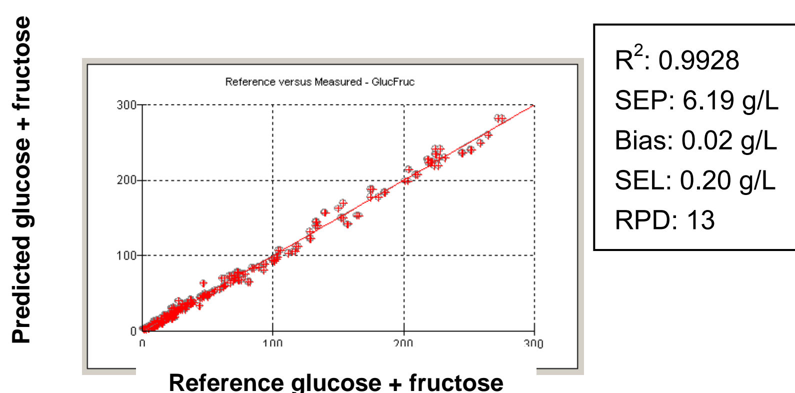

Figure 10B Plot of FT-IR predicted values for new glucose + fructose calibration vs. reference values.

Table 6: The validation statistics of the performance of the global WineScan calibrations using white- and red fermenting must as independent test sets.

Parameter	SEP[a]	bias	R^{2b}
Ethanol %v/v	0.19	-0.21	0.9980
pH	0.09	0.03	0.6676
VA[c] g/L	0.09	-0.10	0.8909
TA[d] g/L	0.68	-2.85	0.4563
Glucose + fructose[e] g/L	17.04	-7.17	0.9560

[a]Standard error of prediction; [b]Coefficient of correlation; [c]Volatile acidity expressed as acetic acid; [d]Titratable acidity expressed as tartaric acid; [e]Total glucose plus fructose

Table 7: Summary of the calibration and validation statistics for the evaluation of the new fermenting must calibrations of pH, VA, TA, glucose + fructose, glucose, fructose and YAN

	Calibration statistics			Validation statistics					
Model	**N[a]**	**PLS[b] factors**	**SEC[c]**	**Sample no.**	**SEL[d]**	**SEP[e]**	**R^{2}[f]**	**bias**	**RPD[g]**
Ethanol %v/v	211	10	0.19	105	0.04	0.15	0.9988	0.04	30
pH	256	12	0.04	127	0.01	0.04	0.9225	-0.01	4
VA[h] g/L	181	10	0.06	90	0.04	0.07	0.8937	-0.01	3
TA[i] g/L	210	12	0.38	105	0.14	0.35	0.7973	-0.004	2
Gluc+fruc[j] g/L	256	10	5.62	127	0.20	6.19	0.9928	0.02	13
Glucose g/L	294	15	4.74	146	0.25	4.88	0.9847	-0.31	8
Fructose g/L	265	11	3.99	132	0.23	4.14	0.9889	0.64	10
YAN[k] mg/L	172	11	14.97	85	0.53	14.10	0.9097	-2.55	6
YAN_F[l] mg/L	113	10	19.87	55	0.45	16.37	0.9117	-1.010	4

[a]Number of samples; [b]Partial least square factors; [c]Standard error of calibration; [d]Standard error of laboratory; [e]Standard error of prediction; [f]Coefficient of determination; [g]Residual predictive deviation; [h]Volatile acidity expressed as acetic acid; [i]Titratable acidity expressed as tartaric acid; [j]Total glucose plus fructose; [k]Yeast assimilable nitrogen; [l]Yeast assimilable nitrogen Formol titration

4.4.6 ESTABLISHMENT OF QUANTITATIVE CALIBRATION MODELS FOR GLUCOSE, FRUCTOSE AND YEAST ASSIMILABLE NITROGEN (YAN).

Several compounds play a key role in problematic wine fermentations, such as sugars (glucose, fructose) and nitrogen substrates (yeast assimilable nitrogen) and therefore glucose, fructose and nitrogen levels need to be measured in wine during alcoholic fermentation to monitor the process and to identify possible problematic fermentations (Urtubia *et al*., 2004). Calibration models for glucose, fructose and YAN are not part of the WineScan FT 120 software. New calibration models were established for the determination of glucose, fructose and YAN in fermenting must. Each calibration model was established using PLS1-regression and validated with an independent validation set. Results are shown in Table 7.

4.4.6.1 New glucose calibration model

The new calibration model for glucose performed very well. The descriptive statistics of the fermenting must used to establish the new calibration model are shown in Table 5 (min: 0.06 g/L; max: 141.30 g/L; mean: 34.99 g/L; SD: 40.32 g/L). For the new glucose calibration model, the sample set (n = 440) was divided in a calibration set (n = 294) and a validation set (n = 146). Wavenumber 1168 cm^{-1} was selected as the first filter that explained 91% of the variance between the samples for glucose. The region associated with glucose is the C-O stretch at 1030 cm^{-1} (Pistorius, 1995; Bevin *et al*., 2008). A total of 15 factors had shown the lowest SEC (4.739 g/L). The bias of the validation of the new calibration model was -0.306 g/L, the R^2 0.9847 and the SEP was 4.88 g/L (Figure 11; Table 7). A total of 91% of the samples had a prediction error smaller than 10.0 g/L. Samples with reference glucose values larger than 100.0 g/L had very large prediction errors (>5 g/L) and it could be related to interferences with the reference method. A RPD value of 8 was obtained and it indicates that the model is suitable for quantification purposes. A SEP smaller than 2 times SDD prove it fit for quantification of glucose in fermenting must.

Figure 11 Plot of FT-IR predicted values for new glucose calibration vs. reference values.

4.4.6.2 New fructose calibration model

The new calibration model for fructose also performed very well. The descriptive statistics of the fermenting must are: min: 2.89 g/L; max: 154.56 g/L; mean: 57.17 g/L; SD: 41.32 g/L (Table 5). The sample set (n = 397) was divided in a calibration set (n = 265) and a validation set (n = 132) for the new fructose calibration model. A total of 11 factors had showed the lowest SEC (3.989 g/L). The bias of the validation of the new calibration model was 0.638 g/L, the R^2 was 0.9889 and the SEP was 4.135 g/L (Figure 12; Table 7). Wavenumber 1168 cm^{-1} was also selected as the first filter that explained 95% of the variance between the samples for fructose. The region associated with fructose is the C-O stretch at 1060 cm^{-1} (Pistorius, 1995; Bevin *et al*., 2008). A total of 85% of the samples had a prediction error smaller than 10.0 g/L. Samples with reference fructose values larger than 100.0 g/L had very large prediction errors (>5 g/L). A RPD value of 10 was obtained and it indicates that the model is suitable for quantification purposes. Factors such as a low R^2 (0.9889), small bias (0.638 g/L) and low prediction error (4.135 g/L) is also an indication that the model is suitable to quantify fructose in fermenting must.

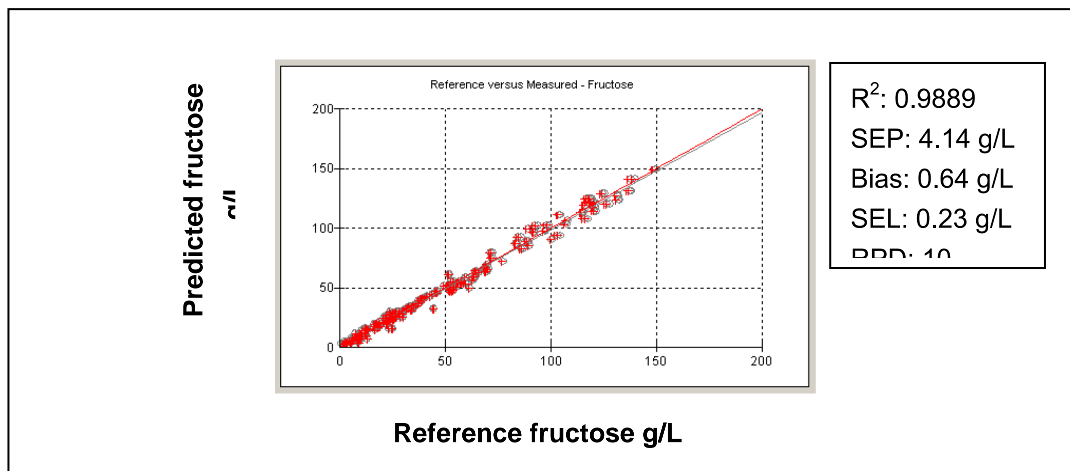

Figure 12 Plot of FT-IR predicted values for new fructose calibration vs. reference values.

4.4.6.3 New yeast assimilable nitrogen (YAN) calibration models using different methods. The new calibration model for YAN using enzyme-linked assay as reference method did not perform so well with red- and white fermenting must and only white fermenting must were used to develop the first model. The prediction of red fermenting must showed extremely large errors between the reference values and the predicted values. A total of only 55% variance between samples was explained when using both red- and white fermenting must. The reason for the poor performance might be the sensitivity of the enzyme-linked assay. A few sample preparation steps were needed for the method. The decolourisation of the red fermenting must could also play a role in the poor performance of the model. PVPP was used, but not all the colour could be removed in one step, therefore several steps were needed before using the fermenting must for testing. The descriptive statistics of the fermenting white must used to establish the new calibration model are shown in Table 5. For the new YAN calibration model, the sample set (n = 257) was divided in a calibration set (n = 172) and a validation set (n = 85). A total of 86% variance between samples was explained by 15 PLS filters. A total of 11 factors had shown the lowest SEC (14.967 mg/L). The bias of the validation of the new calibration model was -2.546 mg/L, the R^2 0.9097 and the SEP was 14.103 mg/L (Figure 13A; Table 7). A RPD value of 6 was obtained and it indicates that the model is suitable for quantification purposes to predict YAN in white fermenting must. An alternative reference method was investigated to develop a model that was suitable to predict both red- and white fermenting must.

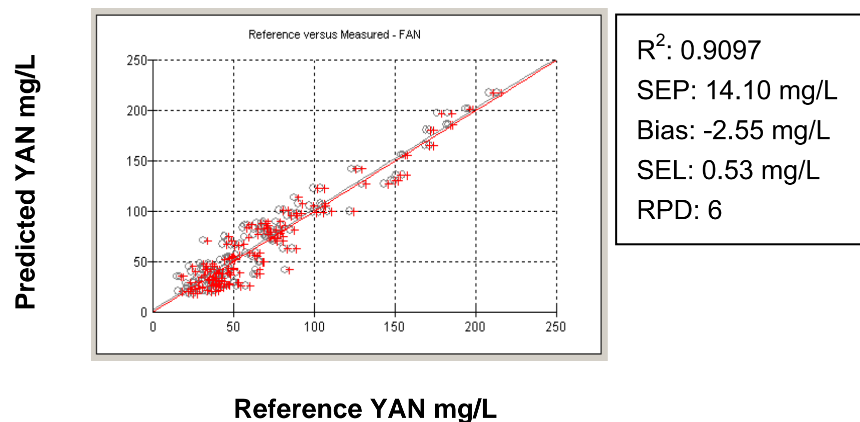

Figure 13A Plot of FT-IR predicted values for new YAN calibration vs. reference values using enzymatic assay method

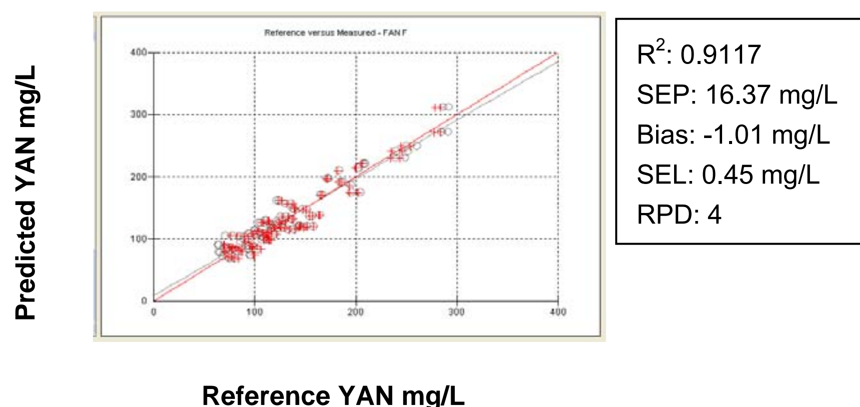

Figure 13B Plot of FT-IR predicted values for new YAN calibration vs. reference values using Formol titration method.

A YAN calibration model was also developed using the Formol titration method (Zoecklein, 1999). Both red- and white fermenting must were used to establish the calibration model. This method requires minimum sample preparation and there was no need to remove the colour of the red fermenting must, but the method is also time-consuming (~15 minutes per sample). A sample set of 168 was used to developed the model and divided in a calibration set (n = 113) and validation set (n = 55). A total of 94% variance between samples was explained by 15 PLS filters and 10 factors had showed the lowest SEC (19,869 mg/L). Vibrational bands between 1700 cm^{-1} and 1500 cm^{-1}, represent the amide backbone of peptides and proteins. Bands around 1740 cm^{-1} and 1400 cm^{-1} are mainly observed in amino acid side chains, due to the C=O stretching in esters and carboxylic acids and the C-O stretching in carboxylates (Pistorius, 1995). The bias of the validation of the new calibration model was -1.101 mg/L, the R^2 0.9117 and the SEP was 16.372 mg/L (Figure 13B; Table 7). A smaller bias could be achieved with the new prediction model, but the SEP was larger. A RPD value of only 4 was obtained and it indicates that the model is only suitable for screening purposes, but it could be considered suitable for quantification purposes if the R^2 (0.9117) was used. A possible reason for the poor performance of the model could be the much smaller sample set that was used to establish the model and expanding the sample set should be considered to develop a prediction model that is suitable for quantification purposes as well. The standard deviation (60.93 mg/L) provides important information on the variance within the sample set; it is influenced by the concentration range of the sample set and can also be consider as a possible reason for the poor performance of the calibration model. The samples were not analysed in duplicate to calculate the SDD, due to the longer time taken to obtain results.

4.4.6.4 Sample selection to establish calibration model for YAN

Table 8: Summary of the calibration and validation statistics for the evaluation of different calibration models for YAN

Calibration statistics				Validation statistics				
Model	**No. of samples**	**PLS[a] factors**	**SEC[b]**	**Sample no.**	**SEP[c]**	**R[2d]**	**bias**	**RPD[e]**
YAN[f]	435	15	73.97	216	53.66	0.6467	4.09	2
YAN[g]	286	10	32.32	142	27.77	0.8093	-0.132	2
YAN[h]	172	11	14.97	168	42.04	0.5240	-49.89	n/a
YAN[i]	172	11	14.97	128	16.02	0.8666	-0.93	n/a
YAN[j]	172	11	14.97	85	14.10	0.9097	-2.55	6
YAN[k]	113	10	19.87	55	16.37	0.9117	-1.01	4

[a]Partial least square factors; [b]Standard error of calibration; [c]Standard error of prediction; [d]Coefficient of correlation; [e]Residual predictive deviation; [f]All samples (enzymatic and Formol); [g]All samples without red fermenting must – enzymatic; [h]Validation white fermenting must model with Formol samples (red and white); [i]Validation white fermenting must model with Formol samples (white only); [j]Model with enzymatic method; [k]Model with formol method.

Different calibration models were developed to choose the most suitable model for both screening and quantitative purposes (Table 8). The model which contains red- and white fermenting must samples of both reference methods performed the poorest and showed substantial systematic errors for YAN (bias = 4.09 mg/L, SEP = 53.66 mg/L, R^2 = 0.6467). An RPD value of 2 was obtained and it indicates that the model was not suitable for screening purposes. The prediction of red fermenting must using enzyme-linked assay method showed extremely large errors between the reference values and the predicted values and it was the reason why the red fermenting must samples were excluded when the calibration model with only white fermenting must samples were developed. A calibration model was then developed without the red fermenting must (enzymatic method), but with both red- and white samples of

the Formol method, and the results showed an improvement (bias = -0.132 mg/L, SEP = 27.77 mg/L, R^2 = 0.8093). The bias indicated a small systematic error in the predicted values, but the large SEP indicated a low accuracy in relation to the independent set. The samples (both red and white) used for the Formol method could not be used to validate the existing calibration model for white fermenting must (bias = -49.89 mg/L, SEP = 42.04 mg/L, R^2 = 0.5240). The bias indicating that the averages of the residuals (difference between reference and predicted values) of the samples were very high. It means that the regression statistics will give a high systematic error as evident by the bias. The low R^2 value also indicates that the model was not suitable for quantification of YAN in both red- and white fermenting must. There was no correlation between the YAN values determined with the reference method and the values predicted by the calibration model. This model was not suitable to predict both red and white fermenting must, but the existing calibration model performed extremely well when validating with only the white fermenting must samples from the Formol method used as an independent validation set (bias = -0.93 mg/L, SEP = 16.02 mg/L, R^2 = 0.8666). This indicates that the existing YAN calibration model can be used to predict white fermenting must samples. The calibration model developed from the reference Formol method prove also to be fit to predict both red and white fermenting must samples (bias = -1.01 mg/L, SEP = 16.37 mg/L, R^2 = 0.9117).

4.4.7 ESTABLISHMENT OF CALIBRATION MODELS FOR GLUCOSE, FRUCTOSE, AND TOTAL GLUCOSE PLUS FRUCTOSE <30 g/L

By establishing calibration models for glucose, fructose and glucose plus fructose with low concentrations (<30 g/L), more accurate results could be obtained when making decisions whether a wine is finished or still fermenting. The descriptive, calibration and validation statistics are shown in Table 9 and 10 respectively. This calibration models could be used as a final step before the fermenting must becomes a finished wine.

Table 9: Descriptive statistics of fermenting must samples used as a reference set to establish new calibration models for glucose, fructose and glucose plus fructose smaller than 30g/L

Parameters	No. of samples (white;red)[a]	Min.[b]	Max.[c]	Mean	SD[d]	SEL[e]
Glucose-dry g/L[f]	274(209;65)	0.06	28.92	7.54	7.87	0.25
Fructose-dry g/L[g]	158(122;36)	2.89	29.98	15.73	8.46	0.23
Gluc+fruc-dry g/L[h]	137(106;31)	3.00	29.74	15.06	8.47	0.20

[a]Sample number (white wine; red wine); [b]Minimum; [c] Maximum; [d]Standard deviation; [e]Standard error of laboratory; [f]Glucose <30 g/L; [g]Fructose <30 g/L; [h]Total glucose plus fructose <30 g/L

Table 10: The calibration and validation statistics for the evaluation of the new calibrations of glucose, fructose and glucose plus fructose smaller than 30 g/L

Calibration statistics				Validation statistics					
Parameter	No. of samples	PLS[a] factors	SEC[b]	Sample no.	SEL[c]	SEP[d]	R^{2e}	bias	RPD[f]
Gluc. –D[g] g/L	183	6	1.25	91	0.25	1.31	0.9705	0.21	6
Fruc.-D[h] g/L	106	5	2.86	52	0.23	1.69	0.9589	0.31	5
Glu+fru-D[i] g/L	92	4	2.41	45	0.20	2.53	0.9102	0.13	3

[a]Partial least square factors; [b]Standard error of calibration; [c]Standard error of laboratory; [d]Standard error of prediction; [e]Coefficient of correlation; [f]Residual predictive deviation; [g]Glucose <30 g/L; [h]Fructose <30 g/L; [i]Total glucose plus fructose <30 g/L.

4.4.7.1 New calibration model for glucose < 30 g/L

The new calibration model for glucose <30 g/L performed very well. The descriptive statistics of the fermenting must used to establish the new calibration model are shown in Table 9. A much smaller SEP (1.31 g/L) was obtained for the model than the full range glucose model, but a smaller sample set was used to develop the model. For the new <30 g/L glucose calibration model, the sample set (n = 274) was divided in a calibration set (n = 183) and a validation set (n = 91). A total of 6 factors had showed the lowest SEC (1.25 g/L). The bias of the validation of the new calibration model was low (0.21 g/L), the R^2 was high (0.9705) and the SEP was small (1.31 g/L) (Figure 14; Table 10). A good model should have a low SEC and SEP, high R^2 and small difference between SEC and SEP. A RPD value of 6 was obtained and it indicates that the model is suitable for quantification purposes.

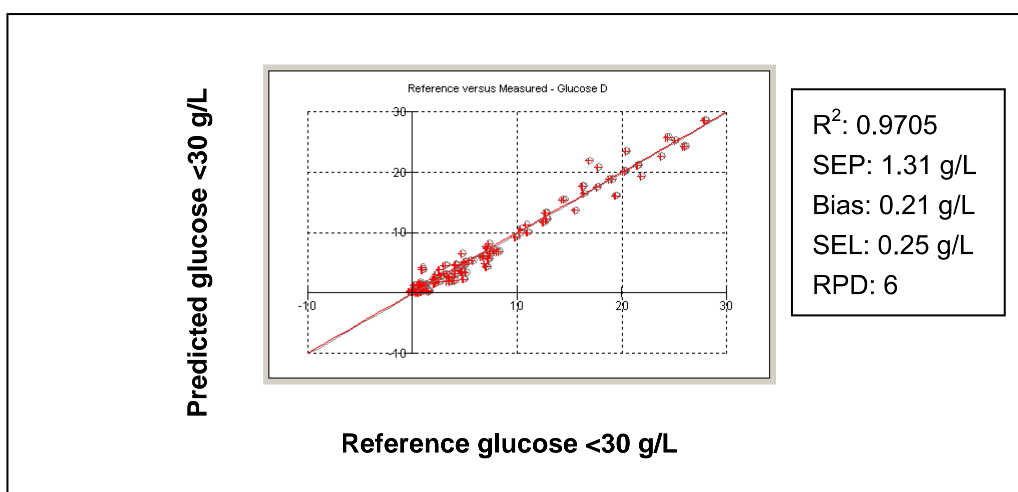

Figure 14 Plot of FT-IR predicted values for new glucose <30 g/L calibration vs. reference values.

4.4.7.2 New calibration model for fructose < 30 g/L

The new calibration model for fructose <30 g/L also performed very well (Figure 15, Table 9). A smaller SEP (1.69 g/L) was obtained for the model than the full range fructose model, but a smaller sample set was used to develop the model. For the new <30 g/L fructose calibration model, the sample set (n = 158) was divided in a calibration set (n = 106) and a validation set (n = 52). A total of 5 factors had showed the lowest SEC (2.86 g/L).The bias of the validation of the new calibration model was fairly low (0.31), the R^2 relatively high (0.9589) (Figure 15; Table 10). A RPD value of 5 was obtained and it indicates that the model is suitable for quantification purposes.

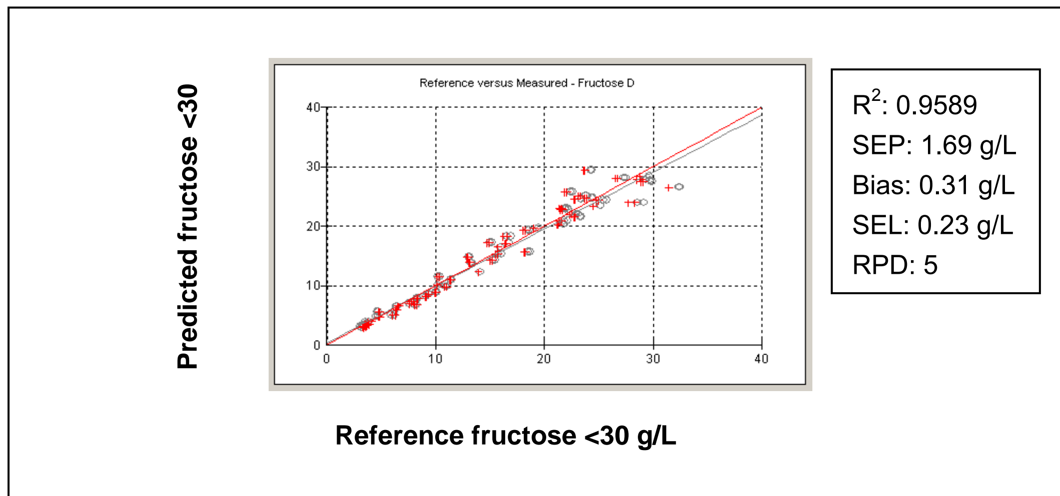

Figure 15 Plot of FT-IR predicted values for new fructose <30 g/L calibration vs. reference values.

4.4.7.3 New calibration model for glucose plus fructose < 30g/L

The new calibration model for glucose plus fructose <30 g/L performed well (Figure 16). The descriptive statistics of the fermenting must used to establish the new calibration model are shown in Table 9. A SEP of 2.53 g/L was obtained for the model, but a sample set of only 137 samples was used to develop the model. For the new <30 g/L glucose plus fructose calibration model, the sample set (n = 137) was divided in a calibration set (n = 92) and a validation set (n = 45). A total of 4 factors had showed the lowest SEC (2.41 g/L). The bias of the validation of the new calibration model was equal to 0.13 g/L, the R^2 0.9102 and the SEP was 2.53 g/L (Figure 16; Table 10). Due to a high prediction error, the RPD was 3, indicating that the model is suitable for screening only. The prediction error had to be decreased to ensure accurate determination of glucose plus fructose in fermenting must. A larger sample set could also be considered as a possible solution to improve the performance of the model. However, the model can also be considered fit for the quantification of glucose plus fructose in fermenting must.

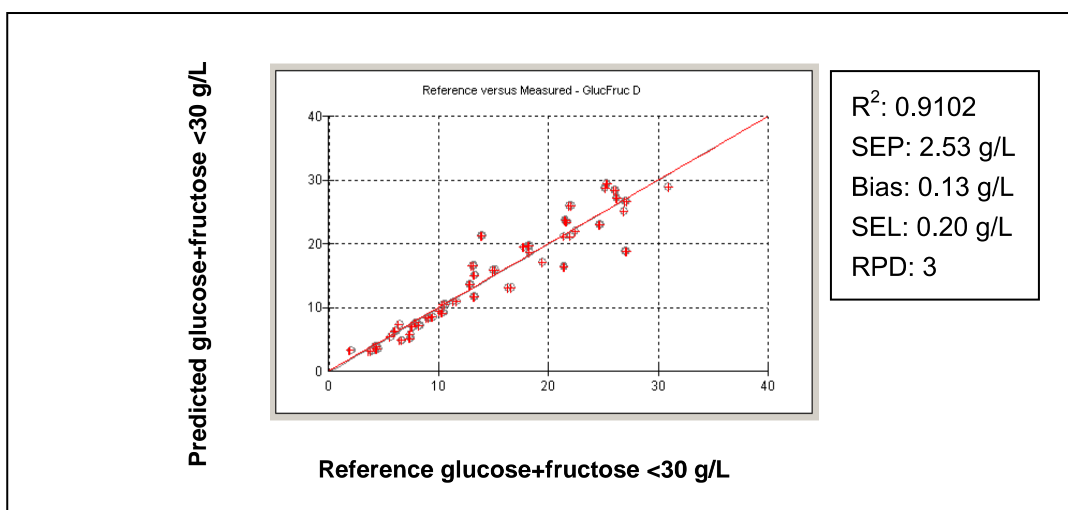

Figure 16 Plot of FT-IR predicted values for new glucose/fructose <30 g/l calibration vs. reference values.

4.4.8 EVALUATION OF THE INFLUENCE OF THE SELECTION OF THE CALIBRATION SET ON THE PERFORMANCE OF THE CALIBRATION MODELS

Table 11 Summary of the calibration and validation statistics for the evaluation of the influence of the selection of calibration set on performance of calibration models.

Calibration statistics				Validation statistics				
Model	Sample no.	PLS[a] factors	SEC[b]	Sample no.	SEP[c]	R^{2d}	bias	RPD[e]
Ethanol %v/v(70/30)	211	10	0.19	105	0.15	0.9988	0.04	30
Ethanol %v/v(50/50)	158	11	0.29	158	0.19	0.9982	0.04	23
Fructose g/L(70/30)	265	11	3.99	132	4.14	0.9889	0.64	10
Fructose g/L(50/50)	198	11	4.27	198	4.32	0.9879	0.10	10
YAN[f] mg/L(70/30)	172	11	14.97	85	14.10	0.9097	-2.55	6
YAN mg/L(50/50)	128	11	14.12	128	15.67	0.8987	-1.99	5
TA[g] g/L (70/30)	210	12	0.38	105	0.35	0.7973	-0.004	2
TA g/L (50/50)	157	8	0.38	157	0.42	0.7089	-0.00	2

[a]Partial least square factors; [b]Standard error of calibration; [c]Standard error of prediction; [d]Coefficient of correlation; [e]Residual predictive deviation; [f]Yeast assimilable nitrogen; [g]Titratable acidity expressed as tartaric acid

Calibration models for each compound were developed by dividing the reference sample set into a calibration and validation set containing 70% and 30% of the samples respectively. The reference set was divided in such a matter to ensure that both the calibration and validation sets cover the entire concentration range and that the calibration set includes more samples than the validation set. The performance of each calibration model was evaluated to establish the influence of a selection of different calibration sets. The reference set of each compound was therefore divided in a calibration and validation set containing 50% and 50% of the samples respectively. This test was done with ethanol, fructose, YAN and TA (Table 11). The results showed that there was a difference of 0.1 %v/v in prediction between the two models for ethanol, but that should be expected, because the ethanol (70/30) model has more samples in the calibration set than the ethanol (50/50) model. The same results were shown with the fructose (70/30) and fructose (50/50) models, but a better bias (0.10 g/L) could be achieved with the fructose (50/50) model. The results with the YAN (70/30) and YAN (50/50) models showed just the opposite than the latter two. The YAN (50/50) model showed better prediction than the YAN (70/30) model and a better bias (-1.99 mg/L) was also achieved. The results of the TA (70/30) and TA (50/50) models showed the same results, but more factors (12) were needed for the TA (70/30) model to achieve the same results as TA (50/50) with 8 factors. Usually, the more samples used for calibration, the better the prediction ability of the equation obtained. However, it is not only the number of samples which is important, but also how the samples are selected. The selection of calibration samples provides all the necessary variation in the spectral properties, therefore, the variation in the concentration range of the property of interest is very important (Naes *et al*., 2002).

The next step of the implemented models is to maintain and keep them alive with different types of samples each vintage. Variations can lead to changes in fermenting must quality parameters, which place further demands on calibration updates. Thus ensuring accurate analysis of future unknown samples, calibration maintenance must be seen as a continuous quality process. For this study robustness can be described as a calibration model's capacity to remain unaffected by any small variations which provides a good indication of the model's reliability during normal usage.

4.5 CONCLUSIONS

The methods usually employed to monitor fermentation processes are slow and labor-intensive and use reagents with a potential environmental impact (Blanco *et al*., 2004). In this study, FT-IR was used in combination with multivariate techniques to accomplish the rapid, reliable and affordable determination of ethanol, glucose, fructose, YAN, and acidity in samples from an alcoholic fermentation process. FT-IR spectroscopy in the simultaneous measurements for ethanol, pH, VA, TA, glucose, fructose, glucose plus fructose and YAN in fermenting must shows potential for accurate analysis and quality control purposes in an industrial cellar. To conclude the results of this study all compounds cannot be quantified with FT-IR, as seen with fermenting must used in this study. The RPD values obtained for ethanol, glucose, fructose, glucose plus fructose and YAN calibrations proved it fit for prediction of fermenting must samples. The RPD values obtained for pH and VA calibrations proved it fit for screening purposes, but these models could be considered for quantification purposes in fermenting must. The global calibration as well as the new calibration model for TA proved it unfit for either quantification or screening purposes for fermenting must, using the RPD as evaluation criterion. The new calibration model for TA showed rather good precision, had a low bias, high R^2 and a SEP which was smaller than 2 times SDD when looking at other evaluation criteria, therefore, the calibration model for TA proved it fit for quantification purposes. Two reference methods (enzyme-linked and Formol titration) were evaluated to determine the YAN content in fermenting must. A calibration model was developed from samples tested by the enzymatic method to predict only white fermenting must and another calibration model was developed from samples tested by the Formol titration method to predict both red- and white fermenting must. Rapid analyses of these parameters will lead to higher throughput of fermenting must samples in laboratories during harvest time.

The study had shown that major chemical compounds of wine can be measured by FT-IR spectroscopy quantitatively. The predictive ability of the FT-IR spectrometry is highly dependent on the composition of the sample set. When designing a data set, it is important to consider the number of samples, the concentration range covered by the samples, and the distribution of samples within this range (Versari *et al*., 2008). The sample size used in this study did permit the development of several calibrations (ethanol, glucose, fructose and glucose plus fructose) suitable for routine use in an analytical laboratory, but further development with larger data sets will be required for the FT-IR calibrations of pH, TA, VA and YAN to become stable.

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Chapter 5

Research results

Qualitative off-line batch monitoring of alcoholic fermentation

5. RESEARCH RESULTS

5.1 ABSTRACT

It is important as a first step to quantify quality-indicating parameters using classical analytical parameters during alcoholic fermentation. A logical next step is to monitor fermentation based on multivariate calibration, whereby important compounds are quantified indirectly through mathematical prediction. Although these two approaches are important to interpret the behaviour of fermentation, quantification of all the quality determining compounds is not always possible, due to time delay and cost. In this study, off-line multiple statistical process control (MSPC) charts were developed using FT-IR spectra generated on samples taken from actively fermenting musts. A small set of fermentations were graphically projected using SimcaP+ software, to identify characteristic patterns associated with alcoholic fermentation in industrial winemaking. These MSCP charts of normal wine fermentations were then used to identify possible problem fermentations. For the purpose of this work, industrial vinifications of 21 red- and white wine tanks conducted during 2008 and 2009 were monitored. The strategies used to try and induce problem fermentations (or fermentations showing abnormal patterns in alcoholic fermentation) under experimental conditions, were spontaneous (uninoculated) fermentations, low pH (2.80) of must and the addition of a high dose of SO_2 (200 ppm) to the must at the onset of fermentation. Results of the multivariate modelling of spectra provided a graphic presentation of normal and abnormal fermentation behaviour.

5.2 INTRODUCTION

The term process analytical technology (PAT), describes the field of process analysis and measurement technologies that have been developed to include several physical, chemical and data analytical tools to characterise chemical and biological process (Urtubia *et al.*, 2007). Alcoholic fermentation is a rather complex bioprocess, that is influenced by several biological and physicochemical factors, both of an intrinsic and extrinsic nature, and in industrial wine cellars, winemakers are well aware of the unpredictable nature and variation in the duration of alcoholic fermentations. Currently, in wine industries, alcoholic fermentation is mostly monitored off-line through chemical analysis on samples removed from fermentation ranks, in the laboratory. This approach is costly and also time-consuming, since in large wineries, several hundred fermentation tanks are operated simultaneously. A disadvantage of this approach is that it is impossible to monitor critical process parameters in real time, due to the time lapse from sampling, until relevant chemical data are obtained.

Some process properties can often not be directly measured by chemical analysis. Examples of such properties include: the identification of compounds or complex mixtures, and the identification of interactions between different process parameters (Varmuza, 2003). In most cases, a univariate approach by considering one or two variables at a time is insufficient to capture the desired data or to provide the required information. Therefore a multivariate approach is necessary for many problems in process analytical technology. Large wineries can benefit from an improved analysis of fermentation data based on the use of advanced multivariate data analysis, and in that way reduce some uncertainty pertaining to the fermentation process (Urtubia *et al.*, 2007).

The measurement and monitoring of wine and alcoholic fermentations have been the subjects of various research laboratories in different countries (Urtubia *et* al., 2004, 2008; Cozzolino *et* al., 2006; Nerantzis *et al*., 2007). Stuck and sluggish alcoholic fermentations still occur frequently today and cause the producing cellar considerable problems. Abnormal fermentation behaviour can lead to much longer fermentation times and high residual fermentable sugars in dry wines, resulting in downgrading of quality of the final product and financial losses to the producing cellar (van Vuuren and Wingfield, 1986). The reduction of the incidence of stuck and sluggish fermentations requires a monitoring system that can detect and classify problems at an early stage. Correct planning and monitoring of fermentations will go a long way to avoid sluggish or stuck fermentations.

The potential of Fourier transform infrared (FT-IR) spectroscopy as an analytical tool to analyse wine has attracted considerable attention in the last decade (Patz *et al*., 1999; Dubernet and Dubernet, 2000; Nieuwoudt *et al*., 2006; Soriano *et al*., 2007). This technology is based on the measurement of the absorbance of radiation in the mid-infrared region (4000 - 400 cm^{-1}) by molecules that contain chemical bonds such as C-C, C-H, O-H, C=O and N-H (Willard *et al*., 1988; Holler *et al*., 2007). Since many compounds absorb in the infrared region, FT-IR spectroscopy captures a large amount of qualitative information pertaining to the matrix (Willard *et al*., 1988; Vonach *et al*., 1998; Holler *et al*., 2007). The advantages of FT-IR spectroscopy for rapid wine screening and quality control during winemaking have already been reported by several authors (Dubernet and Dubernet, 2000; Kupina and Shrikhande, 2003; Patz *et al*., 2004).

The aim of this study was to follow the progress of alcoholic fermentation in industrial wine cellars, by using multivariate modelling of FT-IR spectra only. The objective was to observe the typical patterns, associated with the normal alcoholic fermentation stage of different cultivars at the winery. It was also of interest to see if the strategy using only FT-IR spectra for this analysis could identify problem fermentations that were experimentally induced. This work evaluated the use of SimcaP+ software (www.umetrics.com) that is designed for analysis of measurements on batch data, through the construction of multivariate statistical process control (MSPC) charts. The results presented here are a pilot study and a small number of fermentation tanks, 21 in total, were monitored.

5.3 MATERIALS AND METHODS

5.3.1 ALCOHOLIC FERMENTATIONS

Samples, 100 ml aliquots, were sourced from 21 industrial (4 000L – 280 000L) fermentation tanks at Spruitdrift- and Vredendal Wineries, SA during the 2008 and 2009 vintages. Grapes used for the fermentations were harvested from commercial vineyards in the Olifants River region. Vinifications of Cabernet Sauvignon (2), Shiraz (3), Colombar (5) and Chenin blanc (1) in 2008 and Merlot (2), Extra Light (2), Chenin blanc (1) and Colombar (5) in 2009 were sampled during the alcoholic fermentation stage at 8- to 12 hourly intervals, and approximately 10 – 45 samples were taken per fermentation, depending on the duration of the vinification. The alcoholic fermentation was carried out at temperatures between 10 - 15°C for the white musts (Colombar, Chenin blanc and Extra Light) and between 23 - 28°C for the red musts (Cabernet Sauvignon, Shiraz and Merlot). A total of 443 samples were obtained (Cabernet Sauvignon 2008 = 45, Shiraz 2008 = 66, Colombar 2008 = 151, Chenin blanc 2008 = 20, Extra Light 2009 = 21, Chenin blanc 2009 = 11, Colombar 2009 = 92 and Merlot 2009 = 37). The samples were transported in cooler boxes to the wine laboratory and prepared for the spectroscopic analysis. It was attempted to induce four problem or “abnormal” alcoholic fermentations experimentally,

and these included the following treatments: two fermentations were not inoculated with yeast starter cultures (Merlot), one fermentation of juice with pH 2.80 (Colombar), and one fermentation with a high dose of SO_2 (200 mg/L) (Colombar), added at the onset of alcoholic fermentation. Temperature was controlled at 10°C for the white wine fermentations and at 27°C for the red wine fermentations. The pH of the Colombar fermentation was adjusted with 1 N sodium hydroxide (40 g of sodium hydroxide (NaOH) pellets (Merck, Cape Town, SA) in 1 L of distilled water), before inoculation with wine yeast. SO_2 of 200 mg/L was adjusted using a 1000 mg/L SO_2 (1.483 g sodium bisulfate in 1 L of distilled water) solution (Merck, Cape Town, SA).

5.3.2 SPECTROSCOPIC MEASUREMENTS

Upon reception at the laboratory, 50 ml aliquots of the fermentation samples were centrifuged (2 minutes; 5000 rpm) using a Hermle 200A centrifuge (LASEC, Cape Town, SA). Samples were treated to remove excess CO_2 by manual shaking for 5 minutes, followed by degassing for 5 minutes in an ultrasonic bath (UMC 5, Krugersdorp, SA), (In-house chemical laboratory Standard Operating Procedure, Namaqua Wines, 2007). After centrifugation and degassing, the samples were scanned immediately using a WineScan FT 120 spectrometer, under instrumental conditions described before. These included a cuvette with a path length of 37µm, sample temperature set at 40°C, and the scanning interval set from 930 to 5011 cm^{-1} at 4 cm^{-1} intervals. These instrumental parameters have been set by the manufacturer and can not be changed by the user (FOSS Analytical, Denmark). The number of repeated scans per sample was set at 20. Cleaning of the instrument, using proprietary solutions, was automatically programmed to occur 30 seconds after samples were analysed. The instrument was zeroed before samples were analysed, using the zeroing solution (P/N 1015912, Foss Analytical, Denmark). The FT-IR spectra were obtained in duplicate for each sample and the mean of the two measurements was used.

5.3.3 BATCH MODELLING OF FERMENTATION DATA

Spectra were exported from the WineScan instrument to Excel (Windows 2005) and then imported into the SIMCAP+ software (SimcaP+, Umetrics AB, Umeå, Sweden. www.umetrics.com). Batch data obtained from all the measurements were subjected to multivariate data analysis, using principal component analysis (PCA) on the FT-IR spectra, and partial least squares regression (PLS) with time as the *y*-variable. The **t** scores obtained from PCA of batch data were used to construct $\mathbf{t_1t_2}$ graphical plots, to compare the fermentation behaviour of the various fermentation processes. The **u** scores obtained from PLS batch data analysis, were used to construct $\mathbf{u_1u_2}$ plots in order to model the collective behaviour of all fermentations over time (User Guide, SimcaP+, Umetrics AB, Umeå, Sweden. www.umetrics.com)

5.4 RESULTS AND DISCUSSION

The data collected during vintage 2008 and 2009 consisted of FT-IR spectra taken at 8- or 12 hourly intervals from 17 fermentation tanks that progressed normally for both red- and white must. These tanks were all inoculated with rehydrated active dry wine yeast using inoculae of $>10^6$ cells / mL. During 2009, four tanks that were subjected to treatments at the onset of alcoholic fermentation, that were aimed at inducing problem, or “abnormal” fermentations, were also included in the analysis. For the purpose of this study, these four tanks are referred to as “abnormal”.

5.4.1 MONITORING OF ALCOHOLIC FEMENTATION USING FT-IR SPECTRA

The FT-IR spectra showed variation between the different fermentations of actively fermenting red- and white musts, especially in the areas 1800 – 930 cm^{-1} and 3100 – 2500 cm^{-1} respectively (Figure 1). These areas are known to be associated with the fundamental vibrations of organic molecules (Holler *et al*., 2007). The regions 1710 cm^{-1} – 1545 cm^{-1} and 3620 cm^{-1} – 2968 cm^{-1} are known to be associated with the absorption of water and were excluded from the analysis, as these areas are noise areas, where important information can be masked due to the large variation in the water signals (Holler *et al*., 2007).

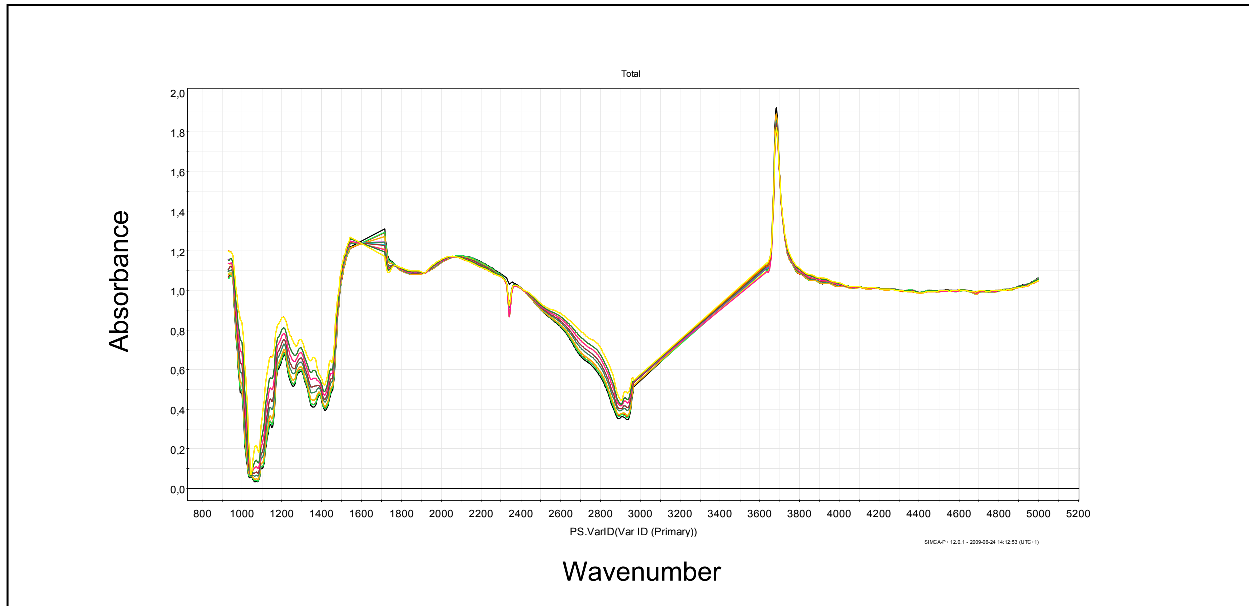

Figure 1 FT-IR spectra of different red- and white fermenting must samples that were sampled during the active alcoholic fermentation stage, at 8- or 12 hourly intervals.

5.4.2 PCA MODELLING OF ALCOHOLIC FERMENTATION IN ALL RED- AND WHITE WINES

Principal component analysis was done on the FT-IR spectra of all samples taken at 8-or 12 hourly intervals from the actively fermenting tanks. Water absorbing regions were excluded in the analysis, as described before.

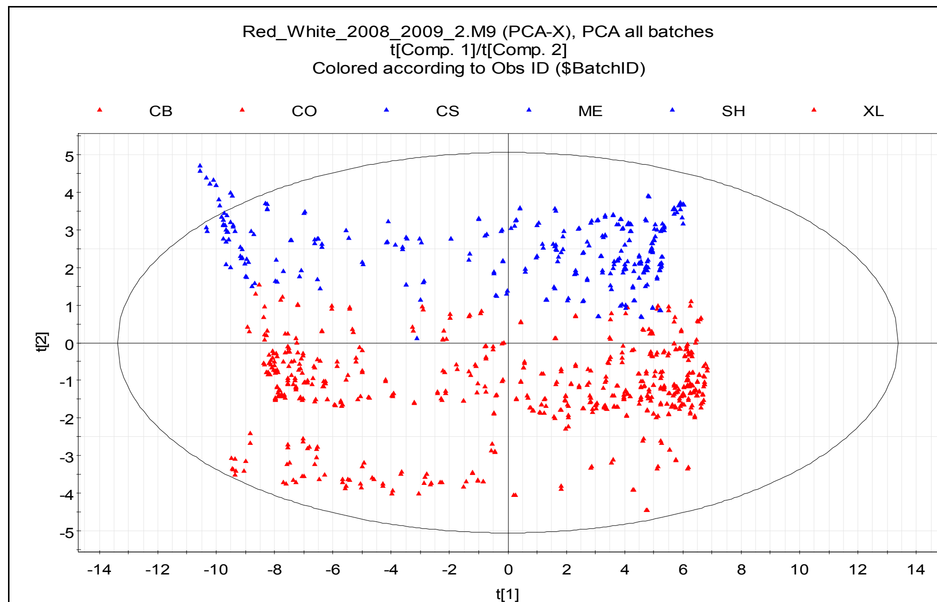

Figure 2 PCA plot (**t1** and **t2**) of FT-IR spectra of samples taken from fermentation tanks of 21 wine fermentations, during alcoholic fermentation at 8-or 12 hourly intervals, of white fermenting must (red markers) and red fermenting must (blue markers). The ellipse indicates a 95% confidence interval.

The PCA plot showed that there was a clear separation between the red- and white fermentations (Figure 2). The data of 21 fermentation tanks, including 17 normal fermentations and 4 problem fermentations were used. This was a clear indication that white- and red must fermentations should be modeled separately. Therefore, from this point onward all models were developed separately for red- and white wines. It was clear that the PCA plot with both red- and white must fermentations did not show the separation of the 4 problem fermentations at this point.

5.4.3 PCA MODELLING OF ALL RED WINES ACCORDING TO VINTAGE AND CULTIVAR

PCA analysis was done to obtain information on the data structure and to investigate possible influences in the grouping of the fermentations done over two consecutive vintages, 2008 and 2009. It was of particular interest to investigate possible vintage or cultivar effects.

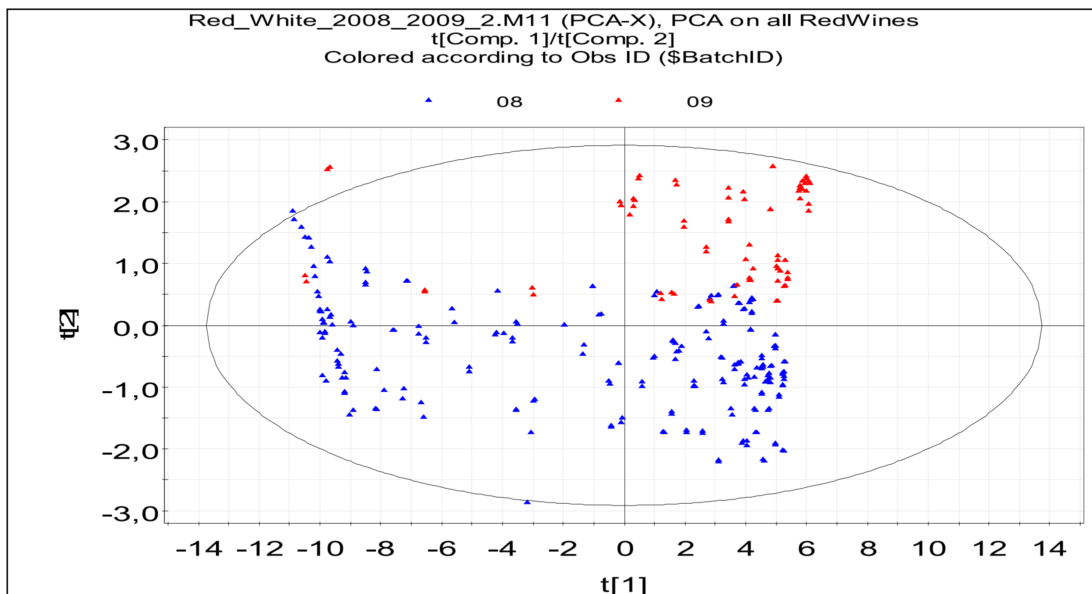

Figure 3 PCA plot (**t1** and **t2**) of FT-IR spectra of samples taken from fermentation tanks during alcoholic fermentations in red wine production in 2008 (blue markers) and 2009 (red markers). Each marker represents a FT-IR spectrum of a sample taken from a fermentation tank at 8-or 12 hourly time intervals. The ellipse indicates a 95% confidence interval.

The location of samples in the **t1** and **t2 plot** (Figure 3) could be interpreted in terms of the different cultivars used in 2008 and 2009. Cabernet Sauvignon and Shiraz tanks were monitored in 2008 and Merlot was used in 2009. Based on the small number of fermentations, the limited data do not support any conclusions regarding vintage effects. Merlot 2009 fermenting must (Figure 4) separated from the other two red cultivars and it could be for several reasons; Merlot must was the only cultivar that was fermented during 2009, it was also the only cultivar where the fermentation has been abnormal. Therefore it is not possible to confirm that Merlot separated due to vintage, cultivar or abnormal behaviour. To obtain a fingerprint of a “typical” alcoholic fermentation associated with Merlot, a few normal fermentation runs obtained from future fermentations, should be included. Cabernet Sauvignon and Shiraz fermentation batches did not separate from each other according to vintage or cultivar (Figure 4), and it could be the result of the normal fermentation of the batches monitored in this study. These cultivars should also be compared with samples taken from future vintages as well as possible abnormal fermentations (from the same cultivars) to extend the model.

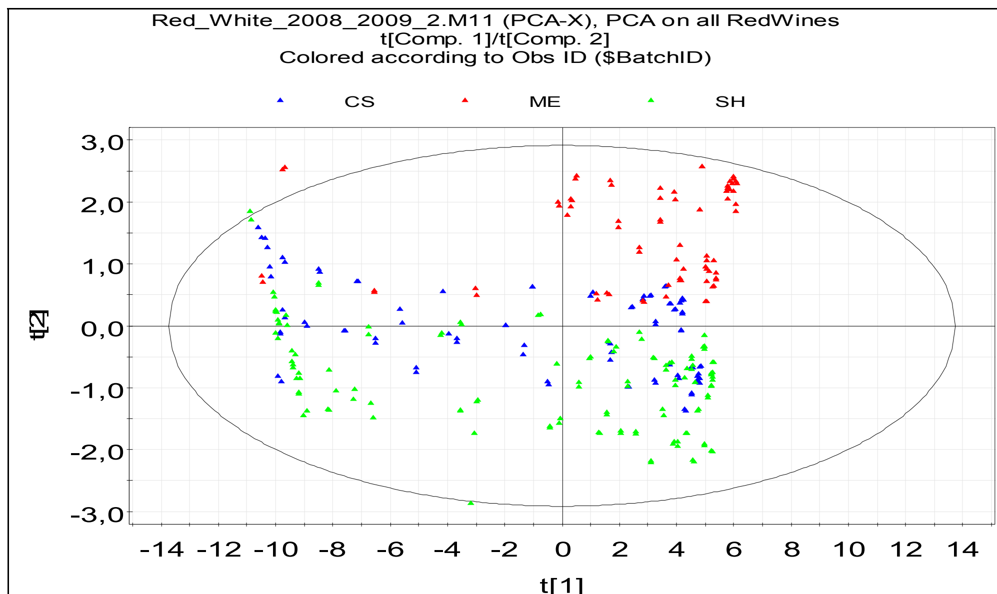

Figure 4 PCA plot (**t1** and **t2**) of FT-IR spectra of samples taken from fermentation tanks during alcoholic fermentation of Cabernet Sauvignon (blue markers), Merlot (red markers) and Shiraz (lime markers) cultivars. Each marker represents a FT-IR spectrum of a sample taken from a fermentation tank at 8-or 12 hourly time intervals. The ellipse indicates a 95% confidence interval.

5.4.4 PCA MODELLING OF ALL WHITE WINES ACCORDING TO VINTAGE AND CULTIVAR

As seen with the red wines, a separation in the PCA score plot between the fermentations done in 2008 and 2009 for white wines was also observed (Figure 5). The wines that separated from the main cluster were the Extra Light wine and Colombar (pH 2.80), from vintage 2009, with the abnormal fermentation behaviour. These samples located outside the 95% confidence interval (Figure 6). It also appeared that the PCA batch model based on **t1** and **t2** scores, grouped all the normal fermentations from both vintages in one large cluster. While the Colombar fermentation conducted at pH 2.8 located outside the 95% confidence interval, the Colombar fermentation conducted with an addition of 200ppm SO_2 at the onset of the fermentation, located inside the circle, and was not classified by the batch model as abnormal. Extra Light is a specific wine style, where the grapes are harvested at a low °Brix, and the wine is produced with much lower final ethanol content (<10.5 %v/v), than normal alcohol strength table wines. In conclusion, the results obtained in the **t1** and **t2** PCA scores plots, could be interpreted that the normal fermentations from different vintages could be grouped in the same cluster if the fermentations follow the same pattern. Different fermentation patterns or abnormal fermentations should therefore potentially be identified using the **tt** plots. It is however also clear, that a library of fermentations pertaining to different cultivars and wine styles, will need to be established, in order to establish a typical “process signature” for each style.

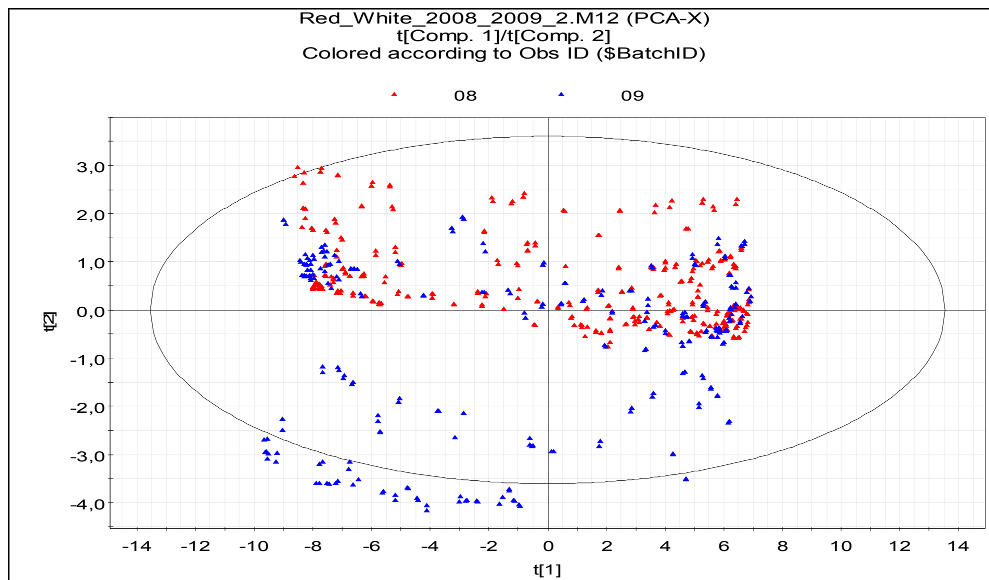

Figure 5 PCA plot (**t1** and **t2**) of FT-IR spectra taken from white fermentation during 2008 (red markers) and 2009 (blue markers) at 8-or 12 hourly time intervals. The ellipse indicates a 95% confidence interval.

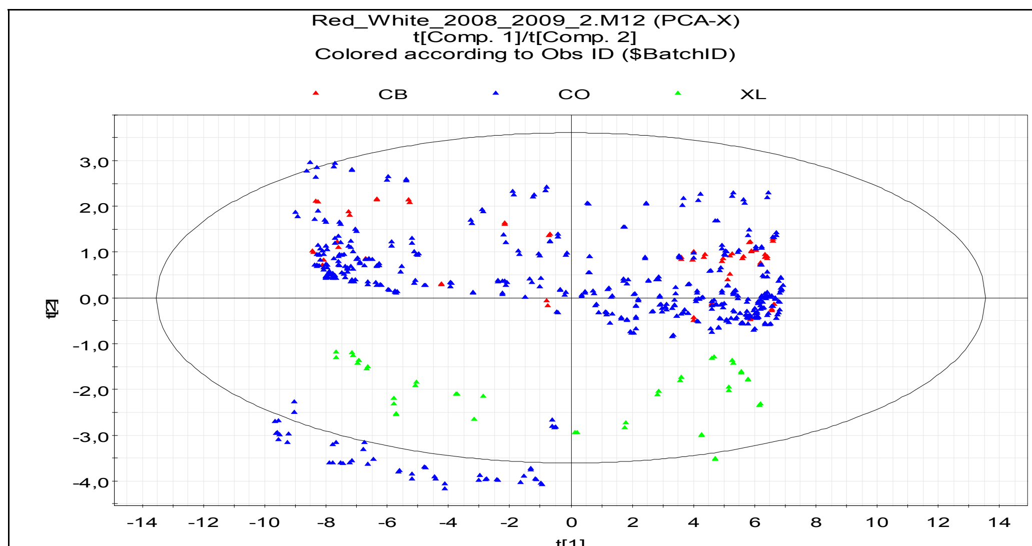

Figure 6 PCA plot (**t1** and **t2**) of FT-IR spectra taken from wine fermentations of Colombar (blue markers), Chenin blanc (red markers) and Extra Light (lime markers), Colombar with pH 2.80 (outside circle). Each marker represents a FT-IR spectrum of a sample taken from a fermentation tank at 8-or 12 hourly time intervals. The ellipse indicates 95% confidence interval.

5.4.5 BATCH MODEL OF NORMAL WHITE WINES USING TIME AS *Y* VARIABLE

The plot of **t** scores versus time of white wines with normal alcoholic fermentation is shown in Figure 7. The batch model is characterised by several features. These include: a fairly similar starting point for all the fermentations with a slight natural variation; major variation during mid-fermentation; and, a shared endpoint that correlated to the complete consumption of grape sugar by the yeast. This pattern could be explained in terms of the practices used for the wine production. The variation seen in the mid-fermentation stage can be expected, since the fermentations will evolve faster or slower in different tanks, depending on several internal and

external factors. These factors can include fluctuations in fermentation temperatures, yeast vigor, or the nutrient status of the fermenting must. At the endpoint, very similar ethanol concentrations are expected for the normal table wines, since the grapes are harvested at similar sugar content, and similar fermentation patterns can be expected, provided the fermentation behavior is normal.

In the model shown in Figure 7, the upper and lower control limits were established based on 3 x SD of the **t** scores (indicated by red lines). The relatively large distance between the upper and lower red lines obtained in this model, clearly shows the large variation between the fermentations at the mid-fermentation stage, although the low number of fermentations that were used to establish the model, could also contribute to the observation. If the MSPC charts are established for only one specific wine style, or one cultivar, 2 x SD or 1 x SD could be used to set the upper and lower control limits. Such an approach would also be used in instances when the final wine product must meet very narrowly defined quality specifications.

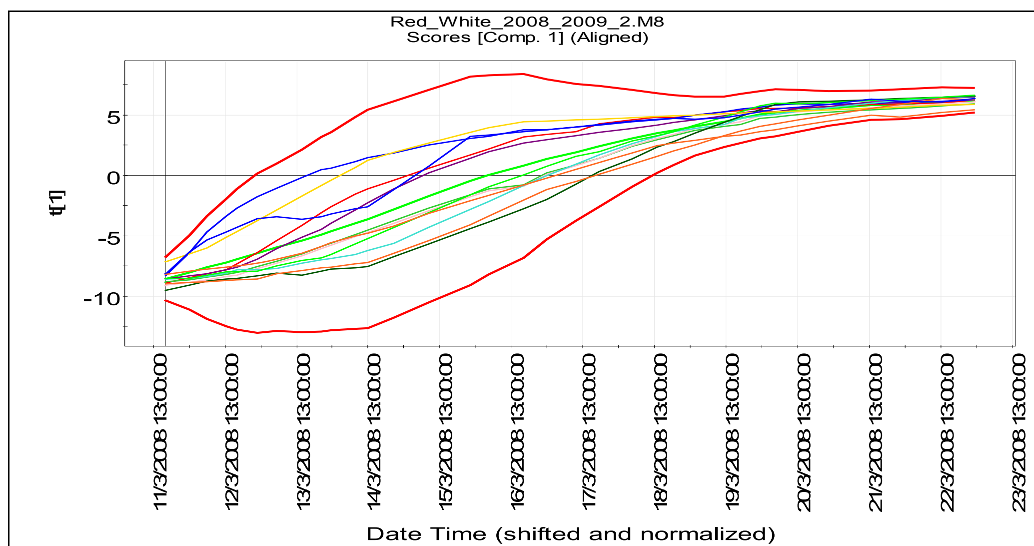

Figure 7 MSPC chart of the normal progress of white wine alcoholic fermentation in an industrial winery (aligned data). Average trend (green line) and 3 x SD (red lines) which indicate the upper and lower tolerance limits.

A challenge in modelling data from wine fermentations is that the durations of the different batches are not the same. A comparison of all batches in terms of starting point and end point therefore requires that the batches are aligned through stretching or shrinking all the batch scores to median batch length (User Guide, SimcaP+, Umetrics AB, Umeå, Sweden. www.umetrics.com). If the differences in batch length are more than 20%, it is recommended that a maturity variable, for instance the final ethanol concentration, is used as **y**-variable. This option was not followed in this study, since the objective was to evaluate batch modelling using the FT-IR spectra only, for the purposes of monitoring alcoholic fermentation.

5.4.6 PREDICTION OF ABNORMAL BATCHES OF WHITE WINES

The white wine fermentations that had normal patterns for alcoholic fermentation, were used as a training set to establish a PLS batch model of normal fermentations (as shown in Figure 7), using aligned fermentation time as *y*-variable. To predict abnormal fermentation batches a prediction set consisting of the problem fermentations were used, and these were Colombar 2009 with high (200ppm) SO_2 added during fermentation and Colombar 2009 with low (2.80) pH

with the start of fermentation. The Colombar wine with the low pH (indicated by the purple line) was positively identified as an abnormal batch, as seen on the MSPC chart (Figure 8) and fermentation rate evolved slower at the end of the fermentation than the normal batches. This wine also did not finish fermentation and got stuck at ethanol content lower than 10 %v/v. The other Colombar wine with the high SO_2 (blue line) was not detected as abnormal. Although the fermentation started at a slower rate (Figure 8), as seen at the start of fermentation a similar endpoint to the normal batches was reached. It is important to note that the purpose of the work was not to establish a good model tested with several test batches, but rather to do a preliminary evaluation of the discrimination capacity of the model. More abnormal fermentations should be considered to confirm these results and establish a batch model to detect different abnormal fermentations.

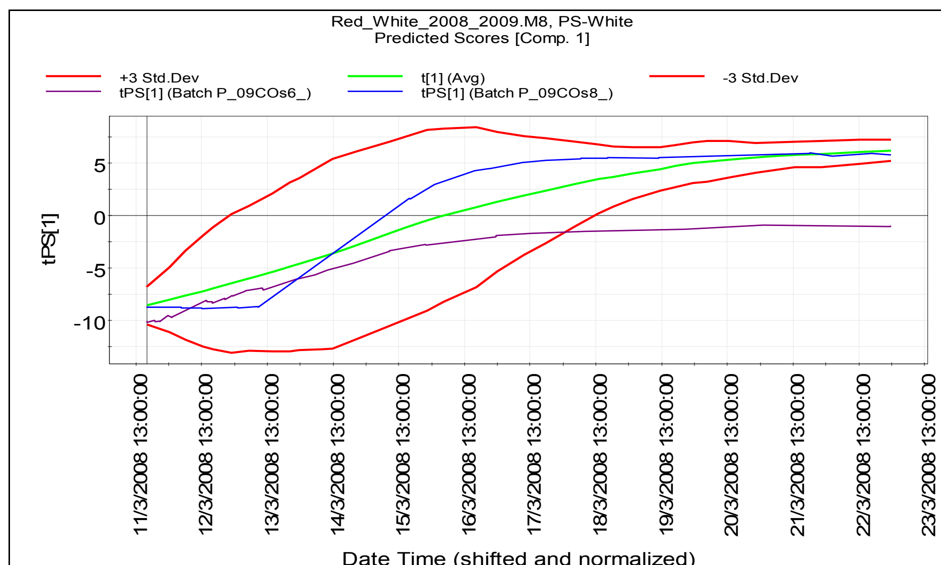

Figure 8 MSPC chart of abnormal Colombar fermentation. Possible abnormal fermentation with 200ppm SO_2 (blue line) and abnormal fermentation with 2.80 pH (purple line). Average trend (green line) and 3 x SD (red lines) which indicate the upper and lower tolerance limits.

5.4.7 PCA MODEL ON BATCH LEVEL OF ALL WHITE WINES

A PCA model was constructed on the batch level to see if there were any differences or similarities between different white wine cultivars (Figure 9 A and B). As seen in Figure 9A, the Extra Light fermentations located far away from the other fermentations. These wines were characterised with high titratable acidity (TA), low pH and low ethanol content. The wine fermentation with the low pH was clearly identified in the score plot as an abnormal batch (outside the 95% confidence interval), (Figure 9B), whereas, the fermentation with the high SO_2 did not seem to differ from the normal batches.

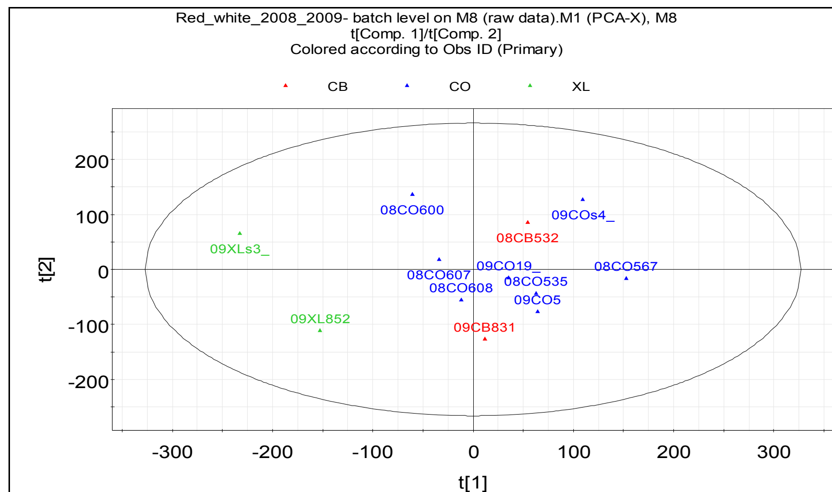

Figure 9A PCA model based on batch level of all white wines of 2008 and 2009. Colombar (blue markers), Chenin Blanc (red markers) and Extra Light (green markers).

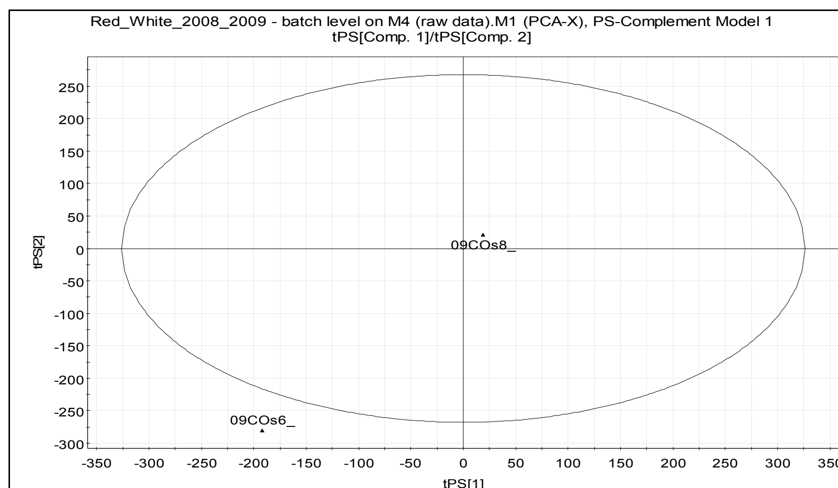

Figure 9B PCA batch model of abnormal Colombar fermentations. Run 09Cos6 was fermented at low pH (pH 2.8), and 09Cos8 was treated with SO_2 as explained in the text. The ellipse indicates 95% confidence interval.

5.4.8 BATCH MODEL OF ALL COLOMBAR WINES USING TIME AS *Y* VARIABLE

The MSPC plot in Figure 10 showed all the Colombar wine fermentations. As shown previously for all the white wines, the Colombar model also showed that the batches had fairly similar starting points, major variation in the middle and similar endpoints. The plot shown in Figure 10A was also aligned through stretching or shrinking all the batch scores to median batch length as described before. In Figure 10B unaligned data were plotted, to highlight the variation in duration of the different fermentations.

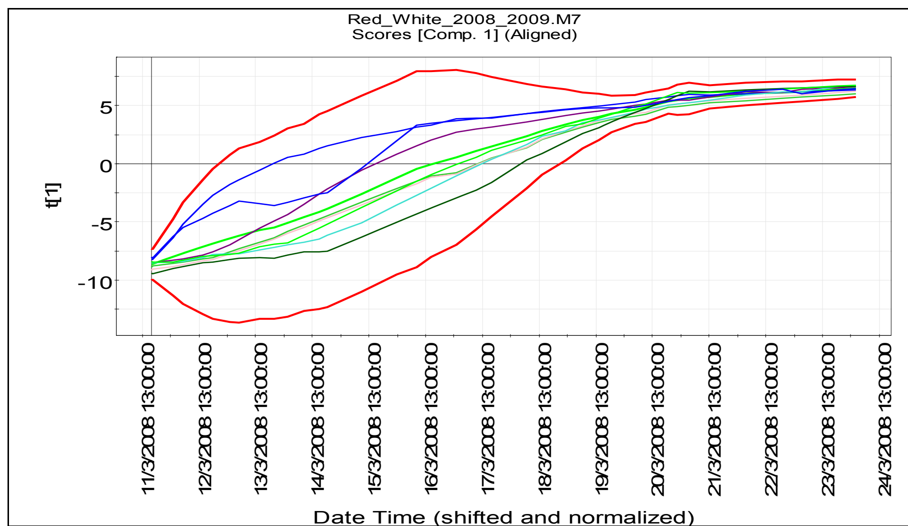

Figure 10A MSPC chart of the normal progress of Colombar alcoholic fermentation in an industrial winery (aligned data). Average trend (green line) and 3 x SD (red lines) which indicate the upper and lower tolerance limits.

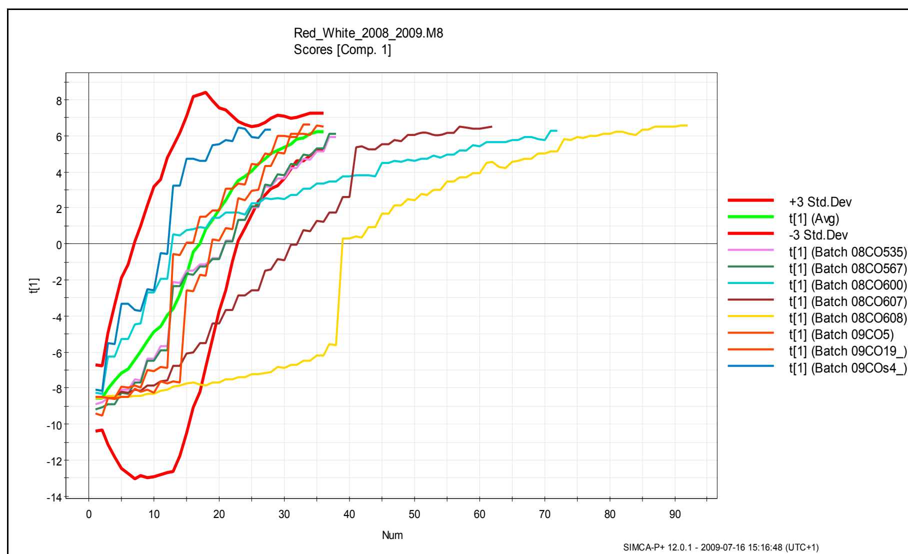

Figure 10B MSPC chart of the same Colombar alcoholic fermentation with unaligned data.

The plot showed that the fermentation rate of three batches were very slow. These three Colombar batches were fermented at very low temperature (10ºC) and the rate of fermentation was clearly much slower, compared to the other Colombar fermentations. Although this big difference in fermentation length is problematic for batch modelling, winemakers prefer these conditions to achieve a more fruity flavour in the wine, in instances when this is required (D. van der Merwe, winemaker, Namaqua Wines, Vredendal, SA. personal communication, 2009). Due to the inherent variation in the duration of alcoholic fermentation in industrial wine production, it is also not necessarily a major problem if a fermentation tank takes long to ferment to dryness, as long as the desired sugar level is reached. However, external factors such as long periods of very high temperatures during grape harvest can put high demands on availability of fermentation tank space. There is also the problem that some fermentations is completed in a shorter period than expected, and this can be due to the cooling plant not working properly or unforeseen power failures. The wine quality of the very fast fermentations is usually

compromised. Therefore, in terms of quality control in the industrial cellar, MSPC charts can be helpful to predict if fermentation progresses too slow or fast, and this can provide the winemaker with additional information to make the necessary adjustments.

5.4.9 BATCH MODEL OF NORMAL RED WINES USING TIME AS *Y* VARIABLE

The red wines showed the same pattern in batch modelling of fermentation spectra, as found for the white wines. The much shorter lengths in fermentation time with the red wines are apparent (Figure 11A). Normal white wine fermentations take 10 - 14 days to ferment to dryness, while normal red wine fermentation takes 5 - 7 days. There is the fairly similar start point, variation in the middle and a similar endpoint, as observed with the white wines. One batch (purple line) (Figure 11B) clearly deviated considerably from the general trend of the other batches, and it could be expected that this batch contributed to a significant amount of variation in the mid-fermentation stage. The reason for this deviation in fermentation behaviour was not immediately clear.

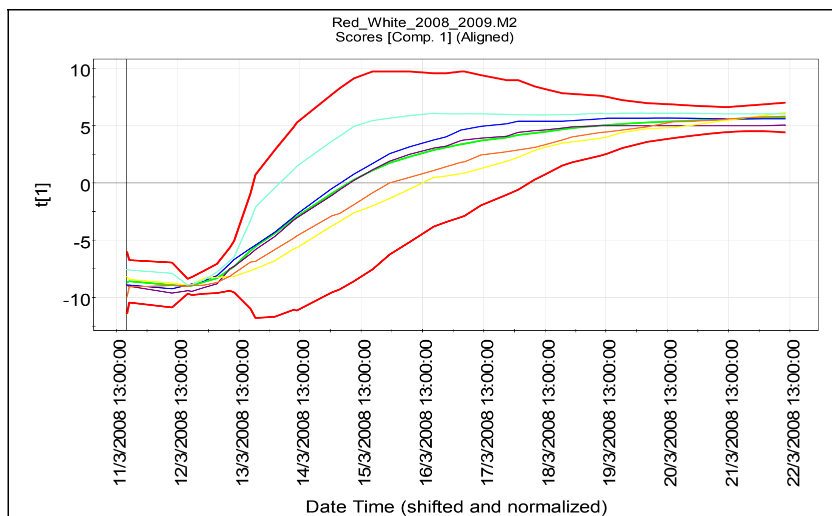

Figure 11A MSPC chart of the normal progress of red wine alcoholic fermentation in an industrial winery (aligned data). Average trend (green line) and 3 x SD (red lines), the upper and lower tolerance limits, are shown.

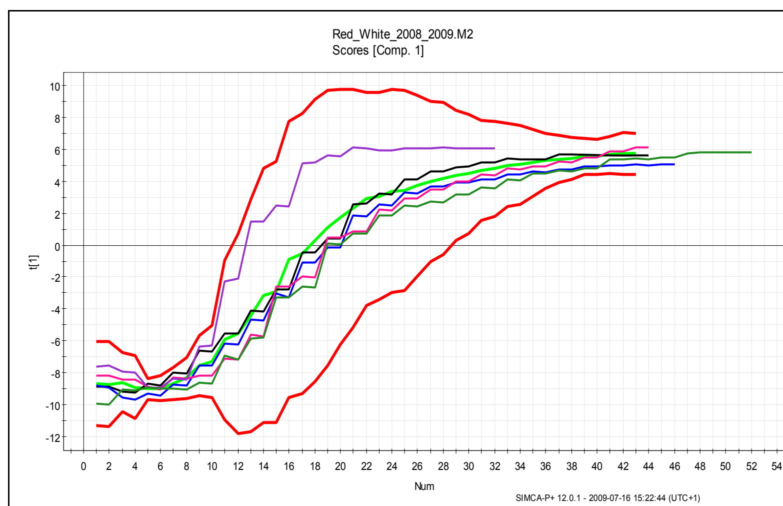

Figure 11B MSPC chart of the normal red wine fermentation with one outlier fermentation (purple line). Average trend (green line) and 3 x SD (red lines), which indicate the upper and lower tolerance limits (unaligned data).

5.4.10 PREDICTION OF ABNORMAL BATCHES OF RED WINES

The red wine fermentations that had normal patterns for alcoholic fermentation, was used as a training set to establish a PLS batch model of normal red fermentations, using aligned fermentation time as *y*-variable. This model was then used to predict the abnormal, or in this case, uninoculated red wine fermentations (Figure 12).

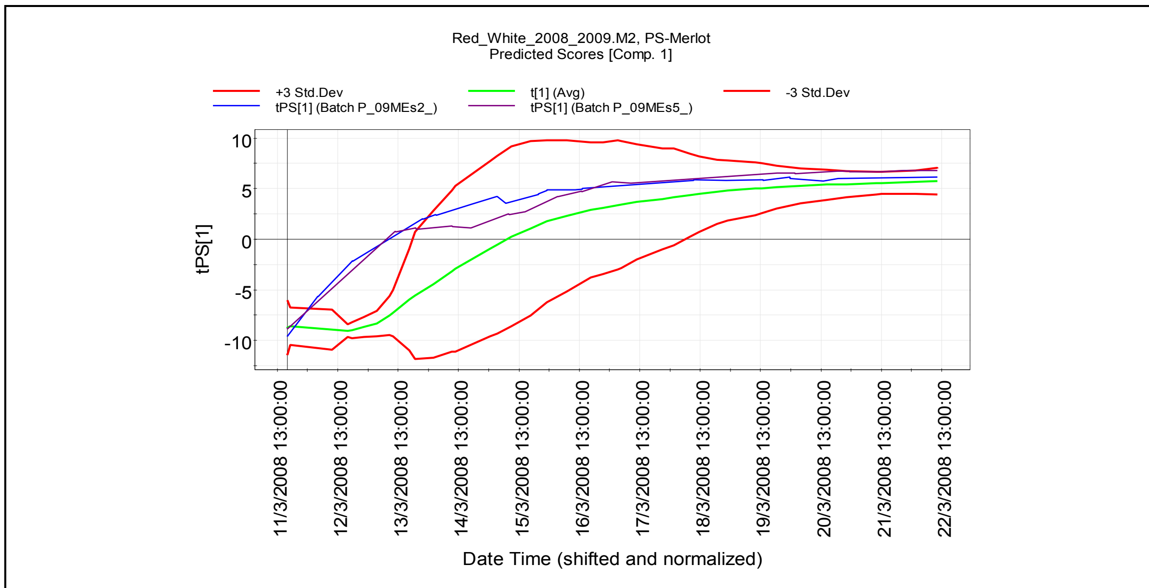

Figure 12 MSPC chart of abnormal Merlot fermentations, indicated by a purple and blue line respectively. Both fermentations were uninoculated. Average trend (green line) and 3 x SD (red lines) are shown.

The two uninoculated Merlot fermentations (indicated by a purple and blue line respectively) were detected as abnormal in the mid-fermentation stage, as indicated by the PLS scores locating above the upper tolerance limit (red line) of the model (Figure 12). Based on the small number of fermentations modeled, it is not possible to interpret this observation in terms of spontaneous fermentations. Nevertheless, the results are interesting on the basis of showing the modelling potential of the qualitative approach used in this project, for monitoring alcoholic fermentation.

5.5 CONCLUSION

Different components of the wine matrix can change during fermentation, but spectroscopy and chemometrics can offer simplified methods to monitor the process, allowing better control to achieve a desired outcome (Goshen *et al*., 2005). The results showed that it was possible to detect changes that occur during alcoholic fermentation, and to classify abnormal behaviour within fermentation, without considering the classical analytical parameters such as ethanol content or sugar levels. Different white- and red wine fermentations were used to predict abnormalities during alcoholic fermentation. The PCA **tt** score plots showed that there was a difference between the white- and red wines, and possibly an influence of cultivar, in the location of the samples in the score plots. The plots of the white wines showed that there were differences between the Extra Light wine and the other white wines, but no separation could be made between the Colombar and Chenin blanc wines. No clear vintage effects could be seen in

vintages 2008 and 2009. The batch models for the white- and red wine fermentations respectively, could be used to predict the abnormal fermentations within each group. PLS score plots were used to establish training sets for normal fermentations, using time as *y*-variable. The prediction of the abnormal fermentation white wine fermentation conducted at pH 2.8, was easily detected by the training model for white fermentations, while the white wine fermentation conducted with the addition of SO_2 to the must, was not identified as abnormal. The prediction of the uninoculated Merlot wines was also well predicted by the model. The approach used in this study was based on using time as *y* variable in the PLS models. One problem associated with using time as *y* variable, is that normal variation in fermentation patterns over time, is an inherent characteristic of wine production. In fact, fermentation time is frequently deliberately manipulated by the winemakers, in order to obtain specific style characteristics in the final product. The most important criterion in bioprocess monitoring for most wineries, is that the wine should ferment until dryness (<5 g/L sugar). Monitoring only the sugar levels in fermenting must, is clearly not enough, because abnormalities in the behaviour of alcoholic fermentation can not be detected early enough. Results obtained in Chapter 4 of this thesis, showed that quantitative data can be generated with high accuracy for the sugar and ethanol content. Multivariate modelling of the FT-IR spectra of samples taken from actively fermenting must, together with interpretations based on quantitative data, can provide much more information than either approach alone. FT-IR spectroscopy has the potential to significantly reduce analytical time and cost of chemical and physical measurements that are currently made during alcoholic industrial wine production, and the spectra obtained from fermenting must have the potential to predict abnormal fermentation in some instances. This can be a powerful strategy for quality control at wineries. Advantages of the approach include fast generation of results which is very important, since abnormalities can be identified almost immediately. The presentation of the MSC plots is also characterised by simplicity of interpretation, allowing better control to achieve a desired outcome. The work reported here is an exploratory study and requires further development with more fermentations before its potential may be realised by the wine industry.

5.6 ACKNOWLEDGEMENT

The author would like to thank Ing-Marie Olssen and Gitte Holm from Umetrics AB, Malmö, Sweden for their enormous contribution to process the data in this chapter.

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Chapter 6

General discussion and conclusions

6. GENERAL DISCUSSION AND CONCLUSIONS

This study had three major aims. The first aim was to evaluate the commercial available FT-IR calibration models for ethanol, pH, TA, VA and total glucose plus fructose content, for quantification purposes in fermenting must, using local South African samples. Although the ethanol calibration model performed very well, a new calibration model was developed, because some of the SA samples fell outside the concentration range of the commercial model. Predictions for pH, TA, VA and glucose plus fructose, did not perform well at all on the commercial models, and therefore new calibration models were developed. The new model of total glucose plus fructose performed extremely well and can be used for quantification purposes in an industrial cellar. The new models for pH and VA also performed well, and can be used for screening purposes and considered for quantification. The calibration model for TA was not considered fit for quantification, and fairly satisfactory for screening. The model showed good precision, however.

The second aim of this study was to developed and established new calibration models for glucose, fructose and YAN. The models for glucose and for fructose performed very well and can be used for quantification purposes. Two calibration models were developed for YAN using different reference methods. The first model was developed to predict only white fermenting must, and enzyme-linked spectrophotometer assays were used as the reference method. This model seemed to be fit for quantification in white fermenting musts, but large prediction errors in red fermenting must were found. The reason for this discrepancy is not clear, but could be related to interferences with the reference method in red fermenting musts. The second model was developed to predict YAN in both red- and white fermenting must, using the Formol titration method as reference method. This model seemed fit for screening purposes only. It is possible that the prediction performance of this calibration model can be improved in future, by increasing the number of calibration samples. In comparison to the 257 samples used for the enzyme-linked assay, only 113 calibration samples were used for the Formol method. The advantage of the second model is that it can be used to predict both red- and white fermenting musts, and by expanding the sample set in future work, this model can definitely be considered for quantification in fermenting must.

In order to maintain long-term instrument calibrations, it is very important to have a system in place in the wine analytical laboratory, for continuous and improved updates of the calibration database, by including diverse sample types collected during each successive season. Therefore, future updating of calibration models; maintenance of these models and associated quality assurance in the evaluation of their performance, are of crucial importance and must be seen as a continuous process. Calibration updates can be done by using mainly annual harvest check samples that cover the natural variation in fermenting must, and where the FT-IR spectra are collected according to a predefined procedure.

Part of the calibration model development phase of the project, was to investigate the use of samples stored frozen, in order to be able to expand the calibration sample sets. Frequently, it is not possible to complete all the reference analysis during harvest, due to time constraints. PCA analysis of fresh and frozen samples respectively, showed that samples stored frozen for up to three months can be used to expand calibration models for ethanol, pH, TA, VA, glucose, fructose and total glucose plus fructose. YAN was not evaluated for this purpose.

The opinion has been voiced, that control of the alcoholic fermentation process in wine production, can only be achieved if both substrate (for example, sugars, ethanol, acidity, nitrogen) and product quality can be monitored accurately and rapidly (Cozzolino *et al*., 2006). The results obtained in this study support this opinion and illustrated that FT-IR spectroscopy is

indeed a powerful tool for rapid evaluation of the alcoholic fermentation process in the industrial cellar. The proposed FT-IR spectroscopy models are thus effective alternatives to the analytical methods traditionally used to monitor fermentation processes. The technology is not without challenges and pitfalls however, and the timing of the sampling during fermentation, and the reliability of the data used for calibration, are among the critical factors for the successful application of FT-IR spectroscopy. In addition, the challenging aspect of representative sampling remains a factor that can not be ignored.

The third aim of this study was to do a small-scale investigation into the use of only FT-IR spectra to provide an informative overview of the pattern of the fermentation process in several tanks, and to possibly identify normal and abnormal behaviour of alcoholic fermentation. Results obtained, showed that red- and white wine fermentations should be modeled separately, which follows naturally, given the major differences in the fermentation process patterns associated with each group. The research explored the use of novel software, SimcaP+ (www.umetrics.com) for this purpose, and different graphic presentations were evaluated to provide an overview of the process behaviour, and to identify problem fermentations. The latter were experimentally induced at the onset of fermentation in the following ways: by lowering the initial must pH to 2.80; through addition of SO_2 to the must at the start of fermentation; and by not inoculating the fermentation with wine yeast starter cultures. The fermentation of low pH must was easily detected as abnormal when compared to the normal batches, while the addition of SO_2 did not seem to affect the fermentation behaviour that much. The spontaneous fermentation also showed abnormalities compared to other normal fermentations. The work reported here is a pilot study aimed at exploring the chosen strategy. Although the results show good potential to detect some problem fermentations, further analysis with considerably more fermentations of different varieties and vintages are needed, before the full potential of the development may be realised, and before this approach can be adopted in the industrial cellar. Clearly, an extended database of “normal fermentations” for each set of conditions must first be established, before the can be used as reference against which to evaluate problem fermentations.

Although a significant amount of the investigations in this study yielded very positive results, some aspects can be improved. The performance of the calibration model for TA was not satisfactory, due to the high prediction errors, ~ 0.35 g/L. Since this parameter is of great importance in monitoring alcoholic fermentation, a thorough investigation should be performed to identify the possible reasons for this somewhat poor performance. One of the disadvantages of the reference method (automatic titration with NaOH and pH electrode), is the requirement that all excessive CO_2 in the fermenting must must be removed prior to titration of the must sample, since this is a known source of error in TA analysis (Zoecklein, 1995, 2002). During the most active phase of alcoholic fermentation, must contains huge amounts of CO_2 and it is challenging to remove all and keep the sample matrix stable. The determination of YAN with the enzyme-linked spectrophotometric assay also showed large prediction errors. It appeared that the Formol titration method was more repeatable and less prone to matrix interferences. The calibration model for YAN using the Formol titration, should however be expanded to improve the regression statistics. Although the Formol titration is described as precise, but not extremely accurate (Zoecklein, 2002) the method can still be used to give an indication of the nitrogen content in fermenting must.

The positive contributions that were made during this study include the development and establishment of FT-IR spectroscopy-based quantitative calibration models that achieved good levels of accuracy, precision, repeatability and robustness for different components, and in many different sample types. Through these contributions, the potential applications on the

WineScan FT-IR spectrometer that is used widely in the SA wine industry were expanded considerably. Graphic models in the form of MSPC charts provided the first overview of the process patterns associated with alcoholic fermentation in industrial-scale tanks, and this approach shows great potential to be used to identify some problem fermentations. This development also added an extra dimension to the current applications of the WineScan instrument. An advantage of using only FT-IR spectra for the construction of the MSPC charts, is that historic data can be used, and a graphic database of alcoholic fermentation patterns be established that can be a useful source of information for interpretation of the process patterns of alcoholic fermentations in future harvest seasons. The next logical step following this off-line approach would be on-line monitoring of alcoholic fermentation, and real-time construction of control charts. An ideal tool for such an approach is near-infrared spectroscopy.

The direction of the developments and positive outcomes of this study, are illustrated in Figure 1. Although wet chemical tests will always be required for some analyses, the number of tests required can be drastically reduced during fermentation monitoring, through exploitation of the advantages of FT-IR spectroscopy and advanced multivariate data analysis methods.

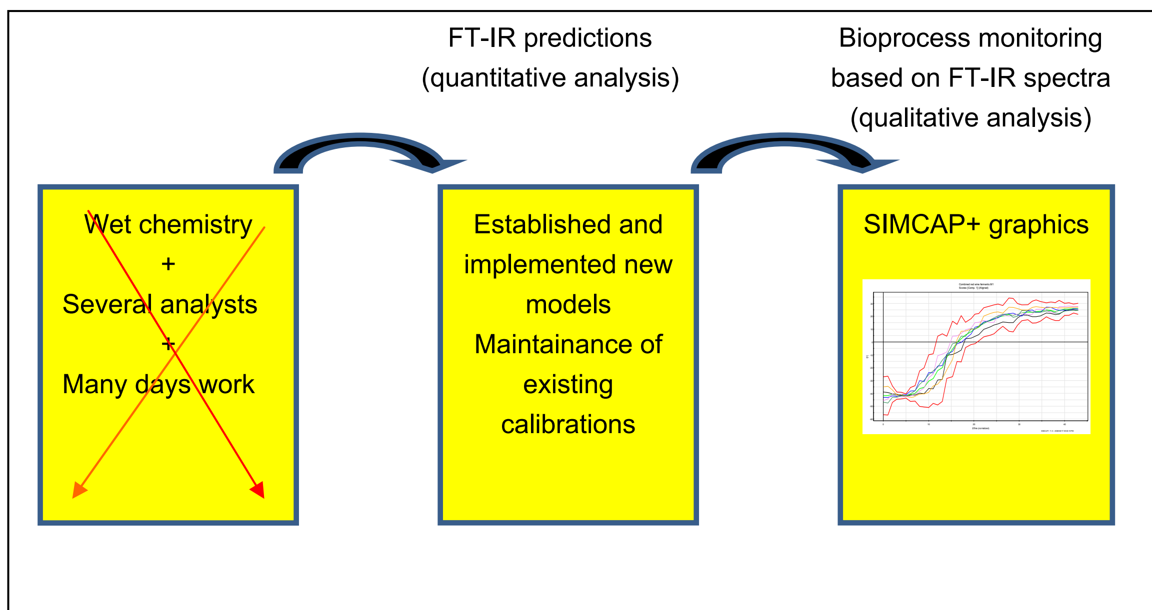

Figure 1. Diagrammatic presentation of the direction of developments and positive outcomes of this study.

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Addendum A

Enzyme-linked assays for fermenting must

Reactions and calculations

ENZYME-LINKED ASSAYS

D-FRUCTOSE AND D-GLUCOSE (MEGAZYME CAT. NO. K-FRUGL 11/05, www.megazyme.com)

In the wine industry, the D-glucose and D-fructose content, is of the most important quality parameters and should be monitored at each stage of the alcoholic fermentation process. These sugars can be measured either independently or simultaneously.

The measurement of D-glucose and D-fructose is accomplished in a three step reaction. The D-glucose and D-fructose are phosphorylated in the presence of the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) respectively, with the simultaneous formation of adenosine-5'-diphosphate (ADP), (1, 2).

$$\text{D-glucose} + \text{ATP} \xrightarrow{HK} \text{G-6-P} + \text{ADP} \quad (1)$$

$$\text{D-fructose} + \text{ATP} \xrightarrow{HK} \text{F-6-P} + \text{ADP} \quad (2)$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (3).

$$\text{G-6-P} + NADP^+ \xrightarrow{G6P-DH} \text{D-gluconate-6-phosphate} + \text{NADPH} + H^+ \quad (3)$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose present in the fermenting must samples and is measured by the absorbance at 340 nm.

After completion of the reaction (3), F-6-P is then converted to G-6-P by phosphoglucose isomerase (PGI) (4).

$$\text{F-6-P} \xleftrightarrow{PGI} \text{G-6-P} \quad (4)$$

The G-6-P formed reacts in turn with $NADP^+$ forming gluconate-6-phosphate and NADPH, leading to a increase in absorbance that is stoichiometric with the amount of D-fructose present in the fermenting must samples and is measured by the absorbance at 340 nm.

PROCEDURE FOR THE ENZYME-LINKED ASSAY IN FERMENTING MUST SAMPLES

Pipette into cuvettes	Blank	Sample
distilled water	2.10 mL	2.00 mL
sample	-	0.10 mL
imidazole buffer	0.10 mL	0.10 mL
$NADP^+$/ATP	0.10 mL	0.10 mL
Mix, read the absorbance of the solutions (A_1) after approx. 3 min and start the reaction by addition of:		
HK/G6P-DH	0.02 mL	0.02 mL
Mix, read the absorbance of the solutions (A_2) at the end of the reaction (approx. 5 min).		
PGI	0.02 mL	0.02 mL
Mix, read the absorbance of the solutions (A_3) at the end of the reaction (approx. 8-10 min).		

CALCULATION OF GLUCOSE + FRUCTOSE CONCENTATION IN FERMENTING MUST SAMPLES

$\Delta A_{D\text{-glucose}} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$

$\Delta A_{D\text{-fructose}} = (A_3\text{-}A_2)_{sample} - (A_3\text{-}A_2)_{blank}$

The values of $\Delta A_{D\text{-glucose}}$ and $\Delta A_{D\text{-fructose}}$ were more than 0.100 absorbance units to achieve accurate results.

The concentration of D-glucose and D-fructose was calculated as follows:

$$C = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of D-glucose or D-fructose [180.16 g/mol]
ε = extinction coefficient of NADPH at 340nm [6300 l x mol^{-1} x cm^{-1}]
d = light path length [cm]
v = sample volume [mL]

If the sample was diluted during preparation, the final result was multiplied by the dilution factor, F.

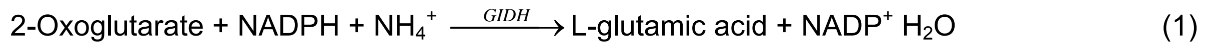
Table 1. Dilution table for the fermenting must samples

Estimated concentration of D-glucose + D-fructose (g/L)	Dilution with water	Dilution factor (F)
<0.8	No dilution required	1
0.8-8.0	1 + 9	10
8.0-80	1 + 99	100
>80	1 + 999	100

AMMONIA (RAPID) (MEGAZYME CAT. NO. K-AMIAR 11/05, www.megazyme.com)

In the wine industry, the measurement of ammonia is important in the calculation of yeast assimilable nitrogen, which forms a very important part of alcoholic wine fermentation. Ammonia is a natural compound, often produced of microbial protein catabolism, and can serves as a quality indicator in certain products. The addition of nutrient supplements to must during fermentation can be accurately managed by the measurement of yeast assimilable nitrogen (YAN). It is also important, as too little available nitrogen can lead to problem fermentation and the generation of hydrogen sulphide (H_2S). The calculation of YAN content in the fermenting must samples were done by the measurement of both ammonia and primary amino nitrogen.

In the presence of glutamate dehydrogenase (GlDH) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), ammonia (as ammonium ions, NH_4^+) reacts with 2-oxoglutarate to form L-glutamic acid and $NADP^+$ (1).



2-Oxoglutarate + NADPH + NH_4^+ $\xrightarrow{GlDH}$ L-glutamic acid + $NADP^+$ H_2O (1)

The amount of $NADP^+$ formed is stoichiometric with the amount of ammonia present in the fermenting must samples. It is NADPH consumption which is measured by the decrease in absorbance at 340nm.

PROCEDURE FOR THE ENZYME-LINKED ASSAY IN FERMENTING MUST SAMPLES

Pipette into cuvettes	Blank	Sample
distilled water	**2.10 mL**	**2.00 mL**
sample	-	0.10 mL
NADHP/TEA buffer	0.50 mL	0.50 mL
Mix, read the absorbance of the solutions (A_1) after approx. 2 min and start the reactions immediately by addition of:		
GIDH	0.02 mL	0.02 mL
Mix, read the absorbance of the solutions (A_2) at the end of the reaction (approx. 3 min).		

CALCULATION OF AMMONIA CONCENTRATION IN FERMENTING MUST SAMPLES

The value of $\Delta A_{ammonia}$ was more than 0.100 absorbance units to achieve accurate results.

The concentration of ammonia was calculated as follows:

$$C = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \quad \text{x} \quad \Delta A_{ammonia} \qquad [g/L]$$

where:

V = final volume [mL]

MW = molecular weight of ammonia [17.03 g/mol]

ε = extinction coefficient of NADPH at 340nm [6300 l x mol^{-1} x cm^{-1}]

d = light path lenght [cm]

v = sample volume [mL]

No samples were diluted for the ammonia assay.

PRIMARY AMINO NITROGEN (PAN) (MEGAZYME CAT. NO. K-PANOPA 03/06, www.megazyme.com)

The amino nitrogen groups of yeast assimilable acids in the sample react with N-acetyl-L-cysteine and o-phthaldialdehyde to form isoindole derivatives (1)[2].

Amino nitrogen + N-acetyl-L-cysteine + o-phthaldialdehyde $\longrightarrow$ isoindole derivative (1)

The amount of isoindole derivative formed in this reaction is stoichiometric with the amount of primary amino nitrogen. It is the isoindole derivative that is measured by the increase in absorbance at 340nm.

PROCEDURE FOR THE ENZYME-LINKED ASSAY IN FERMENTING MUST SAMPLES

Pipette into cuvettes	Blank	Sample
NAC/buffer	3.00 mL	3.00 mL
Distilled water	0.05 mL	-
sample	-	0.05 mL
Mix, read the absorbance of the solutions (A_1) after approx. 2 min and start the reactions immediately by addition of:		
OPA	0.10 mL	0.10mL
Mix, read the absorbance of the solutions (A_2) at the end of the reaction (approx. 15 min).		

CALCULATION OF PAN CONCENTRATION IN FERMENTING MUST SAMPLES

The value of ΔA_{PAN} was more than 0.100 absorbance units to achieve accurate results.

The concentration of PAN (primary amino nitrogen) was calculated as follows:

$$C = \frac{V \times MW \times 1000}{\varepsilon \times d \times v} \times \Delta A_{PAN} \quad \text{[mg of N/L]}$$

where:

V = final volume [mL]

MW = molecular weight of nitrogen [14.01 g/mol]

ε = extinction coefficient of NADPH at 340nm [6300 l x mol^{-1} x cm^{-1}]

d = light path [cm]

v = sample volume [mL]

No samples were diluted for the PAN assay.

Addendum B

List of references with abbreviations used in this study

LIST OF REFERENCES WITH ABBREVIATIONS

Agriculturae Conspectus Scientificus	*Agric. Conspec.Sci.*
American Journal of Enology and Viticulture	*Am. J. Enol. Vitic.*
Analyst	*Analyst*
Analytica Chimica Acta	*Anal. Chim. Acta*
Analytical and Bioanalytical Chemistry	*Anal. Bioanal. Chem.*
Analytical Chemistry	*Anal. Chem.*
Applied Environmental Microbiology	*Appl. Environ. Microbiol.*
Applied Spectroscopy	*Appl. Spectrosc.*
Australian Grapegrower and Winemaker	*Aust. Grapegrower Winemaker*
Australian Journal of Grape and Wine Research	*Aust. J. Grape Wine Res.*
Australian Society of Viticulture and Oenology	*Aust. Soc. Vitic. Oenol.*
Biochimica et Biophysica Acta	*Biochim. Biophys. Acta*
Biotechnology and Bioengineering	*Biotech. Bioeng.*
Carbohydrate Polymers	*Carbohydr. Polym.*
Chemometrics and Intelligent Laboratory Systems	*Chemom. Intell. Lab. Syst*
Chinese Journal of Analytical Chemistry	*Chinese J. Anal. Chem.*
Computers and Electronics in Agriculture	*Comput. Electron. Agric.*
Food Australia	*Food Aust.*
Food Chemistry	*Food Chem.*
Food Control	*Food Control*
Food Microbiology	*Food Microbiol.*
Food Research International	*Food Res. Int.*
Food Technology Australia	*Food. Tech. Aust.*
Fresenius Journal of Analytical Chemistry	*Fresen. J. Anal. Chem.*
Journal of Agricultural and Food Chemistry	*J. Agric. Food Chem.*
Journal of Analytical Chemistry	*J. Anal. Chem.*
Journal of Applied Bacteriology	*J. Appl. Bacteriol.*
Journal of Biological Chemistry	*J. Biol. Chem.*
Journal of Bioscience and Bioengineering	*J. Biosci. Bioeng.*
Journal of Chromatography A	*J. Chrom. A*
Journal of Fermentation and Bioengineering	*J. Ferm. Bioeng.*
Journal of Food Engineering	*J. Food Eng.*
Journal of Food Protection	*J. Food Prot.*
Journal of Industrial Microbiology and Biotechnology	*J. Ind. Microbiol. Biotechnol.*
Journal of Industrial Microbiology	*J. Ind. Microbiol.*
Journal of Microbiological Methods	*J. Micriobiol. Methods*
Journal of Near Infrared Spectroscopy	*J. Near Infrared Spectrosc.*
Journal of Rapid Methods and Automation in Microbiology	*J. Rap. Methods Auto. Microbiol.*
Journal of the Science and Food Agriculture	*J. Sci. Food Agric.*
Microchemical Journal	*Microchem. J.*
Phytochemistry	*Phytochem.*
Postharvest Biology and Technology	*Postharv. Biol. Techol.*
Practical Winery and Vineyard	*PWV.*
Program in Industrial Microbiology	*Prog. Ind. Microbiol.*
Revue Francaise d'Œnologie	*Rev. Fr. Œnol.*

Spectroscopy and Spectral Analysis	*Spectrosc. Spec. Analysis*
Spectroscopy Europe	*Spectrosc. Europe*
South African Journal of Enology and Viticulture	*S. Afr. J. Enol. Vitic.*
Talanta	*Talanta*
Technical Review	*Tech. Rev.*
The Australian and New Zealand Grapegrower and Winemaker	*Aust. N. Z. Grapegrower Winemaker*
Trends in Food Science and Technology	*Trends Food Sci. Technol.*
Turkish Journal of Agriculture and Forestry	*Turk. J. Agric. For.*
Vibrational Spectroscopy	*Vib. Spectrosc.*
Viticulture and Enology Science	*Vitic. Enol.* Sci.
Vineyard and Vintage View	*Vineyard and Vintage View*
Vineyard and Winery Management	*VWM.*
Yeast	*Yeast*