

The Evaluation of Fourier Transform Infrared (FT-IR) Spectroscopy and Multivariate Data Analysis Techniques for Quality Control at an Industrial Cellar

by

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Declaration

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Summary

The alcoholic beverage industry needs objective analysis of grape juice and liqueur quality. Fourier transform infrared (FT-IR) spectroscopy with multivariate data analysis techniques is widely used in wine laboratories across South Africa for accurate, fast and high sample throughput analyses. In this study the potential of FT-IR spectroscopy is evaluated for the quantification of ammonia in freshly pressed grape juice. FT-IR spectroscopy is evaluated, using two different spectrometers, in respectively attenuated total reflection (ATR) - and transmission scanning modes for the quantification of alcohol, pH and invert sugar in spirit-based liqueurs. The ultimate aim was to implement the PLS regression algorithms developed at an industrial cellar and replace the complex and lengthy reference methods used at the time of this study. Principle component analysis (PCA) was performed prior to the calibration step to identify groupings and patterns within the spectra. The PLS calibration models were developed from samples collected at the cellar and using partial least square (PLS) regression. The models were evaluated using the performance criteria coefficient of determination (R^2) and root mean squared error of cross validation (RMSECV) at calibration stage, and root mean square error of prediction (RMSEP) and residual predictive deviation ratio (RPD) at validation stage.

The average RMSEP (1.88 mg/L) of the ammonia PLS calibration model was in agreement with the standard error of laboratory (SEL = 1.54 mg/L). The R^2 (92.05) and average RPD (3.3) proposed a model with excellent precision for screening purposes that was ready to be transferred for use by the laboratory.

The r^2 values for the alcohol, pH and invert sugar PLS calibration models obtained in ATR and transmission, indicated good to excellent precision ($80 < r^2 < 100$). The alcohol PLS calibration model obtained in transmission was suitable for quality- and process control purposes (RPD = 21.2), while the invert sugar PLS calibration model for quality control purposes ($5 < \text{RPD} < 6.4$). The pH and invert sugar calibration models obtained in ATR were suitable for screening purposes with RPD = 3.6 and RPD = 4.8, respectively. These PLS regression algorithms were implemented at the cellar. The pH PLS calibration model obtained in transmission was suitable for rough screening of samples (RPD = 2.7) and future development was necessary to increase the predictability of the model.

The results obtained in this study made a significant contribution towards validation of FT-MIR as a powerful tool for rapid quantification of quality indicating parameters in wine and spirit-based liqueurs. The contribution is particularly valuable in the context of ongoing research to improve the quality of products at the cellar to meet consumer demands. The knowledge gained on quantification of quality indication parameters of spirit-based liqueurs is novel and this is one of the first reports on implementation of mid-infrared (MIR) spectroscopy for the quality control of South African spirit-based liqueurs.

Opsomming

Die wynindustrie benodig objektiewe analyses van druiwesap- en likeurgehalte. Fourier-transformasie-infrarooi (FT-IR) spektroskopie met multiveranderlike statistiese metodes word gebruik in wynlaboratoriums regoor Suid-Afrika vir akkurate, vinnige en hoë monsterdeurset ontledings. In hierdie studie is die potensiaal van FT-IR spektroskopie geëvalueer vir die kwantifisering van ammoniak in die sap van vars gepaste wyndruiwe. Twee verskillende FT-IR spektroskopie instrumente, in onderskeidelik (verswakte totale refleksie, ATR) - en transmissie skandering is gebruik vir die kwantifisering van alkohol, pH en invertsuiker in spiritus-gebaseerde likeurs. Die uiteindelige doel was om die parsieë kleinste kwadraat (PKK)-regressie algoritmes wat ontwikkel is, by 'n industriële kelder te implementeer en die komplekse en tydrowende verwysingmetodes wat tydens die studie in die kelder gebruik is te vervang. Verskeie multiveranderlike hoofkomponentanalise (MVK) is uitgevoer voor die kalibrasie stap, met die doel om groeperings en patrone in die spektra te identifiseer. Die PKK kalibrasiemodelle is ontwikkel van monsters wat by die kelder versamel is en die spektra is gebruik in die PKK regressies. Tydens die kalibrasiefase is die modelle geëvalueer met behulp van die bepalingseffisiënt (R^2) en gemiddelde kalibrasieprediksiefout en tydens die validasiefase, met behulp van die standaardvoorspellingsfout (SVF) en relatiewe voorspellingsafwyking (RVA). Die gemiddelde SVF (1.88 mg/L) van die ammoniak kalibrasiemodel was in ooreenstemming met die standaard fout van die laboratorium (SEL = 1.54 mg/L). Die R^2 (92.05) en die gemiddelde RVA (3.3) dui op 'n model met uitstekende presiesheid wat gereed is vir oordra en gebruik deur die industrie.

Die R^2 waardes vir die alkohol-, pH- en invertsuiker –kalibrasie-modelle wat met ATR en transmissie vir die likeurmonsters ontwikkel is, dui op goeie tot uitstekende presiesheid ($80 < R^2 < 100$). Die alkoholkalibrasiemodel wat ontwikkel is in transmissie, is geskik vir kwaliteits- en prosesbeheerdoelwitte ($RVA = 21.2$), terwyl die invertsuiker kalibrasiemodel geskik is vir kwaliteitsbeheer doelwitte ($5 < RVA < 6.4$). Die pH en invertsuiker kalibrasiemodelle in ATR is geskik vir vinnige evalueringdoelwitte, met $RVA = 3.6$ en $RVA = 4.8$ waardes, onderskeidelik. Hierdie algoritmes is ook in die kelder geïmplementeer. Die pH kalibrasiemodel in transmissie was geskik vir vinnige evalueringdoelwitte ($RVA = 2.7$) en toekomstige ontwikkeling is nodig om die voorspellingsakkuraatheid van die model te verbeter.

Die resultate van hierdie studie het 'n betekenisvolle bydrae gelewer tot bevestiging van infrarooi spektroskopie as 'n kragtige tegnologie vir die vinnige kwantifisering van gehalteparameters in druiwesap en spiritus-gebaseerde likeurs. Die bydrae is veral waardevol in die konteks van voortgesette navorsing om die kwaliteit van produkte by die kelder te verbeter en aan verbruikerseise te voldoen. Die studie vir die kwantifisering van gehalteparameters in spiritus-gebaseerde likeurs is eerste in sy soort en een van die eerste

gerapporteerde verslae vir die implementering van infrarooi spektroskopie vir gehaltebeheer van Suid-Afrikaanse spiritus-gebaseerde likeurs.

This thesis is dedicated to my family.

“A dream, all a dream, that ends in nothing, and leaves the sleeper where he lay down, but I wish you to know that you inspired it.”

—Charles Dickens

Biographical sketch

Ansunette Hoon was born in Bloemfontein, South Africa on the 10th of April 1982. She matriculated at Central High School, Beaufort West, 2000. Ansunette obtained a BSc degree in Food Science in 2006 at the University of Stellenbosch.

Ansunette is currently working as Product Developer at Klein Karoo International Ltd., Oudtshoorn, South Africa. She enrolled for an MSc degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University in 2010.

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Preface

This thesis is presented as a compilation of five chapters as indicated below.

Chapter 1 **General introduction, problem statement, project aims and related tasks**

Chapter 2 **Literature review**

Section 1 Ammonia in grape juice

Section 2 Important quality indicating parameters of spirit-based liqueurs

Section 3 Spectroscopy and data analysis

Chapter 3 **Research results**

Application of Fourier transform mid-infrared (FT-MIR) attenuated total reflectance (ATR) spectroscopy for the quantification of ammonia in freshly pressed grape juice

Chapter 4 **Research results**

Application of Fourier transform mid-infrared (FT-MIR) spectroscopy, in attenuated total reflection (ATR) and transmission scanning modes, for the quantification of alcohol, pH and invert sugar in spirit-based liqueur products

Chapter 5 **General discussion and conclusions**

LIST OF ABBREVIATIONS USED IN STUDY

| | |
|------------------------------|--|
| NH ₃ | ammonia |
| NH ₄ ⁺ | ammonium ion |
| ATR | attenuated total reflectance |
| <i>B. cinerea</i> | <i>Botrytis cinerea</i> |
| CaF ₂ | calcium fluoride |
| CO ₂ | carbon dioxide |
| C | Celsius |
| cm ⁻¹ | centimetres |
| R ² | coefficient of determination |
| CYN | complex yeast nutrients |
| Cu ²⁺ | copper |
| (°) | degree |
| DAP | diammonium phosphate |
| FIR | far-infrared |
| PC1 | first principle component |
| i.e. | that is |
| FTIR | Fourier transform infrared |
| FT-MIR | Fourier transform mid-infrared |
| FT-MIR ATR | Fourier transform mid-infrared attenuated total reflection |
| FAN | free amino acids |
| GIDH | glutamate dehydrogenase |
| g/L | grams per litre |
| HCl | hydrochloric acid |
| H ⁺ | hydrogen ions |
| H ₂ S | hydrogen sulphide |
| IR | infrared |
| L | litres |
| Mg | magnesium |

| | |
|----------------|---|
| Mn | manganese |
| Max | maximum |
| µm | micrometre |
| MIR | mid-infrared |
| mg | milligrams |
| mg/L | milligrams per litre |
| ml | millilitres |
| mm | millimetres |
| mV | millivolt |
| Min | minimum |
| nm | nanometre |
| NIR | near infrared |
| NADH | nicotinamide adenosine dinucleotide |
| N | normality |
| No. | number |
| OAH | O-acetylhomoserine |
| OAS | O-acetylserine |
| NOPA | o-phthaldialdehyde/N-acetyl-L-cysteine |
| O ₂ | oxygen |
| pp. | page |
| PLS | partial least squares |
| % | percentage |
| K | potassium |
| kBr | potassium bromide |
| PCA | principal component analysis |
| PC | principal component |
| PAT | process analytical technology |
| RPD | residual predictive deviation ratio |
| RMSECV | root mean squared error of cross validation |
| RMSEP | root mean square error of prediction |

| | |
|----------------------|---|
| <i>S. cerevisiae</i> | <i>Saccharomyces cerevisiae</i> |
| PC2 | second principle component |
| NaOH | sodium hydroxide |
| SANAS | South African National Accreditation System |
| SD | standard deviation |
| SECV | standard error of cross validation |
| SEL | standard error of laboratory |
| SEP | standard error of prediction |
| TA | titratable acidity |
| t | tons |
| VA | volatile acidity |
| v/v | volume per volume |
| YAN | yeast assimilable nitrogen |
| Zn | zinc |

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LIST OF CONFERENCE PRESENTATIONS

Hoon, A. & H. Nieuwoudt (2010). Monitoring grape quality with Fourier transform infrared spectroscopy in an industrial cellar. 32nd SASEV Congress, Lord Charles Hotel, 18-19 November, Somerset West. POSTER.

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Chapter 1

**General introduction,
problem statement, project aims
and related tasks**

1. GENERAL INTRODUCTION, PROBLEM STATEMENT AND PROJECT AIMS AND RELATED TASKS

1.1 GENERAL INTRODUCTION

DGB (Pty) Ltd, Wellington, Western Cape, South Africa, is one of South Africa's largest independent wine, liqueur and spirit producers and distributors based on the amount of litres annually sold to the national and international consumer markets. The company was established in 1990 and consists of three cellars in relative close proximity of each other; the mentioned cellar in Wellington, as well as Franschhoek and Boschendal. Routine chemical analysis on grapes, wines and other types of alcoholic beverages of DGB are performed by the analytical laboratory in the Wellington cellar that uses mostly wet chemistry methods. Recently, mid-infrared spectroscopy (in transmission mode) was also phased in for analysis of selected parameters in grapes and wine. The company owns the wine brands Bellingham, Douglas Green, Boschendal, Tall Horse, Franschhoek Cellar, The Beachhouse, Ribshack Red, Culemborg, The Saints, Brampton and Legacy. The well-known spirit-based liqueur brands Zappa, Butlers, Potency, Malibu, Apple Tang, Kiss and Lavoka, as well as some cream liqueurs, are also produced by DGB.

In today's tough economic conditions, wineries are forced to strive hard to meet consumer preferences and at the same time remain profitable. The demand in local and international markets for lower-cost products that are high in quality remains strong. Against this scenario, the importance of suitable actions by the producing cellars to check and control the quality of products through the whole production process is clear. DGB continuously identifies key quality control points along the production and bottling lines of its various product types. The evaluation of the current status and optimisation of the analytical procedures in use at two of these points formed the basis of the research objectives of this thesis.

The first objective was related to wine production; more specifically, for the monitoring of the ammonia (NH_3) content of freshly pressed grape juice prior to onset of alcoholic fermentation. The second objective was to develop rapid methods for the quantification of alcohol, pH and invert sugar in spirit-based liqueur products throughout the production and bottling processes.

In the wine industrial cellar environment, direct and real-time communication between the viticulturist, winemakers, cellar master and the staff in the analytical laboratory is necessary to ensure that wine quality is maintained throughout the production process, and that appropriate corrective steps are taken timeously when quality-related problems arise. Good wine starts in the vineyard and for production of the top quality brands, the aim is therefore to grow, or buy in, grapes of high quality and to preserve the quality through fermentation, maturation and the bottling of the final product. This is a multi-faceted task that requires numerous interventions, including the

accurate measurement of the concentration of NH_3 in grape juice. NH_3 is an important nutrient for the wine yeast; its availability directly affects the fermentation performance of yeast and hence also the final quality of the wine. The enzyme-linked spectrophotometric assay is traditionally used to quantify NH_3 in grape juice in the wine industry and this is also the method used in the chemical laboratory of DGB. If the amount of NH_3 is insufficient for the wine yeast to complete fermentation, diammonium phosphate (DAP) or complex yeast nutrients (CYN) are the supplements used most commonly by winemakers in the industry (Blateyron & Sablayrolles, 2001). DGB annually harvests some 8000 tons (t) of grapes, and the implications to do this volume of analysis with the enzyme assay motivated an investigation into a more cost- and time efficient method to quantify NH_3 in grape juice, while also at the same time provide the winemaker with real-time information for immediate decision making.

Liqueurs are classified as grape-based and spirit-based liqueurs with an alcohol content of less than 60 percent (%) volume per volume (v/v) (Gallignani *et al.*, 2005). Spirit-based liqueurs are made from distilled sugar cane spirit sweetened with sucrose and/or caramel and flavoured with herbs, fruits, spices or other flavourings. Alcohol, pH and invert sugar are important quality control indicators in these beverages and their concentration ranges must meet strict cellar specifications. Taxes are also imposed in different countries on the alcohol content of beverages and very strict regulations apply to alcohol quantification. The concentration results that fall outside the specification or acceptance criteria, has social and economic implications. Therefore, the accurate determination of these parameters is a very important. Analytical methods for the determination of alcoholic strength in spirits have been reported and involve pycnometry, electronic densimetry, densimetry using a hydrostatic balance, hydrometry (Brereton *et al.*, 2003) and biosensor technology (Hnaïen *et al.*, 2010). The method in use at DGB is electronic densimetry. pH is determined with automatic titration, using a combination electrode and a temperature probe. Traditional methods for measurement of invert sugars are based on manual titration methods (Urbano Cuadrado *et al.*, 2005). These wet chemistry methods are lengthy; they generate large quantities of chemical waste and require a fully staffed laboratory. Therefore, the development of new analytical methods and optimisation of existing analytical procedures that are fast, precise, simple and cost saving is necessary for the quantification of alcohol, pH and invert sugar in spirit-based liqueurs.

Fourier transform mid-infrared (FT-MIR) spectroscopy in combination with attenuated total reflectance (ATR) and with transmission scanning modes, are well-established analytical tools for chemical analysis in the beverage industry (Bauer *et al.*, 2008; Cozzolino *et al.*, 2011). This is the result of the technology's speed of analysis (less than 30 seconds per sample), low unit cost per assay, simple or no sample preparation, reliability of instrumentation and simplicity of use. The spectrum produced by infrared (IR) spectroscopy is a compositional fingerprint with chemical information about the sample. Due to the complexity of the information contained in FT-MIR

spectra, an extensive calibration process that involves multivariate data analysis techniques, i.e. principal component analysis (PCA) and partial least squares (PLS) is required to identify trends and variations between spectra (Bro, 2003).

The use of FT-MIR spectroscopy for the prediction of NH_3 in grape juice (Patz *et al.*, 2004) and parameters such as alcohol and density in spirits (Lachenmeier, 2007) has been reported. At DGB, FT-MIR spectroscopy in transmission mode has been implemented recently and PLS calibration models for the quantification of alcohol, volatile acidity (VA), total acidity (TA), and pH in wine and malic acid, TA and pH in fermenting juice have been optimised and are currently used routinely in the analytical laboratory. Research to further optimise and expand the technology at DGB is an on-going process. This prioritised the need for the development of PLS calibration models for the quantification of NH_3 in grape juice and alcohol, pH and invert sugar in spirit-based liqueurs. Of major importance in consideration of infrared spectroscopy as an alternative option to the wet chemistry methods were environmental aspects, particularly because FT-MIR technology generates no chemical waste.

1.2 PROBLEM STATEMENT

At the onset of this project, the WinescanTM Flex instrument (Foss Analytical, Denmark) that measures liquid samples in transmission mode was already in use at DGB. It was of interest to also evaluate the smaller ATR FT-MIR spectrometer, Alpha-P Bruker Optics (Bruker Optics, Bryanston, South Africa), for reasons that include lower instrument acquisition price and maintenance cost, while both solid and liquid samples can be analysed. The Alpha-P instrument is portable, in contrast to the WinescanTM Flex, and the long-term aim was to develop the technology for grape and liqueur analysis so that the different DGB cellars could ultimately benefit from the PLS models developed in this study.

The Alpha-P spectrometer was on loan to DGB for restricted periods of time, based on availability from the supplier, Bruker Optics (Bruker Optics, Bryanston, South Africa) to conduct the project. Therefore, samples obtained for the development of calibration models were based on availability and not pre-selected. The development of an alcohol PLS calibration model for spectra obtained in ATR mode was not possible due to the absence, at the time of the project, of a closed flow injection system on the spectrometer's sample plate that restricts evaporation (www.bruker.com).

In summary, the main objectives throughout this project were to develop the PLS calibration models for NH_3 in grape juice, and alcohol, pH and invert sugar in spirit-based liqueurs using the respective instrumentation, and to evaluate the models using performance criteria for coefficient of determination (R^2), root mean squared error of cross validation (RMSECV), root mean square error

of prediction (RMSEP) and residual predictive deviation ratio (RPD) for implementation in DGB industrial cellars.

1.3 PROJECT AIMS AND RELATED TASKS

1.3.1 To evaluate the potential of Fourier transform mid-infrared attenuated total reflection (FT-MIR ATR) spectroscopy for the quantification of NH₃ in freshly pressed grape juice and to implement the PLS regression algorithm for routine analysis in an industrial cellar.

Related tasks:

- Obtain FT-MIR ATR spectra on Alpha-P spectrometer (Bruker Optics, Bryanston, South Africa).
- Determine reference values and standard error of laboratory (SEL) for the large range of different grape cultivars harvested annually.
- Develop NH₃ PLS calibration model.
- Evaluate the NH₃ PLS calibration model using the performance criteria R², RMSECV, RMSEP and RPD for routine analysis in an industrial cellar.

1.3.2 To evaluate the potential of FT-MIR spectroscopy using two different scanning modes, respectively ATR and transmission, for the quantification of alcohol, pH and invert sugar in spirit-based liqueurs and to implement the PLS regression algorithms for routine analysis in an industrial cellar.

Related tasks :

- Obtain liqueur FT-MIR ATR spectra on Alpha-P spectrometer (Bruker Optics, Bryanston, South Africa).
- Obtain liqueur FT-MIR spectra on Winescan™ Flex spectrometer (Foss Analytical, Denmark).
- Determine reference values and SEL for large range product types produced.
- Develop alcohol, pH and invert sugar PLS calibration models.
- Evaluate alcohol, pH and invert sugar PLS calibration models using the performance criteria R², RMSECV, RMSEP and RPD for routine analysis in an industrial cellar.

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Chapter 2

Literature review

2. LITERATURE REVIEW

GENERAL INTRODUCTION ON LITERATURE CITED

At the start of the project, a search was conducted on the literature available on ammonia (NH_3) in grape juice and its importance to the wine yeast as a source of available nitrogen during alcoholic fermentation. Yeast assimilable nitrogen (YAN) is a measure of the total nitrogen, including alpha amino nitrogen (NO₃-N) and ammonia (NH_3), as described by $\text{FAN} + \text{NH}_3 = \text{YAN}$. The information pertaining to methods for yeast assimilable nitrogen (YAN) analysis was retrieved and their suitability in the large industrial cellar environment was evaluated through weighing up of advantages and disadvantages of the respective methods. In this review, factors that contribute to sufficient or insufficient levels of YAN available to yeast, and the potential implications of these scenarios for wine aroma and quality, are discussed.

A search was also conducted of the literature that was available on spirit-based liqueurs, with specific focus on the chemical methods used to analyse the quality control parameters alcohol, pH, invert sugar, total acidity (TA), colour and flavour in these product types. A general shortage of data rendered by the search led to information also retrieved from semi-scientific publications and the review is based mostly on practical analytical procedures in the industry.

The review also presents a summary of the application of mid-infrared (MIR) spectroscopy in the alcoholic beverage industry, as well as the data analysis techniques that accompany spectroscopic studies.

2.1 AMMONIA IN GRAPE JUICE

2.1.1 Introduction

Nitrogenous compounds play an important role in the alcoholic fermentation process of wine production. Freshly pressed grape juice, prior to onset of alcoholic fermentation, contains a variety of nitrogen compounds, of which the most important are amino acids, ammonia, and small peptides, known as YAN (O'Kennedy & Reid, 2008). YAN affects yeast biomass production during fermentation (Crépin *et al.*, 2014) and the fermentation rate (Varela *et al.*, 2004). Nitrogen insufficiency is one of the main reasons for stuck or sluggish fermentations (Clement *et al.*, 2013) and can have an impact on wine aroma (Miller *et al.*, 2007).

2.1.2 Importance of available nitrogen for the wine yeast during alcoholic fermentation

During alcoholic fermentation, wine yeasts metabolise sugar through the glycolytic pathway to the major end products alcohol and carbon dioxide (CO₂) (Jackson, 2008). Yeast species include *Kloeckera*, *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, *Hansenula*, *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulasporea*, *Zygosaccharomyces*, *Cryptococcus*, *Debaryomyces*, *Rhodotorula*, *Saccharomycodes* and *Saccharomyces* (Pretorius *et al.*, 1999).

Wine yeasts require nutrients for optimal growth and metabolism (Baroň, 2011). These include nitrogen, sugar, vitamins (thiamin, pantothenic acid, biotin), micronutrients (Zn, Mg, Mn, K, P) and oxygen (O₂) (Stewart, 2014). YAN can be utilised by the yeast in the form of NH₃, (or in NH₄⁺), and/or free amino acids (FAN). Proline is not used as a nitrogen source during the anaerobic alcoholic fermentation process, because the enzyme that catalyses the first step in proline catabolism, proline oxidase, requires O₂ (Henschke & Jiranek, 1993). The restriction of the use of proline as nitrogen source under anaerobic condition impairs the assimilation of arginine, since the assimilation of arginine requires the proline utilisation pathway (Brandriss & Magasanik, 1981).

During fermentation, the yeast transports NH₃ and amino acids from the grape juice into the yeast cell. There they are used to synthesise proteins, such as glycolytic enzymes and membrane transport proteins, known as permeases. Permeases are situated in the cell membrane of the yeast and are responsible for transporting compounds such as amino acids and sugars into the cell. The rest of the proteins are used for cellular constituents, which are important for proliferation of the yeast (O'Kennedy & Reid, 2008).

The nitrogen concentration in juice affects fermentation speed and completion (Bell & Henschke, 2005). In the industrial context, the initial concentration of YAN is measured in freshly pressed grape juice and if the concentration of NH₃ is lower than 50 mg/L, it is considered necessary to add NH₃ for the optimum fermentation performance of the yeast. The addition of NH₃, without prior analysis of the juice to determine whether it is really required, is not recommended (Ribéreau-Gayon *et al.*, 2000) and this practice may lead to organoleptic defects in fermented wine (Jiranek *et al.*, 1995; Vilanova *et al.*, 2012).

2.1.3 Measurement of nitrogenous compounds in grape juice

Real-time information on the initial concentration of YAN is important for effective fermentation control. It is necessary to reduce the incidence of stuck and sluggish fermentations, to increase productivity and efficiency in the cellar, and to exert quality control to ensure the consistency of the wine. Fast methods for analysing NH₃ and the FAN concentration are in demand by modern industries (Cozzolino & Curtin, 2012).

The FAN content is determined by the o-phthaldialdehyde/N-acetyl-L-cysteine (NOPA) assay, described by Ugliano *et al.* (2007). The formol titration is a rapid method for measuring YAN, but the use of formaldehyde, a toxic volatile reagent, requires a well-trained analyst and a fully-equipped laboratory.

In industrial wine laboratories, the NH_3 concentration is determined by an ammonium ion probe or by enzyme-linked spectrophotometric assays (Napa Valley College, 2007). For the method using an ammonium probe, a calibration curve is constructed from known concentration solutions of NH_3 and is then used to estimate the unknown concentration of NH_3 for the juice sample (McWilliam & Ough, 1974).

The enzymatic assay is based on the principle that NH_3 reacts with α -ketoglutarate and reduces nicotinamide adenosine dinucleotide (NADH) in the presence of glutamate dehydrogenase (GIDH) to form L-glutamate and NAD^+ (Ammonia Catalogue number 11 112 732 035, R-Biopharm, Germany). The amount of NADH consumed is measured at 340 nm and is stoichiometrically related to the amount of NH_3 present in the juice. The summation of these two nitrogen measurements, FAN and NH_3 concentration yields YAN. Arginine and proline are the most abundant amino acids in *Vitis vinifera* wine grapes, but are not metabolised during the anaerobic alcoholic fermentation and do not form part of the YAN measurement (Stewart, 2014).

The application of MIR technology to detect early indications of problems with wine fermentation was investigated by Urtubia *et al.* (2008). The method showed potential, but was not ready to apply in a commercial cellar. A comparative analysis of three fermentations with artificial musts was performed: one of normal behavior, one subject to a temperature gradient, and the third deficient in YAN. Fermentations were monitored through changes in spectra in addition to changes in must composition. All measured compounds (glucose, fructose, alcohol, glycerol, succinic and acetic acids) exhibited behavioral changes in nitrogen deficient musts.

Skoutelas *et al.* (2011) validated and compared the formol method and Fourier transform infrared (FTIR) spectroscopy for the quantification of YAN in grape juice. The calibration model developed had a low standard error of prediction (SEP, 6.4 mg/L), a high coefficient of determination (R^2 , 0.99) and a residual predictive deviation ratio (RPD) of 7.8. The accuracy obtained with predictions of future samples using the Fourier transform mid-infrared (FT-MIR) calibration, was comparable to that of the formol method.

Based on the proven success of IR spectroscopy for the quantification of NH_3 in grape juice, the first objective of this study was to evaluate the potential of Fourier transform mid-infrared attenuated total reflection (FT-MIR ATR) spectroscopy for the quantification of ammonia content in freshly pressed grape juice, and to implement the partial least squares (PLS) regression algorithm

in the DGB analytical laboratory, which at the time that the project was undertaken, was relying on the enzymatic assay.

2.1.4 Nitrogen supplementation

The level of nitrogen required for optimum fermentation performance depends on the initial YAN contents of the grape juice, the initial grape juice sugar, the specific yeast strain(s) selected to conduct the fermentation and the timing of the addition (Gutiérrez *et al.*, 2012). Diammonium phosphate (DAP) and complex yeast nutrients (CYN) are the most commonly used supplements for nitrogen deficiencies in grape juice (Blateyron & Sablayrolles *et al.*, 2001). DAP is widely used as a YAN supplement and contains 21% (percentage) nitrogen. For the sake of convenience, 100 milligrams (mg) DAP can be considered to contain 20 mg of YAN (Ugliano *et al.*, 2007). In order to ferment to dryness, clarified juice containing less than 150 mg/L of YAN should be supplemented with nitrogen to at least 150 to 200 mg/L, especially if the vineyard in question has a history of low YAN fermentation-related problems, or if a yeast with a high nitrogen demand has been selected for the fermentation (Ugliano *et al.*, 2007). Large additions of DAP lead to increased yeast biomass and higher maximum heat output due to a greater fermentation rate (Varela *et al.*, 2004).

Table 2.1 shows the recommendations for degree (°) Brix of the grape juice and the target concentration of YAN needed for an optimal fermentation rate (Bisson & Butzke, 2000) in an industrial cellar. From this information it is clear that by monitor of °Brix content frequently to estimate nitrogen requirements, the °Brix can assist the winemaker in early identification of nitrogen insufficiency and stuck fermentations.

Table 2.1: °Brix of grape juice and target YAN for optimal fermentation in wine (Bisson & Butzke, 2000)

| Degree (°) brix of grape juice | Target yeast assimilable nitrogen (YAN) concentration mg/L |
|--------------------------------|--|
| 21 | 200 |
| 23 | 250 |
| 25 | 300 |
| 27 | 350 |

Disadvantages of DAP as a supplement are that overuse can stimulate overproduction of ethyl acetate, acetic acid and volatile acidity, resulting in the suppression of the varietal character of the wine (Ugliano *et al.*, 2007). Residual nitrogen at the completion of fermentation can also increase the risk of microbial instability (Bell & Henschke, 2005). Therefore, care is needed to monitor the initial grape juice NH₃ concentration accurately to ensure completion of fermentation and eventually, high-quality wines.

2.1.5 Relationship between nitrogen status and the production of sulphur-like odours during alcoholic fermentation

Hydrogen sulphide (H_2S) production varies among strains of *Saccharomyces cerevisiae* (*S. cerevisiae*) (Ferreira *et al.*, 2009). Some *S. cerevisiae* strains reduce sulphate to sulphide via the sulphate reduction pathway. The yeast uses sulphide to synthesise the sulphur-containing amino acids, methionine and cysteine (Giudici & Kunkee, 1994; Jiranek & Henschke, 1991). During this process, H_2S is formed. The chemical composition of the grape juice may contribute to the levels of H_2S formed. In the absence of sufficient nitrogen, excess levels of sulphide are formed that combine with hydrogen to form H_2S and is secreted into the medium. This is due to the ongoing reduction of sulphite by the enzyme sulphite reductase, although the nitrogen-containing precursors that react with sulphide, O-acetylserine (OAS) and O-acetylhomoserine (OAH) are exhausted (Jiranek *et al.*, 1995, 1996). H_2S adds a rotten egg odour to wine that is perceived as a negative characteristic in wine.

Suppression of H_2S formation has been obtained by YAN supplementation at the beginning of fermentation (Lambrechts & Pretorius, 2000). The underlying mechanism has been hypothesised to be linked to utilisation of the added nitrogen by the wine yeast to restore amino acid synthesis. If nitrogen is added at the end of fermentation, sulphur-like odours can occur in the wine due to the yeast being inefficient in taking up added nitrogen. If there is no nitrogen supplementation, H_2S can also act as a precursor for the formation of other reduced-sulphur compounds, such as mercaptans, which contribute to the negative impact on wine flavour (Lambrechts & Pretorius, 2000).

Copper (Cu^{2+}) treatments effectively remove H_2S and mercaptans from spoiled wines, but sulphur-containing acetic esters are not affected and remain in the finished wine, where they are eventually hydrolysed to H_2S and mercaptans (O'Kennedy & Reid, 2008).

2.1.6 Factors that affect the level of YAN available to the wine yeast

2.1.6.1 Vineyard specific factors

Ough & Kriel (1985) observed differences between the NH_3 concentrations in grapes from different farms and of different cultivars in the Stellenbosch area, South Africa. Grape samples were analysed for NH_3 content and the vineyards were compared statistically by analysis of variance. Low NH_3 content was ascribed to insufficient levels of NH_3 present in the soil and the uptake thereof by the roots of the vine. Fermentation problems are often vineyard specific and the nutritional requirements of the vineyard must be managed carefully. This involves, amongst other methods, the assessment of the nutrient status of the plant tissue and soil, in order to develop an appropriate fertilising programme for adequate NH_3 supplementation. The manipulation of

grapevine nutrition by increasing the concentration of nitrogenous compounds has the potential to influence grape berry composition and ultimately the composition of the wine, which in turn will influence the quality of wine (Smart *et al.*, 1990). Warnings of low expected YAN levels can be obtained by sampling in the vineyard, one to two weeks prior to harvest, such as during maturity sampling (Ugliano *et al.*, 2007).

2.1.6.2 Ripeness of grapes and fungus infections

NH₃ concentrations are affected by the ripeness of the grapes and infections by *Botrytis cinerea* (*B. cinerea*). NH₃ represents 80% of total nitrogen in grapes and is the form most directly assimilable by yeast (Ribéreau-Gayon *et al.*, 2000). As grapes ripen, the NH₃ content in the grape berries decreases and at the same time, the concentration of amino acids increases. *B. cinerea* consumes amino acids, NH₃ and some vitamins from the berries. This has a negative effect on the fermentation of the juice due to reduced nutrient availability and can result in stuck fermentations and H₂S formation (Ali *et al.*, 2010).

2.1.6.3 Yeast strain-specific NH₃ requirements

Amino acids are not taken up equally by the yeast. Some yeast strains have higher nitrogen requirements than others for optimal performance during fermentation (O'Kennedy & Reid, 2008), ability to ferment to dryness in a high-alcohol content environment, and in releasing fruit-derived aroma flavours (Zoecklein, 2001). Determination of initial concentration of NH₃ in grape juice is essential to indicate the level of nitrogen available at the start of fermentation. For inadequate levels of NH₃ present, supplementation with DAP are necessary. Yeast strains with a low nitrogen requirement may in fact require no further addition of nitrogen (O'Kennedy & Reid, 2008).

2.1.6.4 Alcohol levels and temperature during fermentation process

Nitrogen compounds are incorporated into the yeast cells by means of active transport systems, so-called membrane H⁺-ATPases, which involve a general amino acid permease enzyme and other permeases with a variable degree of specificity for particular sets of amino acids (Henschke & Jiranek, 1993). When alcohol levels produced during fermentation are too high, it increases the yeasts' membrane permeability to protons. The proton-pumping membrane ATPases is inhibited and the active transport mechanisms therefore are diminished or rendered inoperative (Stewart, 2014). Transport of adequate levels of YAN for optimal fermentation by yeasts are not possible.

Carrier-mediated active transport is temperature dependent. The rate of accumulation of amino acids decreases at lower temperatures (Henschke & Jiranek, 1993). The temperature of the fermentation also affects nitrogen requirements. Lower-temperature fermentations require less nitrogen and usually result in more efficient transformation of sugar to alcohol (Ribéreau-Gayon *et al.*, 2000).

Based on the demonstrated importance of NH_3 to the completion of fermentation by yeast and on the quality of wine, the need to accurately monitor the levels in freshly grape juice is clear. Spectroscopy has been successfully established for the quantification of important parameters in the alcoholic beverage industry, as illustrated in Table 2.4 in section 2.3, and was investigated for the determination of NH_3 in grape juice in this project.

2.2 IMPORTANT QUALITY INDICATING PARAMETERS OF SPIRIT-BASED LIQUEURS

2.2.1 Introduction

The South African liquor industry can broadly be segmented into the beer, spirits, liqueur and wine sectors. Liqueurs are classified into grape-based and spirit-based products. Grape-based liqueurs are produced from wine to which herbs or natural flavourings of vegetable origin are added (South African Liquor Products Act 60 of 1989). Spirit-based liqueurs are produced from spirits obtained through fermentation of sugar cane juice. The spirit has no distinctive aroma or taste characteristics, and is colourless. The alcohol content of the spirit should be at least 43% volume per volume (v/v) (South African Liquor Products Act 60 of 1989). Sucrose is added to the spirits base with a mixture of herbs, fruits, spices or other flavourings. This beverage type is one of the fast-growing markets in South Africa and elsewhere in the world. The target consumer groups are younger, active people with a fun lifestyle. According to the South African law, a spirit-based product must be distinctively labeled and easily recognisable with regards to packaging, appearance, content and taste in comparison to other spirit class products.

2.2.2 Production and bottling of spirit-based liqueurs

DGB annually produces an average of four million litres (L) of spirit-based liqueurs. The liqueurs are distributed in 1 L, 750 millilitres (ml) and 500 ml glass bottles, locally and overseas. The liqueur range consists of 20 product types: Zappa red, Zappa original, Zappa green, Zappa blue, Zappa black, Tang bite, Tang, Potency, Malibu, Lavoka Chocolate, Lavoka Caramel, Kiss, Butlers triple sec, Butlers strawberry, Butlers peppermint, Butlers ginger, Butlers coconut, Butlers cherry, Butlers blue and Butlers Banana (www.dgb.co.za). The production and bottling processes of spirit-based liqueurs at the cellar consist out of 17 stages, as illustrated in Figure 2.1. The first nine stages are conducted in the cellar during the production of the product. The following four stages, 10-13, take place after the end product has been pumped from the cellar into the bottling holding tanks and filler tank. The last four stages, 14-17, take place before and during bottling.

During these stages, 8 different quality control points are identified where samples are removed from the production and bottling lines for chemical analysis and sensory evaluation, as shown in Table 2.2. The recipes of the products are restricted property of the cellar, but for the

sake of clarity it suffices to mention that the chemical composition was more or less the same between quality check points 0 to 7 for the different product types.

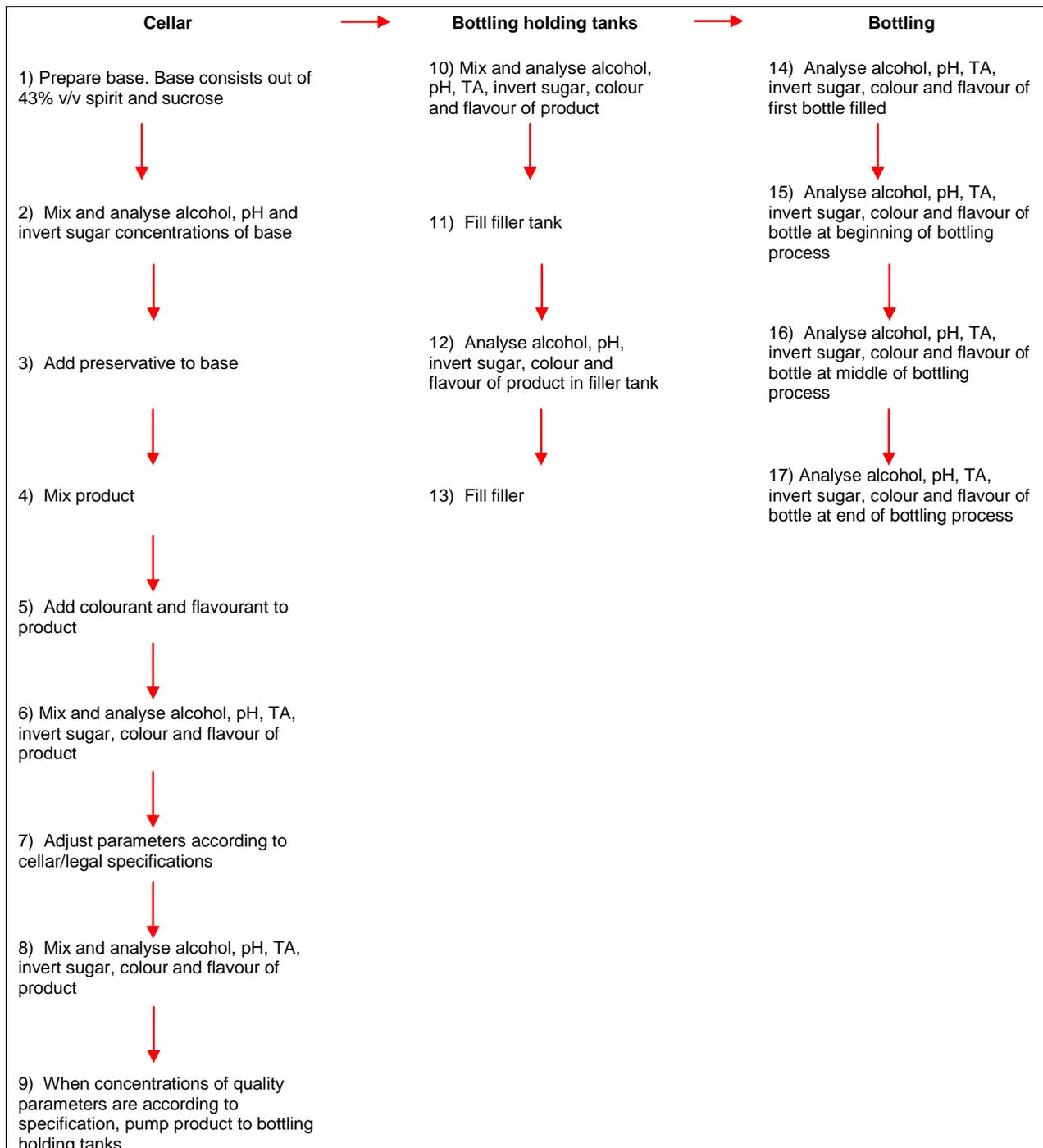


Figure 2.1: The 17-stage production and bottling processes of spirit-based liqueurs (www.dgb.co.za).

Table 2.2: Quality control points at which spirit-based liqueur samples are analysed during the production and bottling processes at DGB cellar (www.dgb.co.za).

| Quality control point | Control point number | Status in the production and bottling process |
|-----------------------|----------------------|---|
| Before bottling | 0 | Last sample tested from production tank before bottling |
| First check | 1 | First bottle in bottling process |
| Last check | 2 | Last bottle in bottling process |
| Line check | 3 | Middle bottle in bottling process |
| Production cellar | 4 | Sample collected during the production of the product |
| Pre-bottling | 5 | Sample collected from bottling tank |
| Pre-filling tank | 6 | Sample collected from filler tank |
| Filler | 7 | Sample collected from filler machine |

2.2.3 Measurement of quality indicating parameters in spirit-based liqueurs using conventional methods

In South Africa, the government implemented an international recognised and effective accreditation and monitoring system, the South African National Accreditation System (SANAS), as the only body for the accreditation of conformity assessment and calibration of good laboratory practices (Accreditation for Conformity Assessment, Calibration and Good Laboratory Practice No19, Act 2006). Real-time control of quality indicating parameters during the production and bottling of spirit-based liqueurs is important to ensure consistent high quality of products. Alcohol, pH and invert sugar has been identified by the cellar as primary quality indicating parameters, whereas TA, colour and flavour are secondary quality indicating parameters.

At the time that the project was undertaken, chemical analysis of these parameters relied on methods that are time consuming and costly. Alcohol, pH and invert sugar were identified as key quality indicating parameters and therefore, the development of high sample throughput and rapid control procedures was priority. MIR spectroscopy, coupled with multivariate data analysis techniques, was evaluated for the purpose of this study. The technique is well-established for the quantification of parameters in the wine industry and its performance was evaluated for its application in spirit-based liqueurs.

At DGB, the cellar master monitors and controls the stages during the production and bottling processes. Each product type has to comply with the cellar product specifications (Table 2.3) for alcohol and invert sugar and also adhere to the regulations of the Liquor Products Act (No 60 of 1989). If a sample does not adhere to specifications, the product is adjusted accordingly and analysed again. Samples are routinely homogenised before measurement with a sonicator (model PS06A, Healthcare Technologies, South Africa).

Table 2.3: DGB spirit-based liqueur specifications for alcohol and invert sugar (www.dgb.co.za).

| PRODUCT | Alcohol (% v/v) | | Invert sugar (g/L) | |
|------------------|--------------------|-----------|-----------------------|-----------|
| | Specification | Tolerance | Specification | Tolerance |
| Zappa | 40.0 | ± 0.5 | 300 | ± 20 |
| Tang Bite | 36.00 | ± 0.5 | 250 | ±20 |
| Lavoka | 30.00 | ± 0.5 | 300 | ±20 |
| Malibu | 24.00 | ± 0.5 | 154 | ± 20 |
| Potency | 24.00 | ± 0.5 | 300 | ±20 |
| Butlers | 24.00 | ± 0.5 | 425 | ± 20 |
| Blue | 24.00 | ± 0.5 | 270 | ± 20 |
| Strawberry | 24.00 | ± 0.5 | 425 | ± 20 |
| Espresso | 24.00 | ± 0.5 | 340 | ± 20 |
| Coconut | 24.00 | ± 0.5 | 180 | ± 20 |
| Tang | 11.7 | ± 0.5 | 250 | ±20 |
| Kiss | 14.50 | ± 0.5 | 200 | ±20 |

2.2.3.1 Alcohol

The alcohol content in spirit-based liqueurs has economic implications with regards to taxes and regulations by governing bodies, making alcohol an important quality parameter for producers. The alcohol stated on the label of the product may not exceed more than 0.5 in numerical value from the actual alcohol content of the liqueur product measured by accredited laboratories (South African Liquor Products Act 60 of 1989). Analytical methods for the determination of alcoholic strength in spirits involve pycnometry, electronic densimetry, densimetry using a hydrostatic balance, hydrometry (Brereton *et al.*, 2003) and biosensor technology (Hnaien *et al.*, 2010). The method in use at DGB is electronic densimetry and expressed as % volume per volume (v/v). To increase the alcohol content, 43% v/v cane spirit is added in the cellar to the product. To lower the alcohol content, distilled water is added to the product.

2.2.3.2 pH, total acidity (TA) and invert sugar

pH and TA are determined with automatic titration, using a combination electrode and a temperature probe. The Rebelein manual titration method, termed copper-ion reduction, is used to determine the amount of reducing sugars present in the sample (Iland *et al.*, 2000). Disadvantage of these titration methods are the dilution of the sample that can contribute to human errors and inaccurate results. The invert sugar content for spirit-based liqueur should legally not be less than 100 g/L. (South African Liquor Products Act 60 of 1989).

2.2.3.3 Colour and sensory evaluation for the determination of flavour in products

Colourimetry is the science of measuring and evaluating of the concentration of colour compounds in a sample (Zwinkels, 1996). Colour is the first characteristic of a product that the consumer assesses, and is therefore an important quality indicator (Blasco *et al.*, 2003). This assessment is based on personal experience and preferences. Colour can be assessed by the evaluation of the visible spectrum of light by the human eye, or by a spectrometer, providing a quantitative measurement by simulating in which the human eye sees an object (McCaig, 2002).

Sensory evaluation includes sample preparation, organoleptic testing, results recording and reporting steps, which are necessary for the scientific evaluation of a product (Piggott, 2012). A sensory panel of four to six trained individuals is responsible for conducting the routine sensory evaluation. The alcohol content of products higher than 25% v/v are diluted using de-ionised or distilled water to a final content of 15 to 25% v/v to reduce the risk of sensory fatigue. Off-taint characteristics that otherwise may have gone undetected are identified. Because of the volatility of some of the flavour components, the dilutions are evaluated at room temperature during organoleptic testing (www.dgb.co.za).

Piggott (2012) described the sensory evaluation of alcoholic beverages using a flavour wheel to identify and categorise the sensory attributes that are related to a product. Figure 2.2 represents a spirit-based liqueur flavour wheel developed by the DGB sensory panel to describe smell and taste. The wheel consists out of 8 flavours that contribute to the characteristics identified by the panel for the spirit-based liqueur range. Fruity flavours include fresh fruit, dried fruit, citrus fruit and artificial fruit flavourings. Sour flavours include vinegary and cheesy flavours. Toasted aromas include burn toast, roasted malt, nuts, coffee, toffee and cocoa. Sweet includes honey, vanilla and caramel flavours (Piggott, 2012). The nose may fatigue and experts refer to sensations such as “nose prickle”, “nose drying”, or “nose burn”, which often are the result of high alcohol concentrations. The panel records the comments and the cellar master is responsible for adjusting the flavour according to the specifications.

These wet chemistry methods are lengthy; they generate large quantities of chemical waste and require a fully staffed laboratory. Real-time monitoring of indicating parameters in spirits products is in high demand by producers. The aims of the study were to evaluate the potential of FT-MIR spectroscopy for the quantification of alcohol, pH and invert sugar in these products during their production and bottling stages and to implement the partial least squares (PLS) regression algorithms developed in this study at the DGB laboratory, which at the time of this study was relying on lengthy wet chemistry methods. To the best of our knowledge, this is the first report of the implementation of MIR spectroscopy for the quality control of South African spirit-based liqueurs.

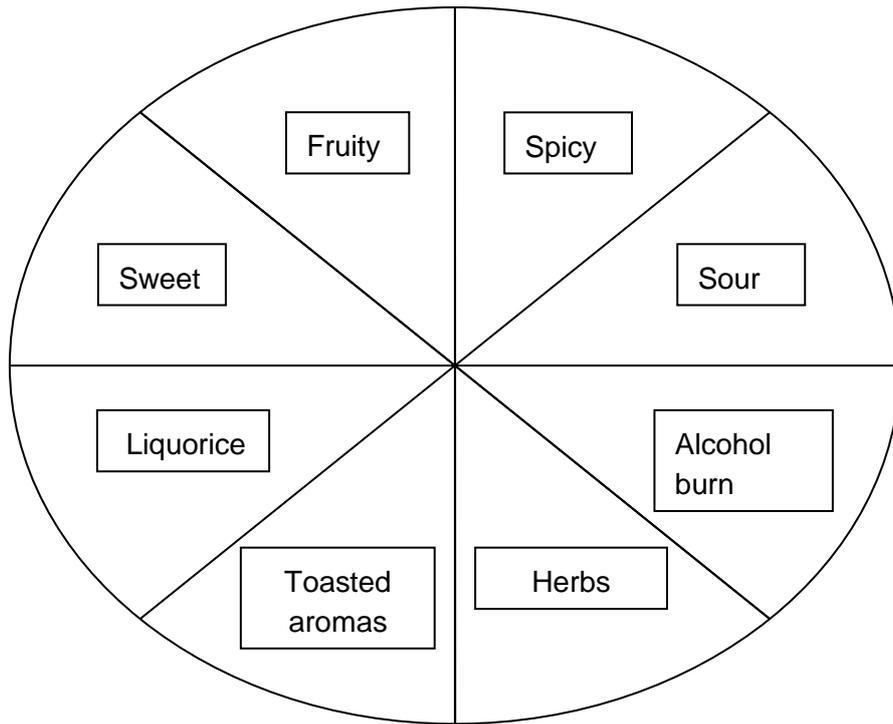


Figure 2.2: Spirit-based liqueur flavour wheel (www.dgb.co.za)

2.3 SPECTROSCOPY AND DATA ANALYSIS

2.3.1 Introduction

The needs of the fast and growing market for products with higher quality and larger production volumes require an analytical method that is accurate, facilitates high sample throughput (Urbano Cuadrado *et al.*, 2004), cost-efficient, easy to operate and require minimum sample preparation. This method must give the winemaker/cellar master real-time information to make fast decisions on improving the quality of the end product. In the alcoholic beverage industry, infrared (IR) spectroscopy is a well-established alternative technique for traditional chemical analysis (Bauer *et al.*, 2008; Cozzolino *et al.*, 2011). Although FT-MIR spectroscopy is widely used in the alcoholic beverage industry, the technology has seen limited quantitative application for spirit-based liqueurs in South Africa.

2.3.2 Infrared spectroscopy

Vibrational spectroscopy is based on the measurement of the stretching, bending and twisting of covalent bonds in functional groups present in a sample upon the exposure to IR radiation. Covalent bonds exhibiting a dipole moment, C-C, C-H, O-H, C=O and N-H, can absorb IR radiation at different characteristic frequencies, depending upon the masses of the atoms and the strength of the bonds (Pavia *et al.*, 2009). The vibrational frequencies are useful for the identification of functional groups and for structural determination. The IR regions of the electromagnetic spectrum are divided into the near infrared (NIR), 14 000 to 4 000 centimetres (cm^{-1}), MIR, 4000 to 400 cm^{-1} , and far-infrared (FIR), 400 to 20 cm^{-1} , regions and may differ depending on the instrumentation used and the specific application the wavenumbers range are customized for (Lin *et al.*, 2009). Wavenumbers are reciprocal to wavelength and like frequency, are directly proportional to energy (De Villiers, 2012). An absorbance spectrum is calculated through a series of statistical and mathematical techniques, known as chemometrics. The MIR region 1400 to 600 cm^{-1} contains information about the chemical composition and physical state of the material under analysis and often referred to as the fingerprint of a sample. This area of the spectrum is visually assessed based on published information for major absorption peaks and bands associated with chemical constituents (Esbensen, 2002).

In Figure 2.3, the FT-MIR ATR spectrum of freshly pressed grape juice sample obtained in this study, shows that characteristic bands at 1650 cm^{-1} related with N-H bending and region 1300 to 1370 cm^{-1} with N-H stretching and correspond to NH_3 (Cozzolino *et al.*, 2009, 2011), are present. The spectrometer is equipped with a diamond ATR crystal absorbed at 1900 to 2300 cm^{-1} (Walker, 1979). In Figure 2.4, FT-MIR spectra of spirit-based liqueur obtained in transmission and converted to absorbance for exploratory data analysis reasons identified in the research chapters

of this study, shows absorption peaks at 1085 and 1045 cm^{-1} , relating to alcohol absorption (Duarte, 2004). The sucrose profile is characterised as a broad band at 1040 to 1000 cm^{-1} (Grassi *et al.*, 2014) and the regions associated with strong water absorption are 1716 to 1543 cm^{-1} and 3626 to 2970 cm^{-1} (De Villiers *et al.*, 2012).

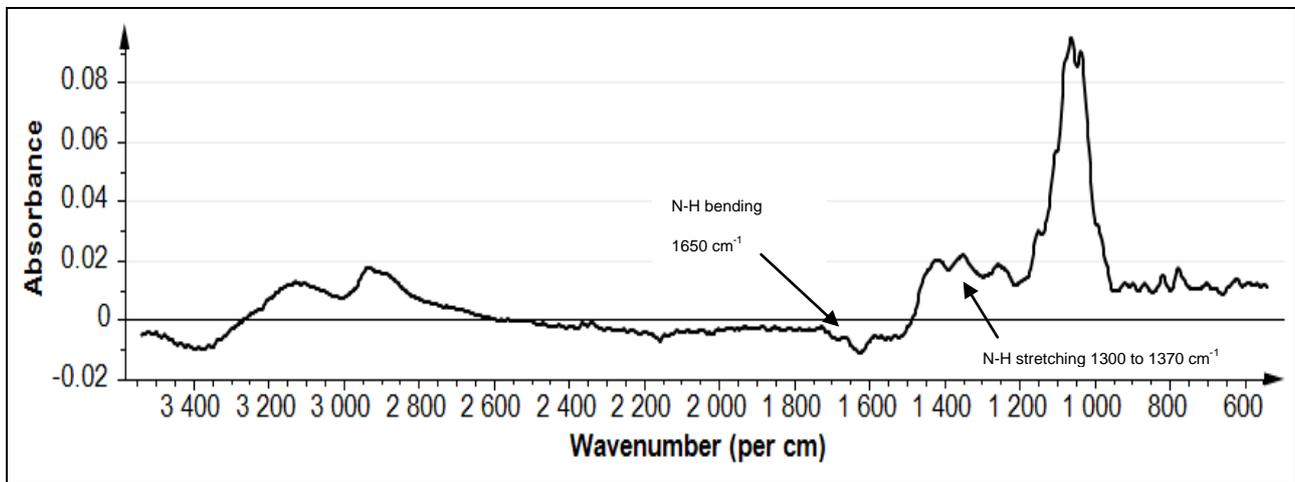


Figure 2.3: Fourier transform mid-infrared attenuated total reflection (FT-MIR ATR) spectrum of a grape juice sample obtained in this study. Interpretation of ammonia absorption is according to Cozzolino *et al.* (2009, 2011) at 1650 cm^{-1} and region 1300 to 1370 cm^{-1} .

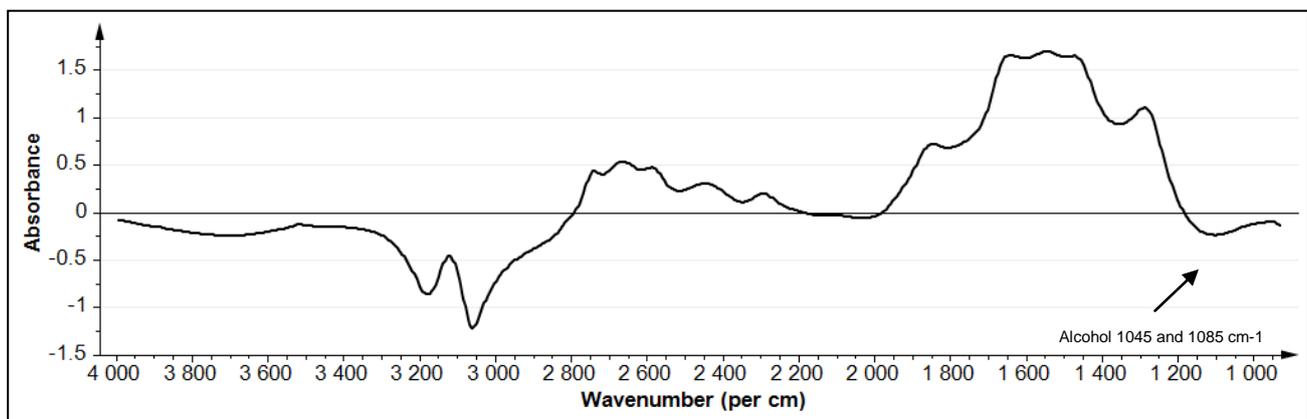


Figure 2.4: Fourier transform mid-infrared spectra of spirit-based liqueur obtained in transmission and converted to absorbance in this study. The regions 1045 and 1085 cm^{-1} associated with alcohol absorption according to Duarte (2004) are illustrated.

2.3.3 Application of spectroscopy in the alcoholic beverage industry

IR spectroscopy has been extensively researched and implemented for various applications in the beverage industry. It has been confirmed to be an accurate method of analysis for the determination of sugars in beverages (Kuligowski *et al.*, 2008) to quantify water-soluble vitamins (Wojciechowski *et al.*, 1998), for the monitoring of grapevine reserves (Schmidtke *et al.*, 2012), the determination of raw sugar cane juices (Cadet *et al.*, 1997), the study of wine production and storage (Bauer *et al.*, 2008) and the monitoring of soluble solids, pH and TA in grape must (Swanepoel *et al.*, 2007).

The use of non-invasive NIR- and Raman spectrometry combined with a principal component analysis (PCA) and -partial least squares (PLS) algorithms was investigated for the non-destructive determination of alcohol content in whisky, vodka and sugary alcoholic drinks through the widest part of 700 mL static bottles by Nordon *et al.* (2005). The proposed methods could be used to calculate the average alcohol concentration over a number of bottles in a bottling line without opening the bottles.

FTIR spectroscopy in combination with multivariate data analysis was introduced for the quality control of 535 spirit drinks and 461 beers by Lachenmeier (2007). The method provide great accuracy for the determination of density, alcohol, methanol, ethyl acetate, propanol-1, isobutanol and 2-/3-methyl-1-butanol ($0.90 < R^2 < 0.98$) in spirit samples and for alcohol, density, original gravity and lactic acid in beer samples ($0.97 < R^2 < 0.98$).

Alcohol models in beer and prior diluted spirit samples were developed by Gallignani *et al.* (1994) using a Perkin-Elmer Model 1750 FTIR spectrometer with a temperature-stabilised detector. The reported results indicated that the derivative FTIR models for the determination of alcohol to be accurate.

Lachenmeier *et al.* (2010) developed a portable multiple-beam IR sensor in combination with a flow-through cell for the determination of alcohol concentration in wine, beer and spirits. The precision of the IR sensor was equal or better than the densimetric or FTIR methods used as reference procedures. The method is used for mobile on-site control in the context of labelling control of wine, beer and spirits and in the monitoring the fermentation process.

Gallignani *et al.* (2005) developed a strategy to determine alcohol in beer, wine and spirits by using on-line liquid-liquid extraction of alcohol with chloroform and FTIR spectroscopy. Spirits with alcohol higher than 15% v/v were diluted with water prior to analysis. The method can be used as an alternative for the determination of alcohol in alcoholic beverages and is suitable for routine control analysis.

Both MIR and NIR spectroscopy have been widely used for applications in the alcoholic beverage industry in Table 2.4, and was therefore the choice of method of analysis for this study. There was limited quantitative application in the literature for spirit-based liqueurs and not any of them were obtained from South African products.

Table 2.4: Review table of the application of IR spectroscopy for quantitative analysis in the alcoholic beverage industry.

| Reference | Objective | Samples | Statistical analysis | Conclusion |
|--------------------------------------|---|---|----------------------|---|
| Nagarajan <i>et al.</i> , 2006 | Quantitative analysis of alcohol, sugar, and tartaric acid in alcoholic beverages using attenuated total reflectance spectroscopy | 100 synthetic samples for calibration model | FT-MIR ATR, PLS | ATR effective for quantifying alcohol, sucrose and tartaric acid in alcoholic beverages. |
| Lobo <i>et al.</i> , 2006 | Calibration models for routine analysis of cider by mid-infrared spectroscopy | Ciders exclusively made from cider-apple pressing, analysed in different stages of the making process, from the end of the fermentation to several months in bottle | MIR, PLS, PRESS | Accurate, precise and robust calibration models, suitable for routine analysis purposes, were optimised for the routine analysis of specific gravity (SG), TA, volatile acidity (VA), pH, alcohol and fructose in ciders. |
| Cozzolino <i>et al.</i> , 2011 | Feasibility study on the use of attenuated total reflectance mid-infrared for analysis of compositional parameters in wine | 130 Commercial Australian red and white wine samples | FT-MIR ATR, PLS | Calibration models suitable for routine analysis in industry. SEP values 0.11% v/v for alcohol, 0.0007 for SG, 0.10 for pH, 0.53 (g/L) for TA, 1.35 g/L for Glucose+Fructose, and 0.12 g/L for VA. |
| Lachenmeier, 2007 | Rapid quality control of spirit drinks and beer using multivariate data analysis of Fourier transform infrared spectra | 535 spirit drinks and 461 beer samples | FTIR, PCA, PLS | Accurate and strong correlation for the spirit parameters density, alcohol, methanol, ethyl acetate, propanol-1, isobutanol and 2-/3-methyl-1butanol ($R^2=0.9$ to 0.98) Lower correlation for beer parameters pH, bitterness unit, and colour, semi quantitative for screening analysis ($R^2=0.97$ to 0.98). Differentiation of deteriorated fruit spirits distilled from microbiologically spoiled mashes was possible. PCA classification for authenticity control is possible. |
| Urbano Cuadrado <i>et al.</i> , 2004 | Near infrared reflectance spectroscopy and multivariate analysis in enology. Determination or screening of fifteen parameters in different types of wines | 180 samples consisting out of red, rose and white wines - young and aged wines of different varieties | NIR, PLSR | Strong correlation for alcohol, volumic mass, TA, pH, glycerol, colour, tonality, lactic acid and total polyphenol index ($R^2>0.8$). Screening capability for VA, organic acid, malic acid, tartaric acid and gluconic acid, reducing sugars and total sulphur dioxide ($R^2=0.432$ to 0.705). |
| Niu <i>et al.</i> , 2008 | Analysis of sugars in Chinese rice wine by Fourier transform near-infrared spectroscopy with partial least square regression | 40 samples of five brewing years (1996, 1998, 2001, 2003 and 2005) | FT-NIR, PLSR | NIR spectroscopy offers screening capability for isomaltose, isomaltotriose, maltose, and panose in Chinese rice wine. |
| Fragoso <i>et al.</i> , 2011 | Quantification of phenolic compounds during red winemaking using FT-MIR spectroscopy and PLS-regression | 5 grape varieties harvest at different ripening stages over 10 days | FT-MIR, PLS | Rapid and valuable tool to monitor phenolic compound extraction during winemaking. |
| Fernández <i>et al.</i> , 2007 | Quantitative analysis of red wine tannins using Fourier transform mid-infrared spectroscopy | 86 red wines from vintages 2004 and 2005 | FT-MIR, PLS | Accurate determination of tannin concentration, but currently under development for use of routine analyses. |

Table 2.4 (cont.)

| | | | | |
|-----------------------------------|---|--|----------------------|--|
| Coldea <i>et al.</i> , 2013 | Rapid quantitative analysis of alcohol and prediction of methanol content in traditional fruit brandies from Romania, using FTIR Spectroscopy and chemometrics | 26 fruit brandies | FT-MIR ATR, PCA, PLS | FTIR offer a good prediction and statistical correlation for methanol quantification in fruit brandies. |
| Swanepoel <i>et al.</i> , 2007 | Optimisation of the quantification of total soluble solids, pH and titratable acidity in South African grape must using Fourier transform mid-infrared spectroscopy | 1170 South African grape must samples | FT-MIR, PCA, PLS | Accurate prediction models for Total soluble solids (TTS) (SEP=0.38 °Brix), pH (0.04) and TA (0.51 g/L). FT-MIR useful for rapid quantification of grape must parameters and for quality control in an industrial cellar. |
| Silva <i>et al.</i> , 2014 | Application of FTIR-ATR to Moscatel dessert wines for prediction of total phenolic and flavonoid contents and antioxidant capacity | 56 Moscatel dessert wines | FT-MIR ATR, PLS | Method suitable for rapid screening of total phenolic and flavonoid contents in Moscatel dessert wines. |
| Garde-Cerdán <i>et al.</i> , 2010 | Employment of near infrared spectroscopy to determine oak volatile compounds and ethylphenols in aged red wines | 510 wines aged with different storage time and in different oak barrels | NIR, PLS | NIR spectroscopy can be used as rapid tool to determine oak volatile compounds and wines ethylphenols in aged red, $R^2 > 0.86$ for all the compounds. RPD > 1.5 for wines aged in French and in American and French oak barrels, and in "reserva" and "gran reserva" wines. |
| Grassi <i>et al.</i> , 2014 | Beer fermentation: monitoring of process parameters by FT-NIR and multivariate data analysis | 2 different <i>S. cerevisiae</i> strains (WLP005 and WLP570), 3 different fermentation temperatures (19, 21 and 24 °C) | FT-NIR, PCA, PLS | Reliable and robust NIR instrumentation possible for on-line implementation to monitor °Brix, pH and biomass evolution during beer fermentation. |
| Nieuwoudt <i>et al.</i> , 2006 | Rapid screening of the fermentation profiles of wine yeasts by Fourier transform infrared spectroscopy | Chenin blanc and synthetic musts | FTIR, PCA, PLS | Excellent quantitative prospects for parameters VA, alcohol, glycerol and residual sugar for Chenin blanc. |
| Gallignani <i>et al.</i> , 2005 | A simple strategy for determining alcohol in all types of and Fourier transform infrared spectrometric detection in the mid-IR | Commercial alcoholic beverages, from beers to spirits alcoholic beverages based on its on-line liquid-liquid extraction with chloroform, using a flow injection system | FTIR | Samples with alcohol higher than 15% v/v required dilution. This methodology represents a valid alternative for the determination of alcohol in alcoholic beverages, and could be suitable for the routine control analysis. |
| Casalta <i>et al.</i> , 2013 | Comparison of different methods for the determination of assimilable nitrogen in grape must | 10 must from 6 different grape varieties from France | FTIR | Can be alternative method for routine analysis, based on convenience, reliability and accuracy. Future updates include more samples to get greater variability of French must in calibration model. |

NIR – near infrared spectroscopy; PLS(R) - partial least squares regression; MIR - mid-infrared; FTIR - Fourier transform infrared; PCA - principal component analysis; ATR - attenuated total reflectance; R^2 - coefficient of determination; SEP – standard error of prediction; RPD - residual predictive deviation ratio; RMSEC - root mean square error of calibration; PRESS – prediction residual sum of squares; (°C) – degree Celsius; % - percentage; *S. cerevisiae* – *Saccharomyces cerevisiae*; > - greater than.

2.3.4 Univariate data analytical tools

Univariate statistics investigates each variable separately or relates a single independent variable x to a single dependent variable y (Esbensen, 2002). The predictive capability of a calibration model is dependent on the accuracy of the reference laboratory result, the range of the constituent being measured, the distribution of samples within the range, the total number of samples in the model, and the sampling and sample preparation (Mark, 1991). Samples included in the calibration sets should cover the range and distribution of values expected in the samples for which the calibrations will be used (Luciano & Næs, 2009). The precision of the analytical methods used in this study as reference methods was evaluated by the standard error of laboratory (SEL) and calculated using the following equation:

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

where y_1 and y_2 are duplicate measurements of a sample, and n is the number of samples (Nieuwoudt *et al.*, 2004).

2.3.5 Multivariate data analytical tools

Multivariate data analytical tools are applied to datasets that are more complex and used for exploration, prediction, classification, discrimination or regression purposes (Esbensen, 2002). Relationships between the variables are identified and interpreted based on definite sample groupings visualised in score and loading plots (Bäckström *et al.*, 2007). In this study the X -data refer to MIR spectra obtained and the y -variable to the reference method analysis results. Techniques used in this study are described below.

2.3.5.1 Principal component analysis (PCA)

PCA is a dimension reduction method for exploratory data analysis that considers the total data structure with all the variables. PCA visualises as much systematic variance as possible in the structure with the fewest PC's (principal components) possible with scatter plots and leaves the unsystematic noise behind (Esbensen, 2002). Samples with similar properties will group together on a score plot, whereas loading plots give valuable input of what causes the specific groupings. PC1 (first principal component) is the linear latent variable with the maximum possible variance. The direction of PC2 (second principal component) is orthogonal to the direction of PC1 and also has maximum possible variance in the scores (Esbensen, 2002). Hotelling T^2 statistic is used in statistical process control to detect out of control status in multivariate processes, but offers no assistance about the origin of the declared faulty status. The Hotelling T^2 statistic is used to calculate the weighted relative distance between each observation and the origin in the model plane (Cedeño Viteri *et al.*, 2012). A significance level of $p = 0.05$ (95% confidence) is used to compute the Hotelling T^2 ellipse displayed in the score plots. Those variables whose distance

measures exceed a 5% threshold value are considered doubtful and should be investigated (Cedeño Viteri *et al.* 2012).

2.3.5.2 Partial least squares regression (PLS)

Pre-processing can be applied to spectra prior to calibration development to eliminate noise that may influence the model. PLS is a linear regression method that is used for multivariate calibration development (Martens & Næs, 1989; Esbensen, 2002). This method finds a correlation between the **X**-data (MIR spectra) and the **y**-variables (in this study, chemical concentrations of reference sample analysis). The **X**-data are transformed into a set of intermediate linear latent variables that are used for regression with a dependent **y** variable. The first components explain the most covariance between scores in **X**-space and scores in the **Y**-space. The complexity of the model contributes to the number of PLS components. The optimal number components is selected based on the lowest error in calibration (Esbensen, 2002).

R^2 is the ratio of the variation explained by the model to the total variation (Esbensen, 2002). Evaluation of the PLS calibration models include coefficient of determination (R^2) when based on calibration set and cross-validation statistics. The criteria proposed in Table 2.5 on the values of R^2 were employed in this study (Urbano Cuadrado *et al.*, 2004). Thus, R^2 values between 70 and 89 indicated good precision, whereas values equal to and higher than 90 indicated excellent precision.

Independent test sets validation statistics are used to test the performance of the PLS algorithm on future unknown samples and hence, also obtain a measure to compare the calibration error achieved to the laboratory error, SEL. The root mean square error of prediction (RMSEP) is used to represent the prediction performance after validation and is expressed in the same unit as the original reference data. The RMSEP is calculated as follows (Esbensen, 2002):

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

where y_i is the reference value for the i^{th} sample, \hat{y}_i is the predicted value for the i^{th} sample and n is the number of samples.

The RPD is defined as the ratio between the standard deviation (SD) of the reference values to the standard error of the predicted values when using independent test set validation (Esbensen, 2002). RPD values was used as a tool to evaluate the prediction ability of the PLS calibration models (Williams & Norris, 2001) (Table 2.5). Values between 5 and 6.4 propose a calibration model suitable for quality control, and a value between 6.5 and 8 for process control. Calibration models with RPD values equal to and higher than 8.1 are suitable for any application.

Table 2.5: Criteria for the interpretation of R^2 (Urbano Cuadrado *et al.*, 2004) and RPD (Williams & Norris, 2001) used to evaluate the performance of the calibration models.

| R^2 ^a | Interpretation |
|--------------------|---|
| 5 to 29 | Not suitable for quantification |
| 30 to 49 | Correct separation between low and high values |
| 50 to 69 | Good separation between low, medium and high values |
| 70 to 89 | Good precision |
| ≥ 90 | Excellent precision |
| RPD ^b | Interpretation |
| 0 to 2.3 | Not recommended |
| 2.4 to 3 | PLS calibration model suitable for rough screening purposes |
| 3.1 to 4.9 | PLS calibration model suitable for screening purposes |
| 5.0 to 6.4 | PLS calibration model suitable for quality control |
| 6.5 to 8 | PLS calibration model suitable for process control |
| ≥ 8.1 | Any application |

^a R^2 : coefficient of determination; ^bRPD: residual predictive deviation ratio

2.3.5.3 Bias

Bias gives an indication of the systematic error of the calibration and is calculated as the average difference between the reference and predicted values and should ideally be equal to zero (Esbensen, 2002).

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

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

2.4 CONCLUSION

The application of IR spectroscopy as a method of analysis in the alcoholic beverage industry, is worldwide well-established. This review on application of IR spectroscopy in the alcoholic beverage industry reflects the use of this method for screening, quality control, quantification and classification purposes. The growing need for low-cost, high-quality products by consumers highlights the importance of suitable actions by the South African cellars to introduce and maintain technology to control the quality of products. The development of FT-MIR calibration models for NH_3 and parameters in spirit-based liqueurs, contribute to the cellars' need and expands the use of IR in the South African alcoholic beverage industry.

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Chapter 3

Research results

Application of Fourier transform mid-infrared (FT-MIR) attenuated total reflectance (ATR) spectroscopy for the quantification of ammonia in freshly pressed grape juice

CHAPTER 3. RESEARCH RESULTS

Application of Fourier transform mid-infrared (FT-MIR) attenuated total reflectance (ATR) spectroscopy for the quantification of ammonia in freshly pressed grape juice

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ABSTRACT

Background Ammonia (NH₃) is an important source of nitrogen for the metabolism of the wine yeast. NH₃ deficiencies in grape juice can lead to stuck or sluggish fermentations and organoleptic defects in wine. Due to these potential negative implications, it was important to develop and implement methods whereby the NH₃ concentrations in freshly pressed grape juice could be determined rapidly and accurately. Traditional methods for quantification of NH₃ in grape juice are lengthy and cumbersome and do not lead to automated analysis.

Objective The aims of this study were firstly to evaluate the potential of Fourier transform mid-infrared (FT-MIR) attenuated total reflectance (ATR) spectroscopy for the quantification of NH₃ content in freshly pressed grape juice, prior to onset of alcoholic fermentation and secondly, to implement the spectrometer and partial least squares regression algorithm developed in this study at an industrial cellar.

Design A sample set of 183 grape juice samples, consisting of 10 different cultivars were collected during the 2010 and 2011 harvest seasons. Reference data for NH₃ were generated on the samples using an enzyme-linked spectrophotometric assay and the NH₃ concentration ranged from 10.85 to 47.10 mg/L. FT-MIR ATR spectra of the samples were generated in the range between 4000 and 400 cm⁻¹.

Results Cross validation was used to test the NH₃ partial least squares (PLS) calibration model, using the performance criteria coefficient of determination (R²) and root mean squared error of cross validation (RMSECV). The R² was 92.05 and the RMSECV was 1.86 mg/L. Subsequently, repeated independent test set validation with 61 samples was done to evaluate the predictive performance of the model based on the root mean square error of prediction (RMSEP) and residual predictive deviation ratio (RPD). The average RMSEP value of 1.88 mg/L was in agreement with the standard error of laboratory (SEL) 1.54 mg/L. The average RPD value of the NH₃ PLS calibration model was 3.3.

Conclusion The NH_3 PLS calibration model gave excellent precision and was fit for screening purposes. The technology was transferred to an industrial wine cellar for accurate, fast and high throughput quantification. The calibration model provided the industry with a tool to detect insufficient yeast assimilable nitrogen (YAN) levels in freshly pressed grape juice and contributed to effective quality control in the wine production activities.

3.1 INTRODUCTION

Wine yeast requires YAN as nutrient to synthesise proteins and nucleic acids for optimal growth and metabolism (Baroň, 2011; Gutiérrez *et al.*, 2012). YAN consists of free amino nitrogen (FAN) and NH_3 (or NH_4^+) (Henschke & Jiranek, 1993). YAN is required by the wine yeast to increase biomass during fermentation (Crépin *et al.*, 2014) and for fermentation activities (Varela *et al.*, 2004).

Insufficient amounts of NH_3 in grape juice are known to be a major cause of slow, stuck and sluggish fermentations (Filipe-Ribeiro & Mendes-Faia, 2007; Gutiérrez, 2013). Slow fermentation refers to a low fermentation rate throughout the fermentation process; stuck fermentation to a low fermentation rate of residual sugars and sluggish fermentation to a late-onset of the fermentation process (Blateyron & Sablayrolles, 2001). Hydrogen sulphide (H_2S) is formed during the synthesis of sulphur-containing amino acids, methionine and cysteine by yeast (O’Kennedy & Reid, 2008). Excess amounts of H_2S are formed when inadequate levels of NH_3 occur in grape juice (Jiranek *et al.*, 1995). Some *Saccharomyces cerevisiae* (*S. cerevisiae*) strains metabolise sulphur containing amino acids, thereby releasing sulphide, which can combine with hydrogen to produce H_2S . H_2S has a rotten egg odour that negatively affects wine aroma and flavour (Giudici & Kunkee, 1994).

The FAN concentration in grape juice is determined by the o-phthaldialdehyde/N-acetyl-L-cysteine (NOPA) assay (Gump *et al.*, 2000). The NH_3 concentration in grape juice is traditionally determined by an ammonium ion probe, or by enzyme-linked spectrophotometric assays. Summation of these two nitrogen measurements, FAN and NH_3 content yields YAN. Arginine and proline are not metabolised during the anaerobic alcoholic fermentation process and do not form part of the YAN measurement (Stewart, 2014). In order to achieve an adequate rate of fermentation to dryness, it is recommended that clarified juice should contain approximately 150 mg/L YAN (Blateyron & Sablayrolles, 2001). If the concentration of NH_3 is lower than 50 mg/L, nitrogen supplementation is necessary for optimum fermentation performance by yeast (Ribéreau-Gayon *et al.*, 2000). Diammonium phosphate (DAP) is frequently used to supplement nitrogen in grape juice in the industry. The increase in NH_3 content of grape juice with insufficient amounts of NH_3 reduces the amounts of heavy sulphur compounds in wines (Moreira *et al.*, 2011).

Fourier transform mid-infrared (FT-MIR) spectroscopy normally ranging from 4000 to 400 cm^{-1} , is a non-destructive analytical technique that is used extensively in the wine industry, based

on its main advantages of accuracy, reliability and fast, high throughput analysis, without the need for costly and time-consuming sample preparation (Lachenmeier, 2007). The method has been used for the determination of YAN in grape juice (Casalta *et al.*, 2013). However, work based on South African grape cultivars is limited. In the context of the large volumes of juice analysed by the DGB analytical laboratory, as well as the lengthy enzyme-linked spectrophotometric assay in use at the laboratory, to quantify NH_3 in grape juice the need to develop an alternative cost- and time effective method was clear. This study therefore reports on a feasibility study using a small, portable FT-MIR spectrometer with attenuated total reflection (ATR) to obtain spectra and use these to develop NH_3 PLS calibration model that could be implemented for quantitative purposes at an industrial cellar.

3.2 MATERIALS AND METHODS

3.2.1 Samples

A total of 183 juice samples from 10 cultivars were collected during 2010 (n=52) and 2011 (n=131) wine grape harvest seasons for development of NH_3 PLS calibration model. The red cultivars (n=83) were Cabernet Sauvignon (n=23), Malbec (n=19), Pinotage (n=15) and Shiraz (n=26). Blanc de Noir (n=16) represented the rosé cultivar. The white cultivars (n=84) consisted of Chardonnay (n=20), Chenin Blanc (n=21), Sauvignon Blanc (n=16), Semillon (n=17) and Verdelho (n=10). Samples were received from 3 contracted wine cellars, respectively Wellington, Franschhoek and Boschendal in the Western Cape, South Africa. The samples were transported in cooler bags with ice cubes to the central analytical laboratory for respective analyses. An aliquot of 200 ml of each sample was centrifuged for 5 minutes at 5000 rpm. Small aliquots of the supernatants were used for spectral analyses as described below, while 100 ml were used to conduct reference values. Samples that could not be immediately analysed were stored frozen at $-8\text{ }^\circ\text{C}$ for maximum 10 days to avoid onset of alcoholic fermentation.

3.2.2 Reference analysis

Samples were analysed for NH_3 content on arrival at the laboratory, or directly after thawing at $0\text{ }^\circ\text{C}$ in the case of frozen samples, using an enzymatic method that is based on the principle that NH_3 reacts with α -ketoglutarate and reduces nicotinamide adenosine dinucleotide (NADH) in the presence of glutamate dehydrogenase (GIDH) to form L-glutamate and NAD^+ (Ammonia Catalogue number 11 112 732 035, R-Biopharm, Germany). The amount of NADH consumed is stoichiometrically related to the amount of NH_3 present in the juice sample and was measured at 340 nm on a spectrophotometer (Hach, Cape Town, South Africa). Strongly coloured red cultivar samples were treated by polyvinylpyrrolidone (PVPP) at a concentration of 2.5-5 g/100 ml sample to prevent inhibition of the reaction by tannins and thereafter clarified with filtration using

filter paper. Samples were analysed in duplicate by the reference method and the SEL was calculated using the following equation:

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

where y_1 and y_2 are duplicate measurements of a sample and n is the number of samples (Nieuwoudt *et al.*, 2004).

3.2.3 Spectral measurements

Since this study was a feasibility study, instrumentation was on loan from the supplier and FT-MIR ATR spectra were collected on two different Alpha-P spectrometers (Bruker Optics, Bryanston, South Africa) for the 2010 and 2011 vintages respectively, as a result of instrument availability. The instruments were fitted with OPUS software (OPUS version 7.0 for Microsoft Windows, Bruker Optics) for calibration development. No further sample preparation as already described was done for spectral analysis and volumes of 0.20 ml supernatant were used. The Alpha-P instrument has a potassium bromide (KBr) beam splitter and a 2x2 mm temperature controllable ATR diamond crystal sample plate, which was set at 40°C. The spectrum of each sample was obtained from 3996.59 to 372.44 cm^{-1} and the average of 64 scans at a resolution of 8 cm^{-1} with a scanner velocity of 10 kHz was recorded. A background measurement against distilled water was conducted after every 10th juice sample.

3.2.4 Data analysis

FT-MIR ATR spectra obtained in this study were visually assessed and peak interpretation was done based on published information as indicated. Explorative data analysis was performed on the spectra to investigate possible influences related to vintage, juice colour and grape cultivar. For this purpose, the spectra were exported from OPUS software to SIMCA software (version 13.0.3, Umetrics, Sweden), a stand-alone chemometrics software with specialist chemometrics functions that were not available in OPUS. Principal component analysis (PCA) is a method suitable for this task (Esbensen, 2002) and was used to investigate and interpret patterns, groupings and similarities in the spectral data. In this study, the **X**-data consisted of spectral measurements, $\mathbf{X} = 1266$, of the grape juice samples and the **Y**-data of one variable, the corresponding NH_3 content of the samples.

PCA-X reduces the original dimensionality in the spectral data to a small number of principal components that visualise the maximum variance in the data. Samples with similar properties will be grouped together in the scores plots, whereas the loading plots provide a means to the correlations between the patterns observed in the scores plots and the wavenumbers (Esbensen, 2002). The spectral data were mean-centered and the y-variable was scaled to unit

variance (UV), by multiplying each y-variable with 1/SD of the full range of reference values for NH₃. The use of PCA for the processing of spectral data to design calibration sets and for the detection and classification of outlier samples has been reported (Cadet *et al.*, 1997; Nieuwoudt *et al.*, 2004; Shen *et al.*, 2012). A quantitative measure of the goodness of fit is given by the parameter R²X (cum) and varies between 0 and 1, where 1 means a perfectly fitting model and 0 no fit at all. A quantitative measure of the predictive power of a model is given by the parameter Q²X (cum), where 1 indicated that the model had good predictive ability. The parameters are used in the evaluation of a PCA model. The Hotelling T² statistic was used to calculate the weighted relative distance between each observation and the origin in the model plane (Cedeño Viteri *et al.*, 2012). A significance level of $p = 0.05$ (95% confidence) was used to compute the Hotelling T² ellipse displayed in the score plots.

PLS regression in this study was used to develop a NH₃ calibration model with OPUS software. Various spectral pre-processing methods were evaluated, i.e. straight line subtraction, vector normalization, first derivative and second derivative, to improve the predictive ability of the PLS models. These methods are standard pre-processing methods and have been described (Esbensen, 2002). PLS finds a correlation between two sets of variables, the **X**-data (MIR spectra) and the y-variables (in this study, the NH₃ concentrations of reference sample analysis). The **X**-data are transformed into a set of intermediate linear latent variables that are used for regression with a dependent y variable. The first components explain the most covariance between scores in the **X**-space and scores in the **Y**-space (Esbensen, 2002).

During the PLS calibration model development stage, different combinations of selected spectral ranges and spectral pre-processing methods were tested by using the optimisation tool in OPUS 7.0 software. Evaluation of the PLS calibration models included R² and RMSECV when based on cross-validation statistics, with 20 samples per segment. The criteria for the interpretation of the R² values were those proposed by Urbano Cuadrado *et al.* (2004) and are summarised in Table 3.1. Best calibration models were selected based on R² and RMSECV values. For the model validation, test set validation, using one third of the samples that were randomly selected as test samples, was used. The test set samples were not included in the calibration stage, but only at the validation stage. Three different independent test set repeats were used to evaluate the model performance. Criteria used to evaluate the precision of predictive capacity of the PLS model were root mean square error of prediction (RMSEP) that was calculated as follows (Esbensen, 2002):

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

where y_i is the reference value for the i^{th} sample, \hat{y}_i is the predicted value for the i^{th} sample and n is the number of samples.

RPD, defined as the ratio between the standard deviation (SD) of the reference values to the standard error of the predicted values (RMSEP) when using independent test set validation (Esbensen, 2002), was also used to evaluate the performance of the models. In Table 3.1 the criteria for the evaluation of RPD values, as proposed by Williams & Norris (2001), in this study, are shown.

Table 3.1: Criteria for the interpretation of R^2 (Urbano Cuadrado *et al.*, 2004) and RPD (Williams & Norris, 2001) used to evaluate the performance of the ammonia PLS calibration model.

| R^2 ^a | Interpretation |
|--------------------|---|
| 5 to 29 | Not suitable for quantification |
| 30 to 49 | Correct separation between low and high values |
| 50 to 69 | Good separation between low, medium and high values |
| 70 to 89 | Good precision |
| ≥ 90 | Excellent precision |
| RPD ^b | Interpretation |
| 0 to 2.3 | Not recommended |
| 2.4 to 3 | PLS calibration model suitable for rough screening purposes |
| 3.1 to 4.9 | PLS calibration model suitable for screening purposes |
| 5.0 to 6.4 | PLS calibration model suitable for quality control |
| 6.5 to 8 | PLS calibration model suitable for process control |
| ≥ 8.1 | Any application |

^a R^2 : coefficient of determination; ^bRPD: residual predictive deviation ratio

3.3 Results and discussion

3.3.1 Reference data

The descriptive statistics for the chemical composition (range, average, standard deviation and standard error of laboratory) of the grape samples used for the NH_3 calibration and validation sets are shown in Table 3.2. The table also contains the ranges of the samples used in the calibration set for the development of the commercial ammonia PLS model (Foss Analytical, Grapescan Application note 180, 2001, Denmark). The ranges of pH, total acidity (TA) and malic acid (MA) are clearly outside the limits for the commercial calibrations, thereby highlighting the need to develop a new model. Swanepoel *et al.* optimised the quantification of total soluble solids, pH and TA in South African grape juice using FT-MIR spectroscopy in 2007. Although the chemical

composition of the juice was similar to the ranges for the samples used in this study, a few differences in the samples for the respective PLS calibration models were noted. Grape samples (11 – 26 °B) from eight cultivars over three consecutive vintage (2003 to 2005) were analysed by Swanepoel *et al.*, whereas samples from twenty cultivars (21.5 – 25.4 °B) during the 2010 and 2011 harvest seasons were analysed during this study. Magerman (2009) developed PLS calibration models for glucose, fructose and YAN in fermenting must. The concept of matrix effects is a well-known principle in quantitative work based on infrared spectroscopy and also for this reason it was necessary to develop SA based algorithms (Nieuwoudt *et al.*, 2004).

Table 3.2: The descriptive statistics for chemical composition (range, average, standard deviation and standard error of laboratory) for the grape juice samples used in the ammonia calibration and validation sets at DGB laboratory and the ranges for the samples used in the ammonia calibration set at Foss (www.foss.dk).

| Parameter | Range Min _{DGB} ^a - Max _{DGB} ^b | Range Min _{Foss} ^c - Max _{Foss} ^d | Average | SD ^e | SEL ^f |
|---------------------|---|---|---------|-----------------|------------------|
| pH | 2.30 - 4.03 | 2.70 - 4.60 | 3.24 | 0.13 | 0.5 |
| Total acidity (g/L) | 2.78 - 9.63 | 0.32 - 10.36 | 5.33 | 0.38 | 0.50 |
| Malic acid (g/L) | 0.69 - 3.69 | 0.20 - 8.54 | 1.89 | 0.13 | 0.20 |
| °Brix | 21.5 - 25.4 | | 23.2 | 0.06 | 0.10 |
| Ammonia (mg/L) | 10.85 - 47.10 | | 24.10 | 7.47 | 1.54 |

^aMin_{DGB}: minimum for samples analysed at DGB; ^bMax_{DGB}: maximum for samples analysed at DGB; ^cMin_{Foss}: minimum for samples analysed at Foss; ^dMax_{Foss}: maximum for samples analysed at Foss; ^eSD: standard deviation; ^fSEL: standard error of laboratory

3.3.2 Spectral analysis

Spectra in the absorption ranges between wavenumbers 3996.59 to 372.44 cm⁻¹ were obtained for the grape juice samples. A baseline off-set between the spectra obtained in 2010 and 2011 seasons was seen (data not shown) and this was ascribed to the use of two different spectrometers during the course of the project. The baseline correction function in OPUS was therefore used to correct for this drift. The full range, baseline corrected spectra are shown in Figure 3.1 (a), with prominent noisy regions at both the lowest and highest wavenumber ends visible. Figure 3.1 (b) shows a FT-MIR ATR line plot of one spectrum with the region 979.93 and 1450.61 cm⁻¹, that is known as the fingerprint area (Nieuwoudt *et al.*, 2004), indicated on the graph. This region contains a significant amount of variation related to the absorbance by molecular chemical groups present in grape juice (Cozzolino *et al.*, 2009, 2011). It is known from literature that absorbance at the wavenumber 1650 cm⁻¹ are related with N-H bending and the region 1300 to 1370 cm⁻¹ with N-H stretching and correspond to NH₃ (Cozzolino *et al.*, 2009, 2011) present in grape juice. A background measurement with distilled water was done to minimise the water absorption regions 1716 to 1543 cm⁻¹ and 3626 to 2970 cm⁻¹ (de Villiers *et al.*, 2012) in the spectra.

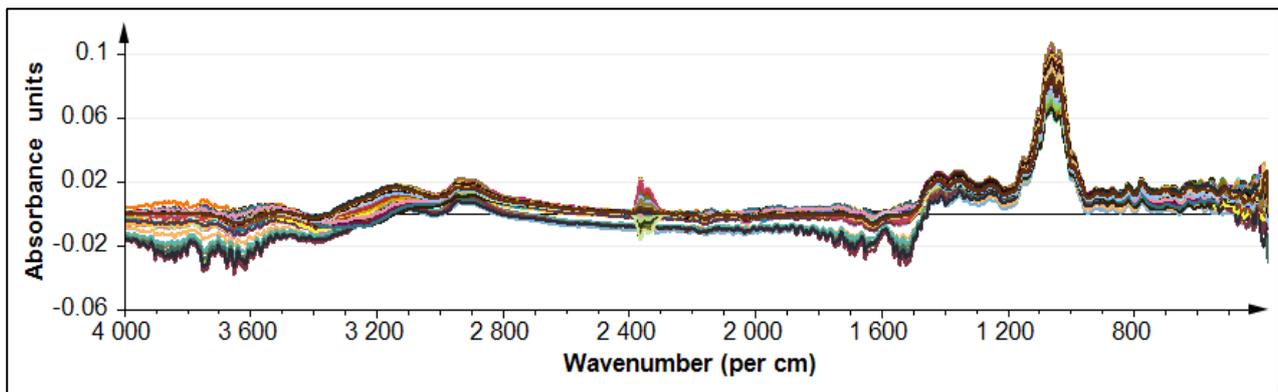


Figure 3.1 (a): Line plot of FT-MIR ATR grape juice spectra obtained between 3996.59 and 372.44 cm^{-1} .

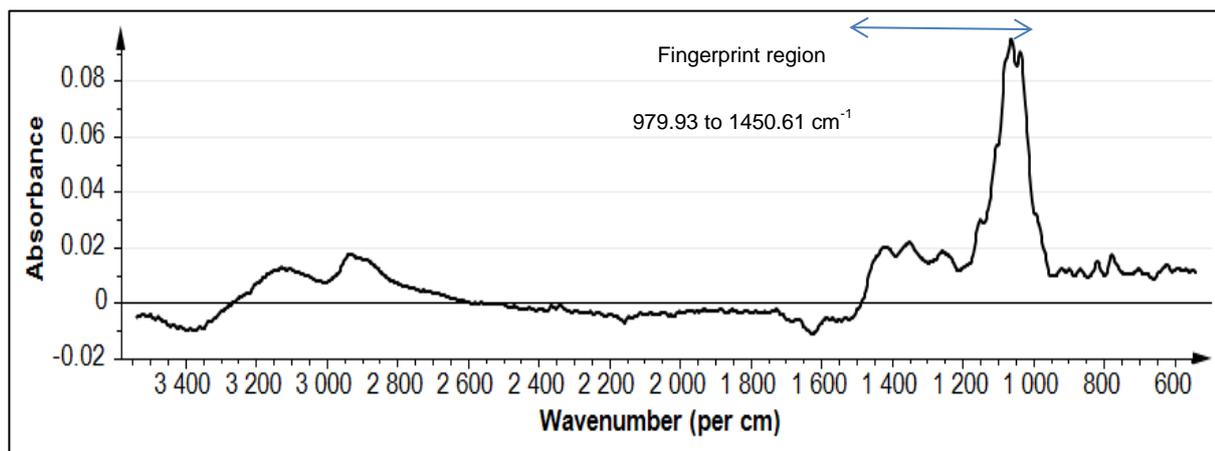
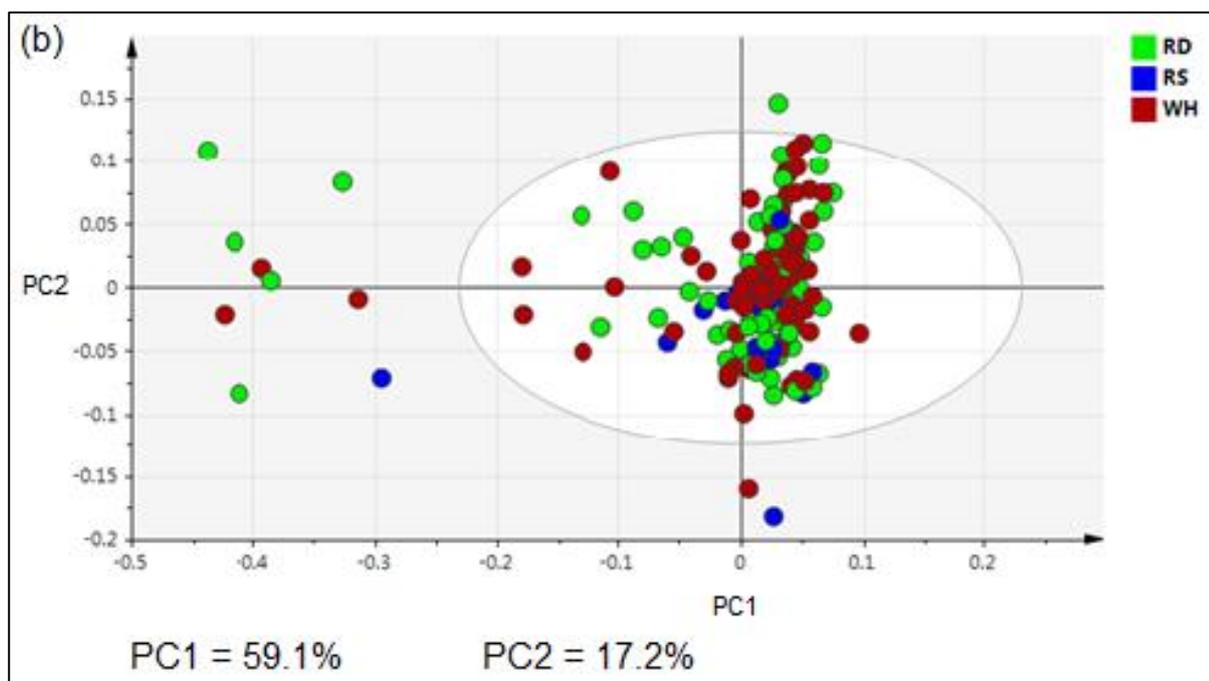
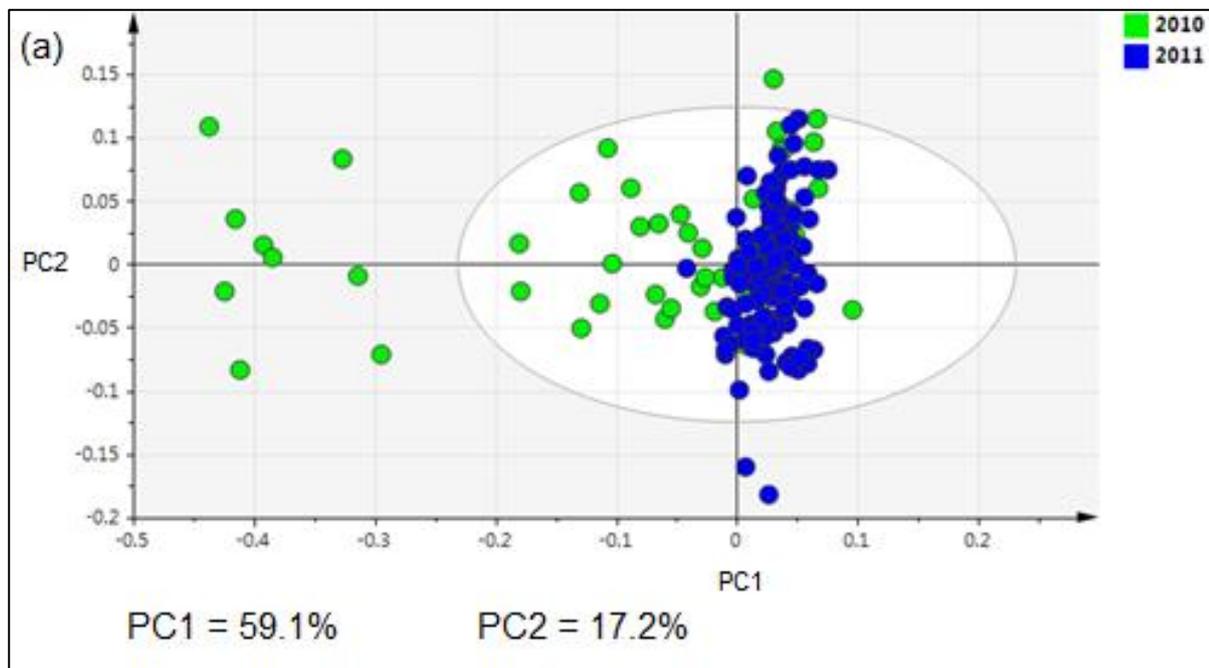


Figure 3.1 (b): Line plot of single FT-MIR ATR grape juice spectrum. Peak annotation is according to Cozzolino *et al.* (2009, 2011). Negative absorption regions are due to the background spectra of water.

3.3.3 Principal component analysis

PCA was used to investigate the influence of vintage, grape cultivar and juice colour on the raw spectra and hence also possibly the predictive abilities of the PLS model. Samples that shared similar chemical properties grouped together in the score plot and the maximum variation between the samples was illustrated by the first components (Esbensen, 2002). In Figure 3.2, results of PCA performed on all the FT-MIR ATR spectra for wavenumbers 538.61 to 3535.34 cm^{-1} are shown. The observations in the scores plots were coloured according to (a) 2010 and 2011 vintages, (b) red (RD), rosé (RS) and white (WH) berry colour and (c) Cabernet Sauvignon (CS), Malbec (MB), Pinotage (PI), Shiraz (SH), Blanc de Noir (BN), Chardonnay (CH), Chenin Blanc (CB), Sauvignon Blanc (SB), Semillon (SE) Verdelho (VD) cultivars and the loading plot of PC1 (59.1% variance explained) illustrated in Figure 3.2 (d). The model was developed with 1048 variables using four principal components that explained 96% of the variance in \mathbf{X} ($R^2\mathbf{X}$ (cum) = 0.93). The $Q^2\mathbf{X}$ (cum) = 0.92 indicated that the model had good predictive ability. The observations showed a degree of separation according to vintage but overlapping still occurred. This indicated that vintages related variation may have an influence on the calibration model and this aspect should be investigated in future research at the cellar. There was no clear clustering on

the basis of juice colour Figure 3.2 (b) or grape cultivar Figure 3.2 (c). The loadings plot for PC1 showed that the absorbance at most wavenumbers contributed to the separation seen in the score plots.



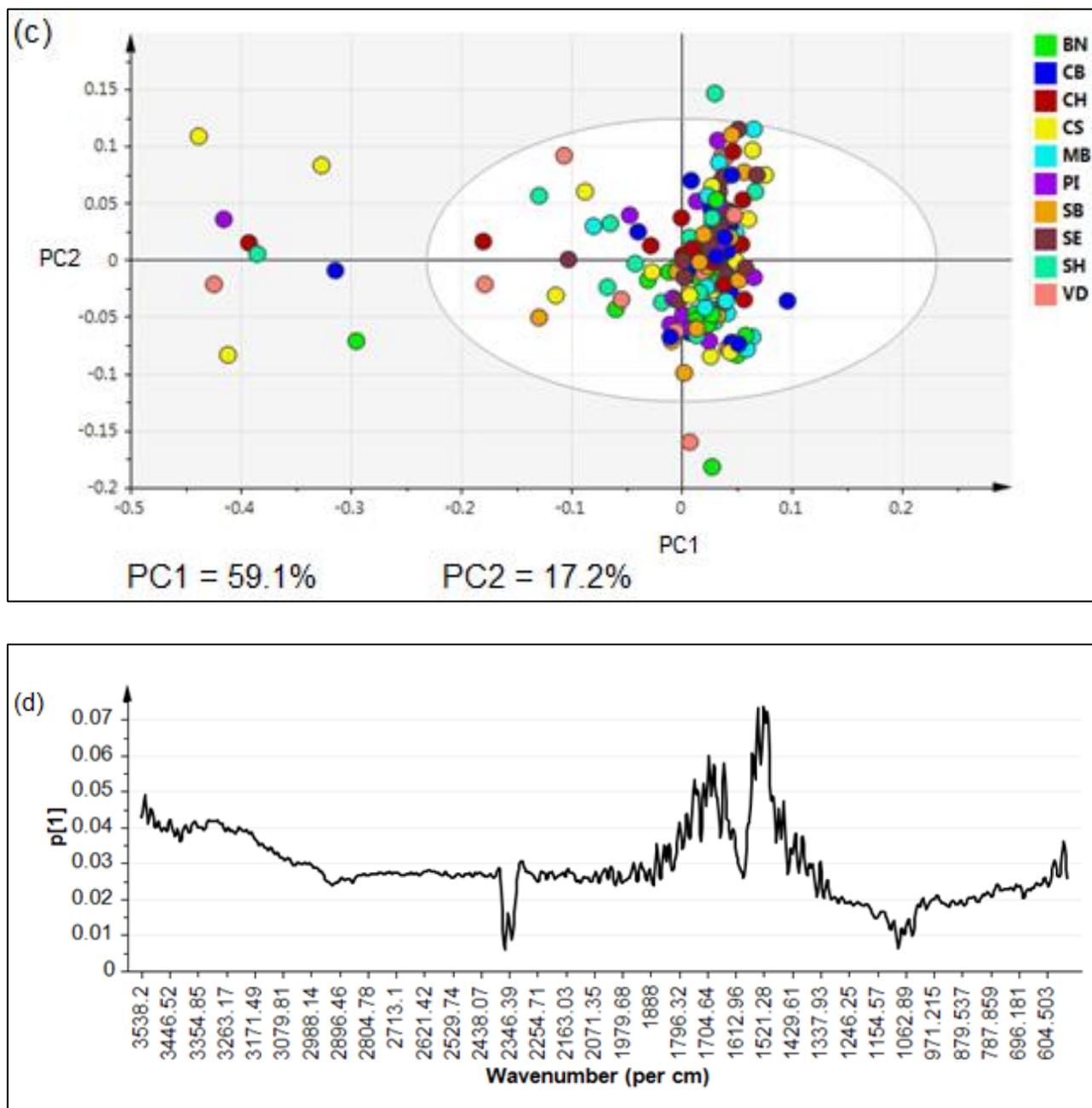


Figure 3.2: PCA score plot of all the grape juice samples obtained between region 538.61 to 3535.34 cm^{-1} coloured according to (a) 2010 and 2011 vintages; (b) red, rosé and white berry colour; (c) 10 cultivars with tolerance ellipse based on Hotelling's T^2 ($p = 0.05$) and (d) Loadings plot of PC1 (59.1% variance explained).

3.3.4 Partial least square regression modelling

Based on the PCA results described in section 3.3.3, for the development of NH_3 PLS calibration model, all the samples in the dataset were used. To guard against overfitting of the model, whilst sufficiently modelling the variance in the data set, the number of components for the calibration was selected based on the highest explained variance with the lowest number of components. Vector normalization (SNV) was selected as pre-processing by OPUS software for the ranges 3535.3 to 2635.7; 2337.8 to 2037; 1739 to 1435.3 and 1140.2 to 836.6 cm^{-1} wavenumbers to eliminate noise in the spectral data. In Table 3.3, the regression statistics for the NH_3 PLS model

with cross validation and the statistics for independent test set validation were reported. The maximum and minimum NH₃ values were in the calibration sets. The performance of the PLS calibration model was evaluated on R² (Urbano Cuadrado *et al.*, 2004) and RPD (Williams & Norris, 2001) criteria in Table 3.1. The predicted versus measured plot for calibration with cross validation (Figure 3.4 (a)) and the independent test set (2) with the lowest RMSEP was shown (Figure 3.4 (b)). The plot illustrated the distribution of the samples along the linear regression line.

Table 3.3: Regression statistics for PLS calibration model with cross validation developed for ammonia in grape juice and independent test set validation statistics.

| | Ammonia | Independent test set validation 1 | Independent test set validation 2 | Independent test set validation 3 |
|--|--------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| N^a (cal) | 183 | 122 | 122 | 122 |
| Min^b (cal) - Max^c (cal) | 10.85 – 47.10 mg/L | 10.85 – 47.10 mg/L | 10.85 – 47.10 mg/L | 10.85 – 47.10 mg/L |
| R² ^d | 92.05 | 92.27 | 89.64 | 90.36 |
| Rank | 8 | 7 | 9 | 7 |
| RMSECV^e | 1.86 mg/L | 1.86 mg/L | 1.78 mg/L | 1.86 mg/L |
| N^f (val) | | 61 | 61 | 61 |
| Min^g (val) – Max^h (val) | | 11.84 – 42.37 mg/L | 12.98 – 39.84 | 12.98 – 39.84 mg/L |
| RMSEPⁱ | | 1.95 mg/L | 1.8 mg/L | 1.88 mg/L |
| RPD^j | | 3.6 | 3.1 | 3.3 |
| Bias | 0.098 | 0.117 | 0.042 | 0.36 |

^a (cal)N: number of samples calibration set; ^b (cal)Min: minimum calibration set; ^c (cal)Max: maximum calibration set; ^dR² coefficient of determination; ^eRMSECV: root mean squared error of cross validation; ^f (val)N: number of samples validation set; ^g (val)Min: minimum validation set; ^h (val)Max: maximum validation set; ⁱRMSEP: residual mean standard error of prediction; ^jRPD: residual predictive deviation ratio

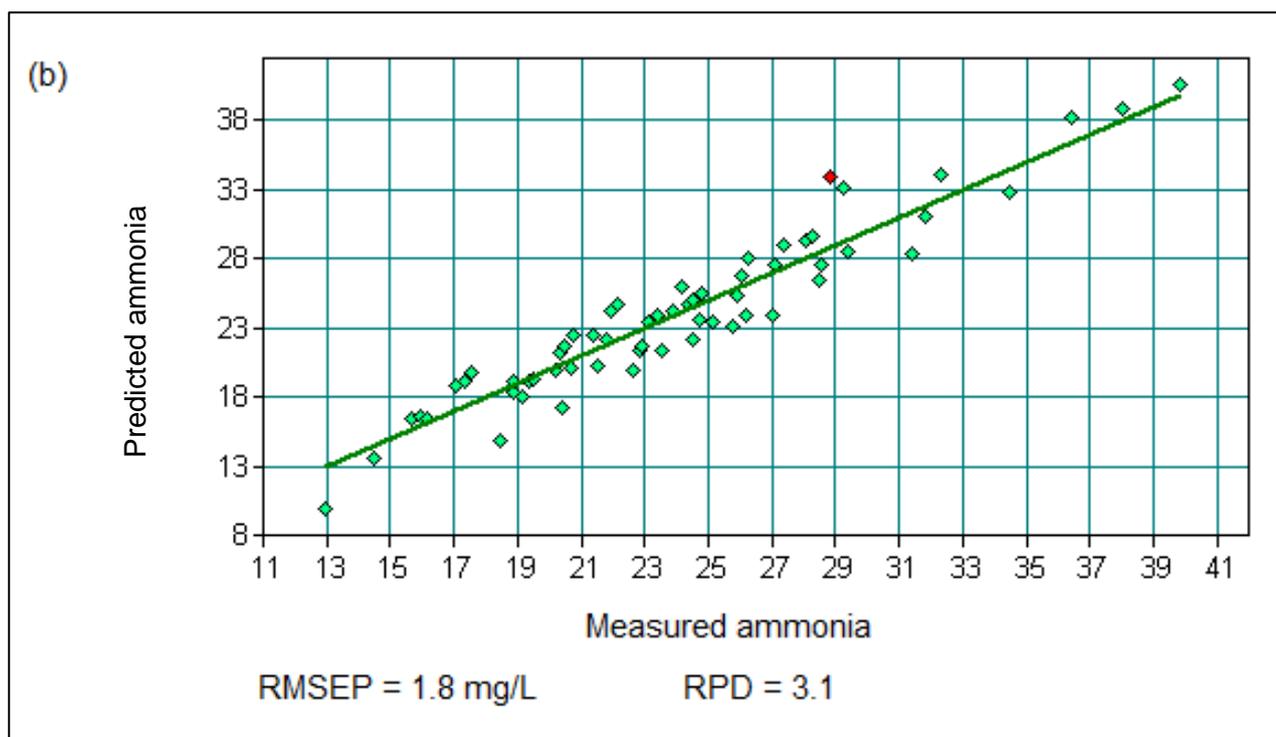
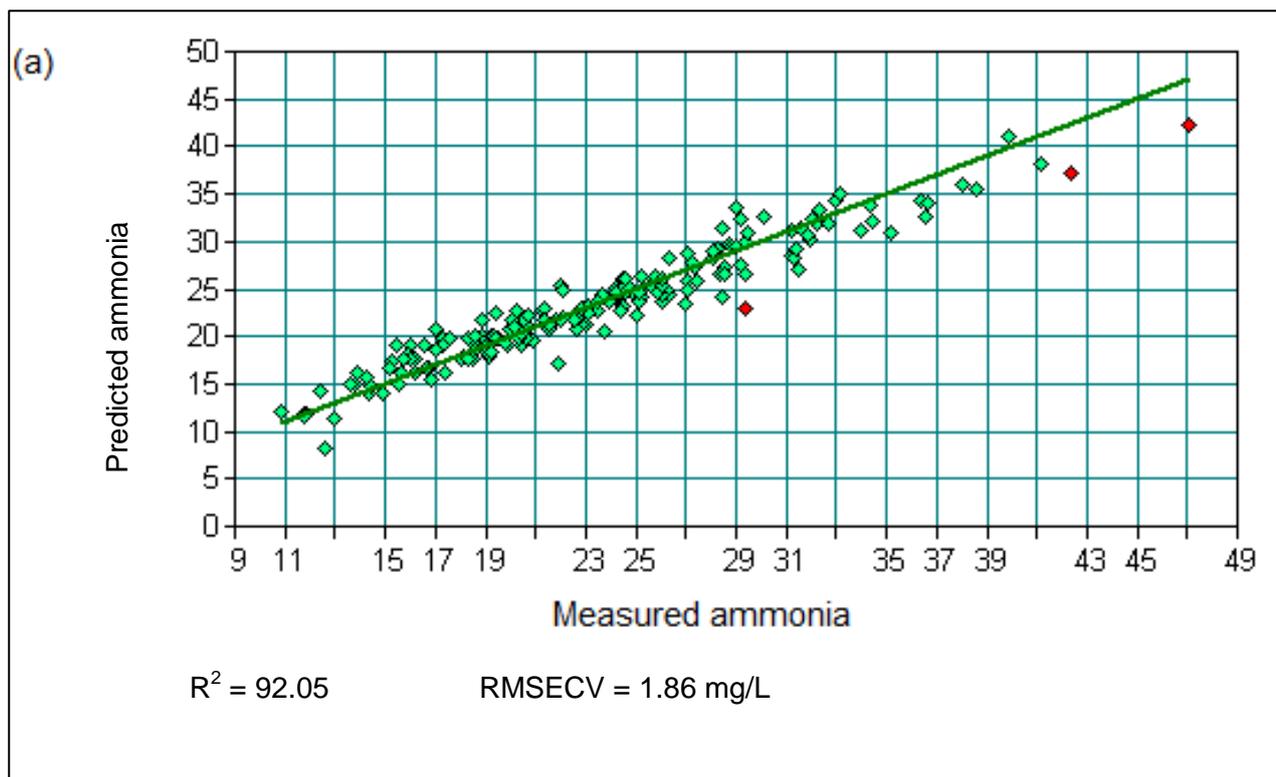


Figure 3.4: Regression plot of predicted versus measured ammonia concentration for (a) calibration with cross validation and (b) test set validation with lowest RMSEP (1.8 mg/L).

In Figure 3.4 the evenly spread distribution of samples along the linear regression line were expected, due to the chemical analysis of the industrial samples used for the calibration and validation sets. The RMSECV was 1.86 mg/L and the R^2 value (92.05) indicated NH_3 PLS model

with excellent precision according to the interpretation guidelines of Urbano Cuadrado *et al.* (2004) in Table 3.1. The average RMSEP of 1.88 mg/L was in agreement with the SEP of 1.54 mg/L and lower than the accuracy of 23.04 mg/L for the commercial calibration model (www.foss.dk). The average RPD value of 3.3 proposed that the PLS model developed is suitable for screening purposes (Williams & Norris, 2001). The cellar accepted the statistical results of the NH₃ PLS calibration model. The calibration model with cross validation that contained optimal amount of samples in calibration set to explain maximum variation in spectra, was implemented for the quantification of NH₃ in grape juice at the cellar.

3.4 CONCLUSION

The aim of the study was to evaluate the potential of FT-MIR ATR spectroscopy for the quantitative determination of NH₃ in freshly pressed grape juice of South African cultivars. In accordance to instrument availability from the supplier, the 183 grape juice spectra were collected on two Alpha-P spectrometers for the 2010 and 2011 vintages with OPUS software. A drift in spectra between the two spectrometers were noted that can influence the accuracy of the model. Baseline correction was used to correct for this drift. For future PLS calibration model development, the standardisation of spectrometers in the cellar are important for accurate results and the transfer of models between spectrometers.

The spectra were exported from OPUS software to SIMCA software for explorative data analysis. Vintage showed a possible influence on the model and should be further investigated in future research by the cellar by adding more samples from different vintages to the calibration model.

The calibration models were developed using PLS regression OPUS software. The NH₃ PLS calibration model gave excellent precision and was fit for screening purposes ($R^2 = 92.05$ and average RPD = 3.3). The average RMSEP value of 1.88 mg/L was in agreement with the standard error of laboratory (SEL) 1.54 mg/L. The technology was implemented by the cellar for accurate, fast and high throughput quantification of NH₃ in grape juice.

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Chapter 4

Research results

Application of Fourier transform mid-infrared (FT-MIR) spectroscopy, in attenuated total reflection (ATR) and transmission scanning modes, for the quantification of alcohol, pH and invert sugar in spirit-based liqueur products

CHAPTER 4. RESEARCH RESULTS

Application of Fourier transform mid-infrared (FT-MIR) spectroscopy, in attenuated total reflection (ATR) and transmission scanning modes, for the quantification of alcohol, pH and invert sugar in spirit-based liqueur products

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ABSTRACT

Background Spirit-based liqueurs are produced from distilled cane spirit that have been sweetened with sucrose and/or caramel and flavoured by herbs, fruits, spices or other flavourings. To date, quality control during production and bottling of these products in South Africa has been done with lengthy and cumbersome wet chemical methods.

Objective The aims of the study were to evaluate the potential of Fourier transform mid-infrared (FT-MIR) spectroscopy using two different spectrometers, respectively attenuated total reflection (ATR)- and transmission scanning modes for the quantification of alcohol, pH and invert sugar in these products during their production and bottling stages. The ultimate aim was to implement the respective instrumentation and the partial least squares (PLS) regression algorithms developed in this study at an industrial cellar.

Design A sample set of 589 liqueur samples consisting out 20 different product types was collected at 8 quality control points of production. Of these, 343 samples comprising of 19 product types were scanned in reflection mode and 246 samples, comprising of 17 product types were scanned in transmission mode. Reference data for alcohol, pH and invert sugar content were generated on the samples using appropriate wet chemistry methods.

Results Cross validation was used to test the alcohol, pH and invert sugar PLS calibration models, using the performance criteria coefficient of determination (R^2) and root mean squared error of cross validation (RMSECV). The R^2 and RMSECV values for PLS calibration models obtained in reflection mode were 86 and 0.7 for pH; 85.8 and 26.1 g/L for invert sugar. The alcohol, pH and invert sugar PLS calibration models obtained in transmission mode had R^2 values of 99.72, 81.5 and 92.4, and RMSECV values of 0.47% v/v, 1.0 and 22.4 g/L, respectively. Thereafter, independent test set validation was used to evaluate the predictive performance of the PLS calibration models, using the performance criteria root mean square error of prediction (RMSEP) and residual predictive deviation ratio (RPD). The RMSEP values for the PLS calibration models were in agreement with the standard error of laboratory (SEL) for all the parameters under study. The RPD values obtained for PLS calibration models in reflection mode were 3.6 for pH and

4.8 for invert sugar. The alcohol, pH and invert sugar PLS calibration models obtained in transmission mode had RPD values of 21.2, 2.7 and 5.7, respectively.

Conclusion The alcohol PLS calibration model obtained in transmission mode provided excellent precision, $R^2 = 99.72$ and RPD = 21.2, and was fit for quality- and process control purposes. The pH PLS calibration models obtained in reflection- and transmission mode provided good precision with R^2 values 86 and 81.5, respectively. The RPD value 3.6 indicated that the pH PLS calibration model obtained in reflection mode was suitable for screening purposes, whereas the RPD of 2.7 for the pH PLS calibration model in transmission for rough screening only. The invert sugar PLS calibration models obtained in reflection- and transmission mode provided good precision with $R^2 = 85.8$ and excellent precision $R^2 = 92.4$, respectively. The invert sugar PLS calibration model obtained in reflection is suitable for screening purposes (RPD 4.8) and the invert sugar PLS calibration model obtained in transmission mode for quality control purposes (RPD 5.7). The results obtained in this study resulted in technology development that was transferred to the industrial cellar and facilitated high sample throughput quantification of important quality indicating parameters. To the best of our knowledge, this is the first report of the implementation of mid-infrared (MIR) spectroscopy for the quality control of South African spirit-based liqueurs.

4.1 INTRODUCTION

The composition of liqueur products must adhere to specifications of the producing cellar and also comply with the strict legal requirements related to alcohol and invert sugar content in different countries. In South Africa, the alcohol stated on the label of the product may not exceed more than 0.5 in numerical value from the actual alcohol content of the liqueur product measured by accredited laboratories. The invert sugar concentration for spirit-based liqueurs should legally not be less than 100 g/L (South African Liquor Products Act 60 of 1989). Inconsistency in the chemical composition of spirit-based liqueurs are early detectable with pH values and identified as a quality indicating parameter by the cellar. It is therefore important that procedures are implemented that facilitate real-time control during the production and bottling processes, to ensure consistent high quality of the products. These procedures include the monitoring of quality indicating parameters to identify defects at the various stages of production (Duchesne *et al.*, 2012). Analytical methods for determination of alcoholic strength in spirits are well documented and include pycnometry, electronic densimetry, densimetry using a hydrostatic balance, hydrometry (Brereton *et al.*, 2003) and biosensor technology (Hnaien *et al.*, 2010). Traditional titration methods are used to determine invert sugar and pH levels in spirit-based liqueurs. These methods include weighing, distillation and titration of products, as well as lengthy procedures that may contribute to opportunities for experimental error, apart from the fact that rapid quality control and high sample throughput are not possible with these methods.

Infrared spectroscopy is process analytical technology (PAT) that has been demonstrated to be cost- and time-efficient (Helmdach *et al.*, 2013). Both MIR, 4000 to 400 cm^{-1} , (Nagarajan *et al.*, 2006; Silva *et al.*, 2014; Cozzolino *et al.*, 2011) and NIR, 14 000 to 4 000 cm^{-1} (Urbano Cuadrado *et al.*, 2004; Niu *et al.*, 2008; Garde-Cerdán *et al.*, 2010) have been widely used for applications in the alcoholic beverage industry. The ranges may differ slightly depending on the instrumentation used and the specific application the wavenumbers range are customized for (Lin *et al.*, 2009). The spectrum is calculated through a series of statistical and mathematical techniques and contains information about the chemical composition and physical state of the material under analysis (Cozzolino *et al.*, 2009). The information captured in the MIR region 979.93 to 1450.61 cm^{-1} is often referred to as the fingerprint of a sample (Nieuwoudt *et al.*, 2004).

FTIR in combination with PLS regression was evaluated and implemented by Lachenmeier (2007) as a screening method for quality control in spirit drinks and beer. Kemsley *et al.* (1992) investigated the technique as a technical tool for on-line monitoring to analyse sugar mixtures in soft drinks and brewing. Accurate PLS calibration models suitable for routine analysis of specific gravity (SG), total acidity (TA), volatile acidity (VA), pH, alcohol and fructose in apple ciders were developed by Lobo *et al.* (2006). The concept of matrix effects is a well-known principle in quantitative work based on infrared spectroscopy and also for this reason it was necessary to develop spirit-based liqueur algorithms (Nieuwoudt *et al.*, 2004).

In today's society, there is a strong demand for high-quality products. The existing wet chemistry methods that are used by the laboratory to analyse these parameters in liqueur are lengthy, complex and generate large amounts of chemical waste. Although FT-MIR spectroscopy is widely used in the alcoholic beverage industry, the technology has seen limited quantitative application for spirit-based liqueurs in South Africa. Therefore, the development of new analytical methods using FT-MIR spectroscopy that is fast, precise, simple and cost saving is necessary for the quantification of alcohol, pH and invert sugar in spirit-based liqueurs. Spectra in transmission mode were obtained on a spectrometer owned by the cellar. The spectrometer was in full-time use during production of wine and frequently occupied when spirit-based liqueurs to be analysed. For this reason, a spectrometer obtaining spectra in reflection mode was borrowed in accordance to availability from the supplier and evaluated for the accurate quantification of parameters in these products. The development of an alcohol PLS calibration model obtained in reflection mode was not possible due to the absence of a closed flow injection system on the spectrometer.

The aim of the project was to establish PLS calibration models for the quantification of alcohol, pH and inverts sugar in South African spirit-based liqueurs with FT-MIR spectra obtained in reflection and transmission mode using instrumentation employed by cellar and combined multivariate techniques.

4.2 MATERIAL AND METHODS

4.2.1 Samples

A total of 343 liqueur spectra were obtained in reflection and 246 spectra in transmission for development of alcohol, pH and invert sugar PLS calibration models, consisting out of 19 and 17 product types, respectively. The full-time use of the spectrometer during the production of wine at the cellar, contributed to fewer spectra obtained in transmission (Table 4.1). The samples were analysed as received by the laboratory at different stages according to their status in the production and bottling processes. In Table 4.2, eight quality control points were identified by the cellar where samples were removed from the production and bottling lines for chemical analysis. The recipes of the products are restricted property of the cellar, but for the sake of clarity it suffices to mention that the chemical composition was more or less the same between quality control points for the different product types. Due to the availability of the spectrometer from the supplier, spectra that were not immediately obtained in reflection were stored at 0 °C for maximum 3 days. Samples were homogenised in a sonicator (model PS06A, Healthcare Technologies, South Africa) at 50% amplitude for 2 minutes before analysis.

Table 4.1: Liqueur samples scanned in reflection and transmission mode for the calibration and validation sets for alcohol, pH and invert sugar in spirit-based liqueurs.

| Product | Abbreviation | N ^a Reflection | N ^a Transmission |
|--------------------|--------------|---------------------------|-----------------------------|
| Zappa Red | ZR | 16 | 16 |
| Zappa Original | ZO | 12 | 14 |
| Zappa Green | ZG | 7 | 13 |
| Zappa Blue | ZL | 23 | 23 |
| Zappa Black | ZB | 20 | 21 |
| Tang Bite | TB | 9 | 9 |
| Tang | TA | 17 | 17 |
| Potency | PO | 37 | 12 |
| Malibu | MA | 9 | 6 |
| Lavoka chocolate | LC | 2 | 0 |
| Lavoka caramel | LR | 29 | 0 |
| Kiss | KI | 24 | 0 |
| Butlers Triple Sec | BT | 20 | 8 |
| Butlers Strawberry | BS | 23 | 17 |
| Butlers Peppermint | BP | 19 | 20 |
| Butlers Ginger | BG | 12 | 16 |
| Butlers Coconut | BO | 15 | 12 |
| Butlers Cherry | BC | 11 | 12 |
| Butlers Blue | BB | 38 | 11 |
| Butlers Banana | BA | 0 | 19 |
| Total | | 343 | 246 |

^aN: number of samples

Table 4.2: Spirit-based liqueur samples analysed at quality control points during the production and bottling processes (www.dgb.co.za).

| Quality control point | Control point number | Status in the production and bottling process | N ^a Reflection mode | N ^a Transmission mode |
|-----------------------|----------------------|---|--------------------------------|----------------------------------|
| Before bottling | 0 | Last sample tested from production tank before bottling | 105 | 60 |
| First check | 1 | First bottle in bottling process | 52 | 35 |
| Last check | 2 | Last bottle in bottling process | 44 | 43 |
| Line check | 3 | Middle bottle in bottling process | 56 | 38 |
| Production cellar | 4 | Sample collected during the production of the product | 23 | 24 |
| Pre-bottling | 5 | Sample collected from bottling tank | 27 | 19 |
| Pre-filling tank | 6 | Sample collected from filler tank | 18 | 7 |
| Filler | 7 | Sample collected from filler machine | 18 | 20 |
| Total | | | 343 | 246 |

^aN: number of samples

4.2.2 Reference analysis

Alcohol strength was determined by using a distillation unit (Glasschem, Cape Town, South Africa) and expressed as % volume per volume (v/v). The procedure involves measurement of the density of the distillate with an oscillation type density meter (model DMA 5000 M, Anton Paar, Cape Town, South Africa). The DMA is calibrated on a daily basis with distilled water and 96% v/v cane spirit.

The invert sugar, sucrose, was hydrolysed into glucose and fructose through addition of 0.1 N hydrochloric acid (HCl) to the sample. The mixture was boiled for 2 minutes. The Rebelein method, termed copper-ion reduction, was used to determine the amount of reducing sugars present by manual titration (Iland *et al.*, 2000). Due to high sugar concentrations of products, samples were diluted 2:1 with distilled water. The titration value on the burette was multiplied with a correction factor to accommodate for the dilution step. A blank sample of distilled water was analysed each day to ensure the concentrations of the chemicals used in this method were correct (Iland *et al.*, 2000).

pH was determined with automatic titration (Mettler Toledo, Serial number 5127361187, Cape Town, South Africa) using a combination electrode and a temperature probe. Certified buffers (Mettler pH 2.00, pH 4.00, pH 7.00 and pH 9.00, Microsep, Cape Town, South Africa) were used to calibrate the electrode on a daily basis.

Samples were analysed in duplicate by the reference methods and the standard error of laboratory (SEL) was calculated using the following equation:

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

where y_1 and y_2 are duplicate measurements of a sample and n is the number of samples (Nieuwoudt *et al.*, 2004).

4.2.3 Spectral measurements

No further sample preparation was necessary for spectral measurements. In accordance to instrument availability from the supplier, Fourier transform mid-infrared attenuated total reflection (FT-MIR ATR) spectra were collected on an Alpha-P (Bruker, Bryanston, South Africa) with OPUS software (OPUS version 7.0 for Microsoft Windows, Bruker Optics) for calibration development. Sample volumes of 0.20 ml were used. The Alpha-P contains a potassium bromide (KBr) beam splitter and a 2x2 mm diamond ATR crystal sample plate controlled at 40 °C. The spectrum of each sample was generated from 3996.14 to 374.73 cm^{-1} by taking the average of 64 scans at a resolution of 8 cm^{-1} with a scanner speed of 10 kHz. A reference (background) spectrum of distilled water was recorded after every 10th sample.

FT-MIR spectra were obtained in transmission from sample volumes of 30 ml on the WineScan™ Flex with WinISI software (Foss Analytical, Denmark). Spectra were generated from 925.92 to 5011.54 cm^{-1} . The instrument is fitted with a Michelson interferometer and a 37 micrometre (μm) calcium fluoride (CaF_2) lined cuvette that was temperature controlled at 40°C. The apparatus was cleaned after each sample with the S-470 Cleaning Agent Solution (Foss Analytical, Denmark) and zeroed automatically every 120 minutes with the Zero Solution (Foss Analytical, Denmark). The user settings of the WinISI software were not available to explore for new PLS calibration development and the spectra were transformed to OPUS software format (OPUS version 7.0 for Microsoft Windows, Bruker Optics) for calibration development.

4.2.4 Data analysis

FT-MIR spectra obtained in this study were visually assessed and peak interpretation was done based on published information as indicated. Spectra were exported from OPUS software to SIMCA software (version 13.0.3, Umetrics, Sweden). Explorative data analysis was performed to investigate the influence of product type and quality control points of production on FT-MIR spectra with principle component analysis (PCA). PCA was used to interpret patterns, groupings and similarities of samples in score plots. Samples with similar properties will be grouped together in score plots, whereas the loading plots give valuable input as to the reasons for the specific groupings (Esbensen, 2002). In this study, the **X**-data in the matrix consisted of spectral measurements of spirit-based liqueurs and the **Y**-variables the corresponding alcohol, pH and invert sugar content of the samples.

The spectral data was mean-centered and the y -variables were unit variance (UV) scaled by multiplying each reference y -variable value with $1/SD$. A significance level of $p = 0.05$ (95% confidence) was used to compute the Hotelling T^2 ellipse displayed in the score plots.

PLS regression was used to develop alcohol, pH and invert sugar calibration models with OPUS software. Various spectral pre-processing methods were evaluated, i.e. straight line subtraction, vector normalization, first derivative and second derivative, to improve the predictive ability of the PLS models.

For PLS calibration model development, the different combinations of spectral ranges selected and spectral pre-processing methods were tested by using the optimisation tool in OPUS 7.0 software. Evaluation of the PLS calibration models included R^2 and RMSECV when based on cross-validation statistics, with 20 samples per segment. The criteria for the interpretation of the R^2 values were those proposed by Urbano Cuadrado *et al.* (2004) and are summarised in Table 4.3. Best calibration models were selected based on R^2 and RMSECV values. For test set validation, one third of the samples were randomly selected as test samples and these were not included in the calibration stage, but only validation stage. Three different independent test set repeats were used in the model validation stage. Root mean square error of prediction (RMSEP) represents the model prediction performance after validation and is calculated as follows (Esbensen, 2002):

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

where y_i is the reference value for the i^{th} sample, \hat{y}_i is the predicted value for the i^{th} sample and n is the number of samples.

The test set with the lowest RMSEP for each model was reported in this study and the predictive abilities of the model evaluated by RPD. In Table 4.3 the criteria for the evaluation of RPD values by Williams & Norris (2001) to evaluate the performance of PLS calibration models in this study, are shown.

Table 4.3: Criteria for the interpretation of R^2 (Urbano Cuadrado *et al.*, 2004) and RPD (Williams & Norris, 2001) used to evaluate the performance of the alcohol, pH and invert sugar PLS calibration models.

| R^2 ^a | Interpretation |
|--------------------|---|
| 5 to 29 | Not suitable for quantification |
| 30 to 49 | Correct separation between low and high values |
| 50 to 69 | Good separation between low, medium and high values |
| 70 to 89 | Good precision |
| ≥ 90 | Excellent precision |
| RPD ^b | Interpretation |
| 0 to 2.3 | Not recommended |
| 2.4 to 3 | PLS calibration model suitable for rough screening purposes |
| 3.1 to 4.9 | PLS calibration model suitable for screening purposes |
| 5.0 to 6.4 | PLS calibration model suitable for quality control |
| 6.5 to 8 | PLS calibration model suitable for process control |
| ≥ 8.1 | Any |

^a R^2 : coefficient of determination; ^bRPD: residual predictive deviation ratio

4.3 RESULTS AND DISCUSSION

4.3.1 Reference data

In Table 4.4 the descriptive statistics for chemical composition (range, mean, SD and SEL) of the spirit-based liqueur samples used for alcohol, pH and invert sugar PLS calibration and validation sets are shown. Kleintjes (2013) developed PLS calibration models for the quantification of ethanol, density, obscuration and colour in brandies, gin, whiskies and vodkas for the alcohol range 33.75 to 46% v/v. The different matrix effects and smaller alcohol range of the samples used in the calibration models was an reason to develop new algorithms (Nieuwoudt *et al.*, 2004) in this study. Although the sample sets for building the reflection and transmission calibration models consisted of different product types at different quality control points, the ranges of the chemical composition of the samples were similar. The sample sets contained a wide selection of product types and therefore the ranges of the chemical composition for each parameter were large. Due to the full-time use of the spectrometer at the cellar, fewer spectra were obtained in transmission mode. The SEL for data obtained in this study is the same as for the SEL of the DGB laboratory as analysis was done by the same technician.

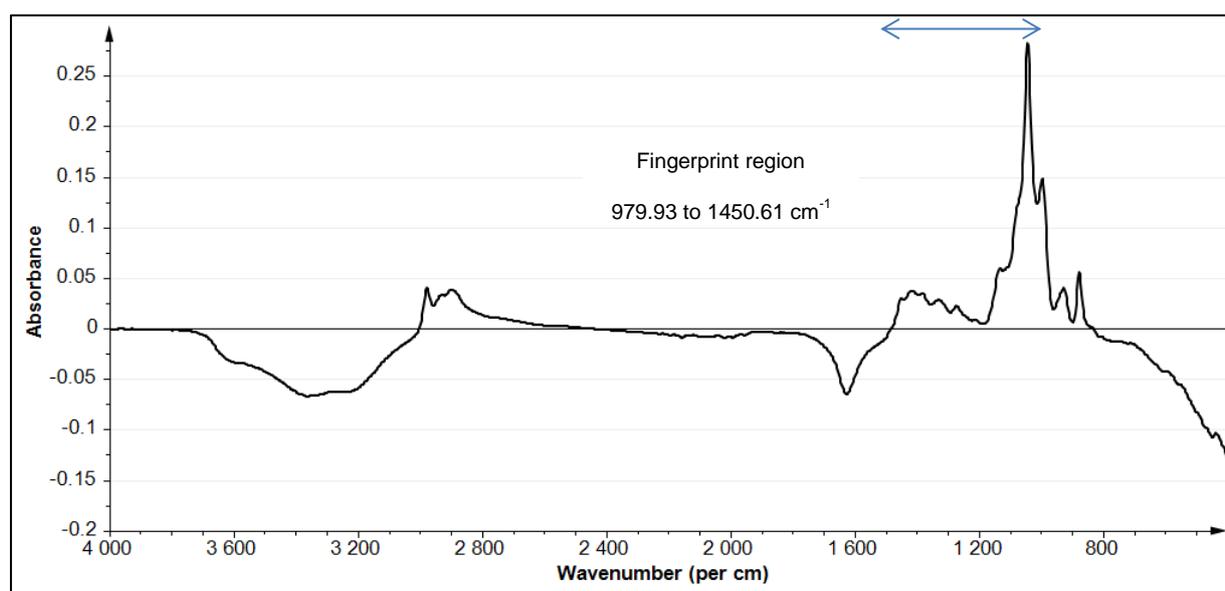
Table 4.4: Descriptive statistics of the samples used for building PLS calibration models for alcohol, pH and invert sugar of spirit-based liqueurs.

| | Reflection scanning mode | | Transmission scanning mode | | |
|------------------------------------|--------------------------|------------------|----------------------------|-----------|------------------|
| | pH | Invert sugar g/L | Alcohol (% v/v) | pH | Invert sugar g/L |
| Min ^a -Max ^b | 2.3 - 7.5 | 150 - 439 | 11.66 - 40.94 | 2.2 - 9.3 | 150 - 451 |
| Mean | 5.5 | 316.1 | 29.46 | 6.1 | 321.2 |
| SD ^c | 2.3 | 75.4 | 8.92 | 2.3 | 81.3 |
| SEL _{DGB} ^d | 0.5 | 10 | 0.39 | 0.5 | 10 |

^aMin: minimum; ^bMax: maximum; ^cSD: standard deviation; ^dSEL_{DGB}: DGB inter-laboratory standard error of laboratory

4.3.2 Spectral analysis

Spectra in the absorption ranges between wavenumbers 3996.14 to 374.73 cm⁻¹ were obtained for the 343 liqueur samples in ATR. Figure 4.1 (a) shows a FT-MIR ATR line plot of one of these spectra. Figure 4.1 (b) shows the FT-MIR line plot of a spectrum obtained in transmission and converted to absorbance for the ranges 3996.89 to 925.92 cm⁻¹. Spectra in transmission were converted to absorbance spectra to visually compare absorption bands and peaks between the two modes of scanning. The region between 979.93 and 1450.61 cm⁻¹ is known as the fingerprint region and contain a significant amount of variation related to the absorbance by molecular chemical groups present in the sample (Cozzolino et al., 2009, 2011), indicated on the spectra. Negative absorption regions are due to the background spectra of water.

**Figure 4.1 (a):** Line plot of FT-MIR ATR spectrum obtained between 3996.14 to 374.73 cm⁻¹.

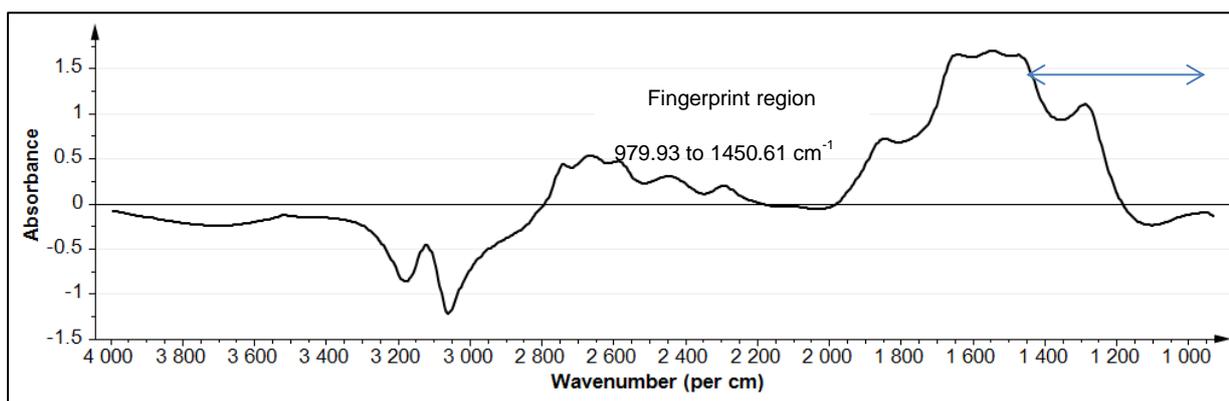


Figure 4.1 (b): Line plot of FT-MIR spectrum obtained in transmission and converted to absorbance between 3996.89 and 925.92 cm^{-1} .

In Figure 4.2 (a) and 4.2 (b), line plots of the same six spectra obtained in reflection and transmission respectively, are visualised. The chemical composition of the samples is shown in Table 4.5. It is known from literature that absorbance at the wavenumbers 1045 and 1085 cm^{-1} correspond to alcohol present in the sample (Duarte, 2004). The alcohol concentration of each sample (Table 4.5) is imitated by the amplitude of the absorption peak at these wavenumbers for the respective samples. Zappa Black samples (ZB131 and ZB121) with alcohol concentrations of 40% v/v show higher absorption peaks at 1045 and 1085 cm^{-1} than Butlers Blue samples (BB101 and BB111) with alcohol concentrations of 24% v/v and Tang samples (TA131 and TA141) with alcohol concentrations of 11% v/v.

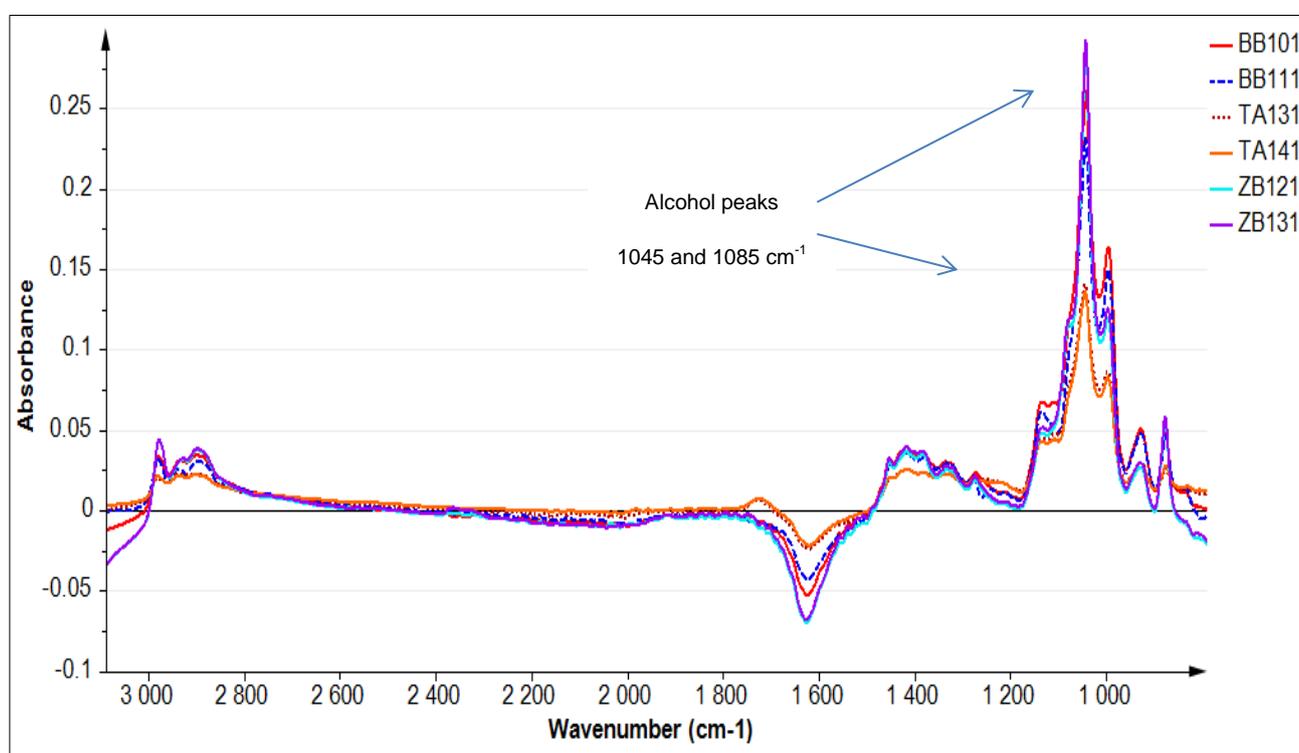


Figure 4.2 (a): Line plot of FT-MIR-ATR spectra obtained for samples in Table 4.5. Peak annotation is according to Duarte (2004). Amplitudes in alcohol peaks are observed at 1045 and 1085 cm^{-1} for the samples and correspond to the alcohol concentrations of samples.

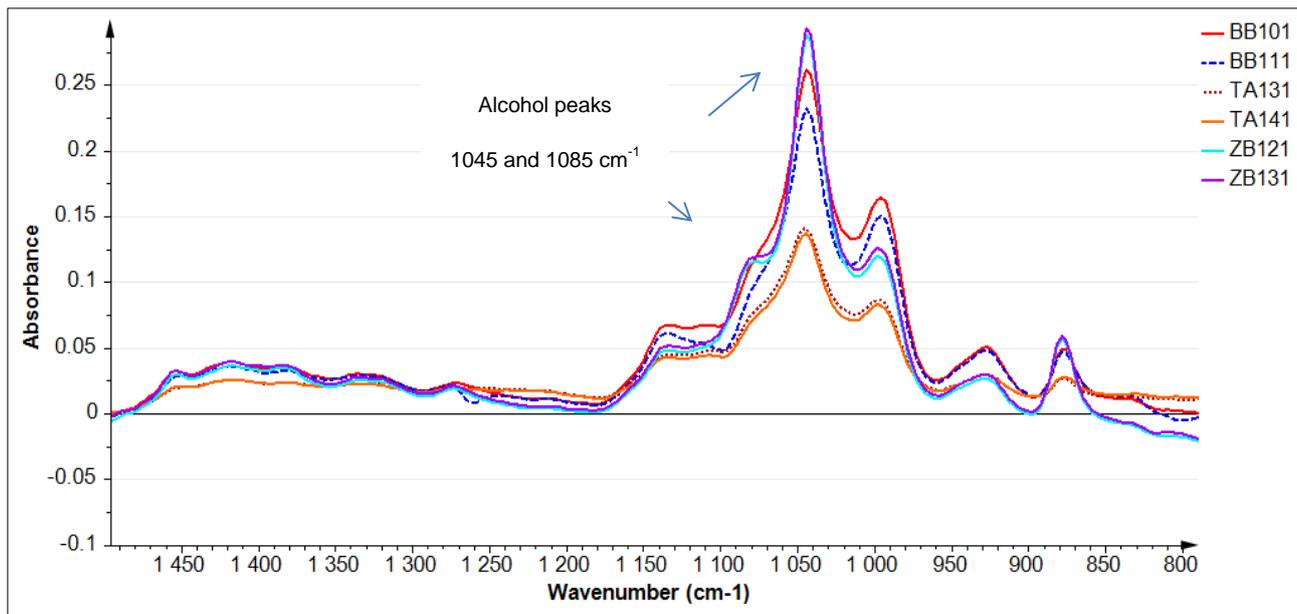


Figure 4.2 (b): Line plot of FT-MIR spectra obtained in transmission. Amplitudes in peaks at 1045 and 1085 cm^{-1} (Duarte, 2004) are noted correspond to alcohol absorption for samples in Table 4.5.

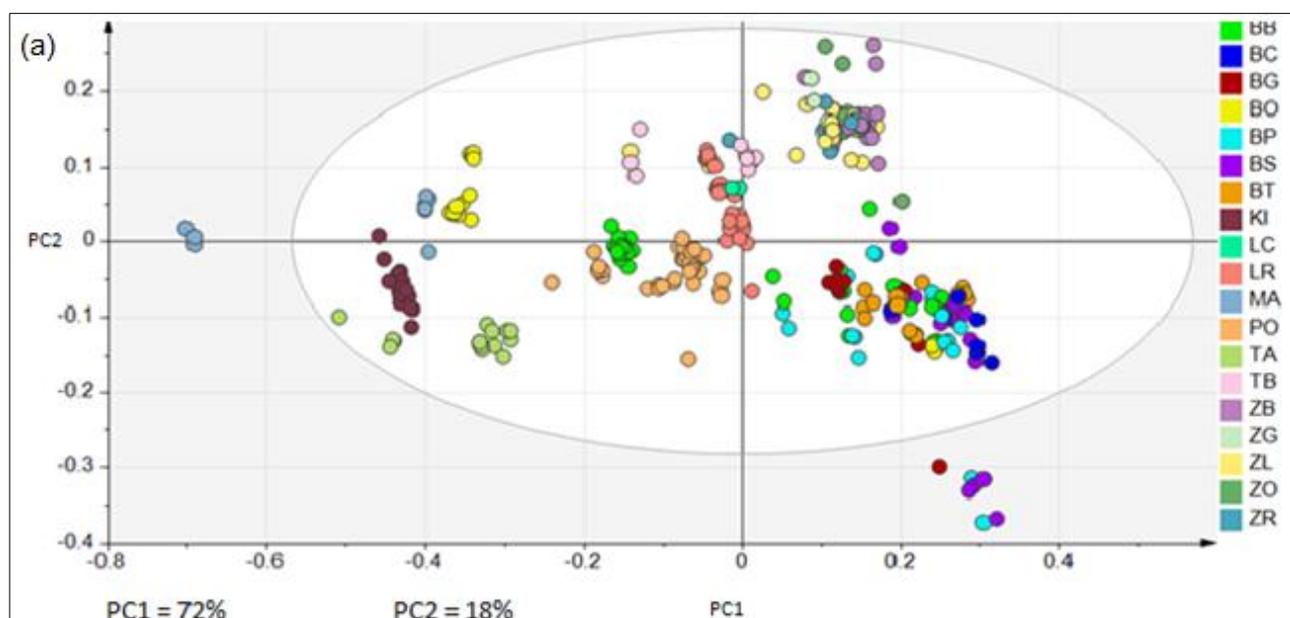
Table 4.5: Chemical composition of samples present in reflection and transmission calibration sets

| Sample number | Product | Alcohol % v/v | pH | Invert sugar g/L |
|---------------|--------------|---------------|------|------------------|
| TA141 | Tang | 11.89 | 2.52 | 230 |
| TA131 | Tang | 11.94 | 2.65 | 230 |
| BB101 | Butlers Blue | 24.32 | 7.68 | 406 |
| BB111 | Butlers Blue | 24.36 | 6.86 | 401 |
| ZB131 | Zappa Black | 40.4 | 6.39 | 280 |
| ZB121 | Zappa Black | 40.6 | 7.27 | 298 |

4.3.3 Principal component analysis

To investigate influence of product type and quality control points of production on the predictive abilities of the models, PCA was used. The noisy regions below 538.61 and above 3535.34 cm^{-1} in reflection, and above 3535.34 cm^{-1} in transmission, were found to contain little useful information and were trimmed off in further data analyses.

In Figure 4.3 (a) and (b), PCA was performed on all the FT-MIR ATR spectra for the trimmed region 538.61 to 3535.34 cm^{-1} and coloured accordance to product type and quality control points in the production process, respectively. Using four components, the total variance of 90.5% was explained with PC1 explaining 72% and PC2 18%, respectively. The observations showed a degree of separation according to product type but overlapping still occurred (Figure 4.3 (a)). This indicated that product type related variation may have an influence on the data and should be investigated in future research at the cellar. There was no clear clustering on the basis of quality control points of production (Figure 4.3 (b)). Observations outside the Hotelling T^2 95% confidence ellipse were not deselected at this stage. To investigate the absorbance peaks and wavenumbers that contribute to the separation seen in the score plots, PCA was performed on all the FT-MIR ATR spectra for the fingerprint region 863.87 - 1493.19 cm^{-1} . Using four components, PC1 and PC2 explain 86.5% and 11.5% of the data variance, respectively. The loadings plot (Figure 4.3 (c)) for PC1 showed that the absorbance at the wavenumber 1045 cm^{-1} correspond to alcohol absorption (Duarte, 2004) and contributes to the separation seen in the score plots.



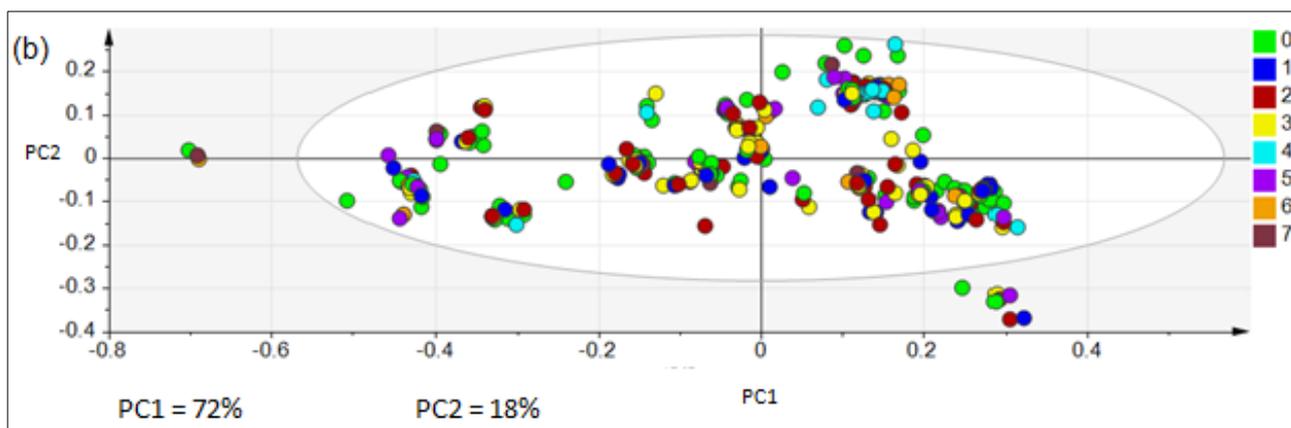


Figure 4.3: PCA score plot of all the samples obtained in reflection for region 538.61 to 3535.34 cm^{-1} coloured according to (a) product type and (b) quality control points of production. Ellipse on the score plot indicates Hotelling T^2 range 95% confidence.

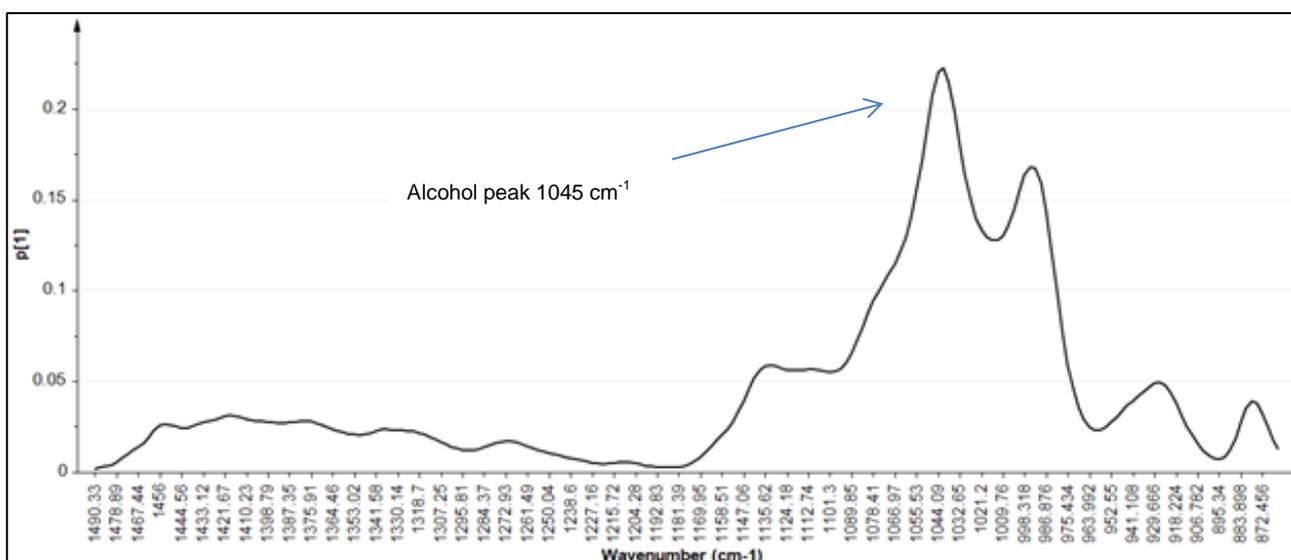


Figure 4.3 (c): Loading plot of PC1 (86.5% variance explained) between region 863.87 - 1493.19 cm^{-1} .

In Figure 4.4, PCA was performed on all the FT-MIR spectra obtained in transmission between region 925.92 to 3535.34 cm^{-1} to investigate the influence of product type (Figure 4. (a)) and quality control points (Figure 4.4 (b)) on the data. Using three components, PC1 and PC2 explained 83.8% and 11.5% of the data variance, respectively. The observations showed a degree of separation according to product type, but overlapping still occurred. There were no observations outside the ellipse defined by Hotelling T^2 statistic. There was no clear clustering on the basis of quality control points. This was expected, as the chemical compositions between quality control points for the different products were similar.

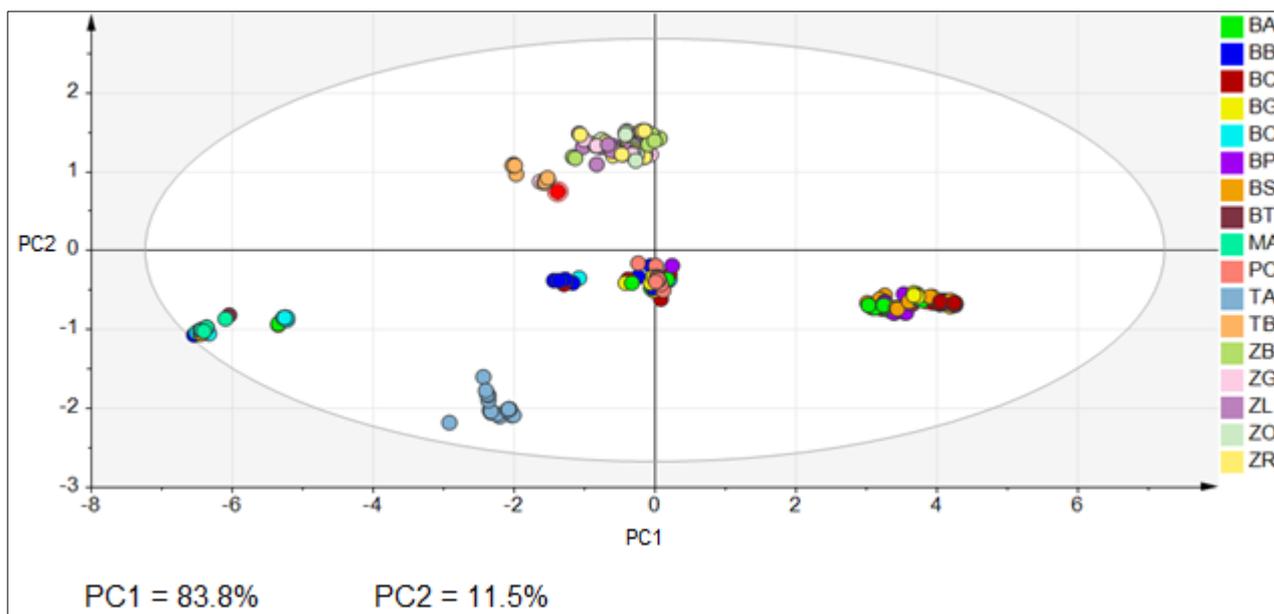


Figure 4.4 (a) PCA on FT-MIR spectra of all spectra obtained in transmission for the region 925.92 to 3535.34 cm^{-1} and coloured accordance to product type. Ellipse on the score plot indicates Hotelling T^2 range 95% confidence.

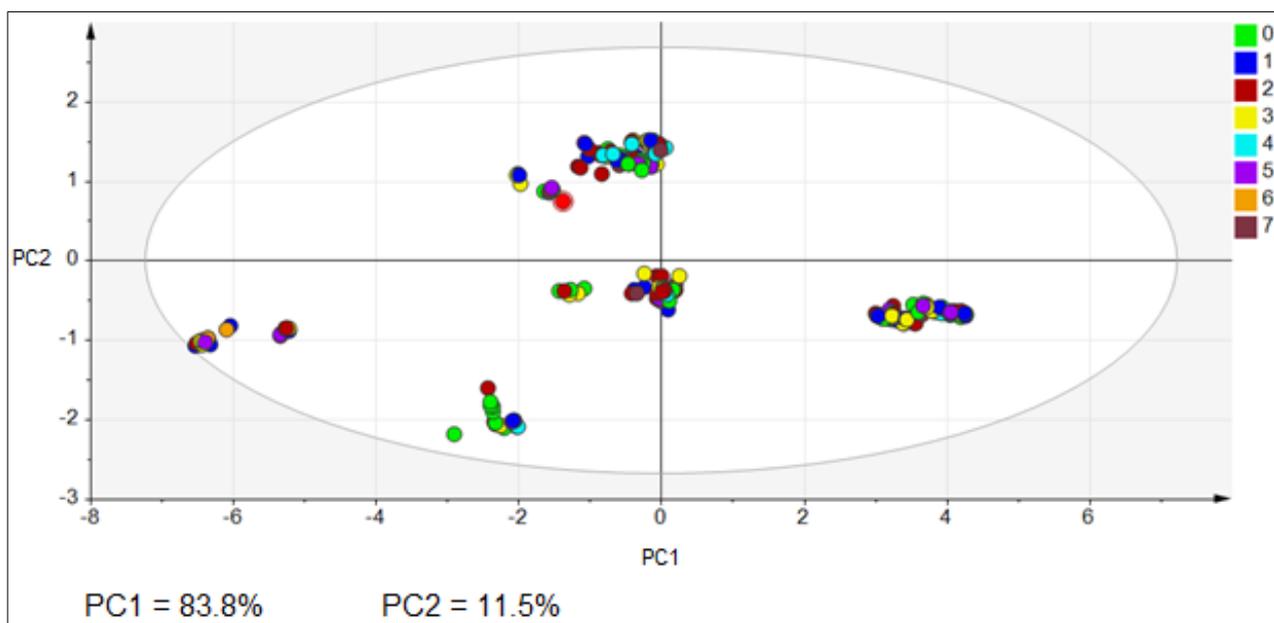


Figure 4.4 (b) PCA score plot of all the spectra obtained in transmission mode for region 925.92 to 3535.34 cm^{-1} coloured accordance to quality control points. Ellipse on the score plot indicates Hotelling T^2 range 95% confidence.

4.3.4 Partial least square regression modelling

Based on the PCA results described in section 4.3.3, for the development of alcohol, pH and invert sugar PLS calibration models, all the samples for the reflection and transmission data sets were used. The number of components per calibration was selected based on the highest explained variance with the lowest number of components to guard against overfitting whilst sufficiently

modelling the variance in the data set. Pre-processing and wavenumber selection for the PLS calibration models under development were selected by OPUS software and illustrated in Table 4.6. The regression statistics for the PLS calibration models with cross validation was reported in Table 4.7 (a), and the independent validation sets in Table 4.7 (b). The performance of the PLS calibration models were evaluated on R^2 (Urbano Cuadrado *et al.*, 2004) and RPD (Williams & Norris, 2001) criteria in Table 4.3 and visualised by predicted versus measured plots in Figure 4.6. The plots illustrated the distribution of the samples along the linear regression line for calibration and test set validation.

Table 4.6: Pre-processing and wavenumbers selected by OPUS software for PLS calibration development of alcohol, pH and sugar of spirit-based liqueurs.

| Parameter | Reflection | | Transmission | |
|--------------|--------------------------------------|---|---|------------------------------------|
| | Pre-processing | Wavenumbers cm^{-1} | Pre-processing | Wavenumbers cm^{-1} |
| Alcohol | | | Minimum-maximum normalization | 1331 to 2152,8 3375,8 to 3788,6 |
| pH | Multiplicative scattering correction | 5008.8 to 3020,7 2362,8 to 1035,5 | Constant offset elimination | 2149 to 2970,7 |
| Invert sugar | Minimum-maximum normalization | 6994 to 4345,1 3684,3 to 3020,7 1038.4 to 374,7 | First derivative and Multiplicative scattering correction | 2557,9 to 4197,5 |

Table 4.7 (a): Regression statistics for PLS calibration models with cross validation developed for alcohol, pH and invert sugar in spirit-based liqueurs

| Component | Reflection | | Transmission | | |
|--|------------|---------------|--------------------|-----------|---------------|
| | pH | Invert sugar | Alcohol | pH | Invert sugar |
| N^a | 206 | 87 | 245 | 172 | 73 |
| Min^b - Max^c | 2.3 – 7.5 | 150 - 439 g/L | 11.66 – 40.94% v/v | 2.2 – 9.3 | 150 - 451 g/L |
| Rank | 5 | 3 | 10 | 10 | 4 |
| R²^d | 86 | 85.8 | 99.72 | 81.5 | 92.4 |
| RMSECV^e | 0.7 | 26.1 g/L | 0.47% v/v | 1.0 | 22.4 g/L |
| Bias | 0.019 | -0.955 | 0.002 | 0.043 | 0.095 |

^aN: number of samples; ^bMin: minimum; ^cMax: maximum; ^d R^2 : coefficient of determination; ^eRMSECV: root mean squared error of cross validation

Table 4.7 (b): Test set validation statistics for PLS calibration models developed for alcohol, pH and invert sugar in spirit-based liqueurs

| Component | Reflection | | Transmission | | |
|--|------------|---------------|-----------------------|-----------|---------------|
| | pH | Invert sugar | Alcohol | pH | Invert sugar |
| N^{a (cal)} | 138 | 59 | 164 | 116 | 50 |
| Min^{b (cal)} - Max^{c (cal)} | 2.3 – 7.5 | 150 - 439 g/L | 11.66 – 40.94% v/v | 2.2 – 9.3 | 150 - 451 g/L |
| Rank | 10 | 5 | 6 | 10 | 3 |
| R^{2 d} | 92.33 | 95.65 | 99.77 | 86.06 | 96.79 |
| RMSECV^e | 0.5 | 12.9 g/L | 0.40% v/v | 0.7 | 13.3 g/L |
| N^{f (val)} | 68 | 28 | 81 | 56 | 23 |
| Min^{g (val)} – Max^{h (val)} | 2.4 - 7.3 | 188 - 433 g/L | 11.68 - 40.84% v/v | 2.4 - 9.2 | 160 - 444 g/L |
| RMSEPⁱ | 0.5 | 14.3 g/L | 0.41 %v/v | 0.8 | 16.7 g/L |
| RPD^j | 3.6 | 4.8 | 21.2 | 2.7 | 5.7 |

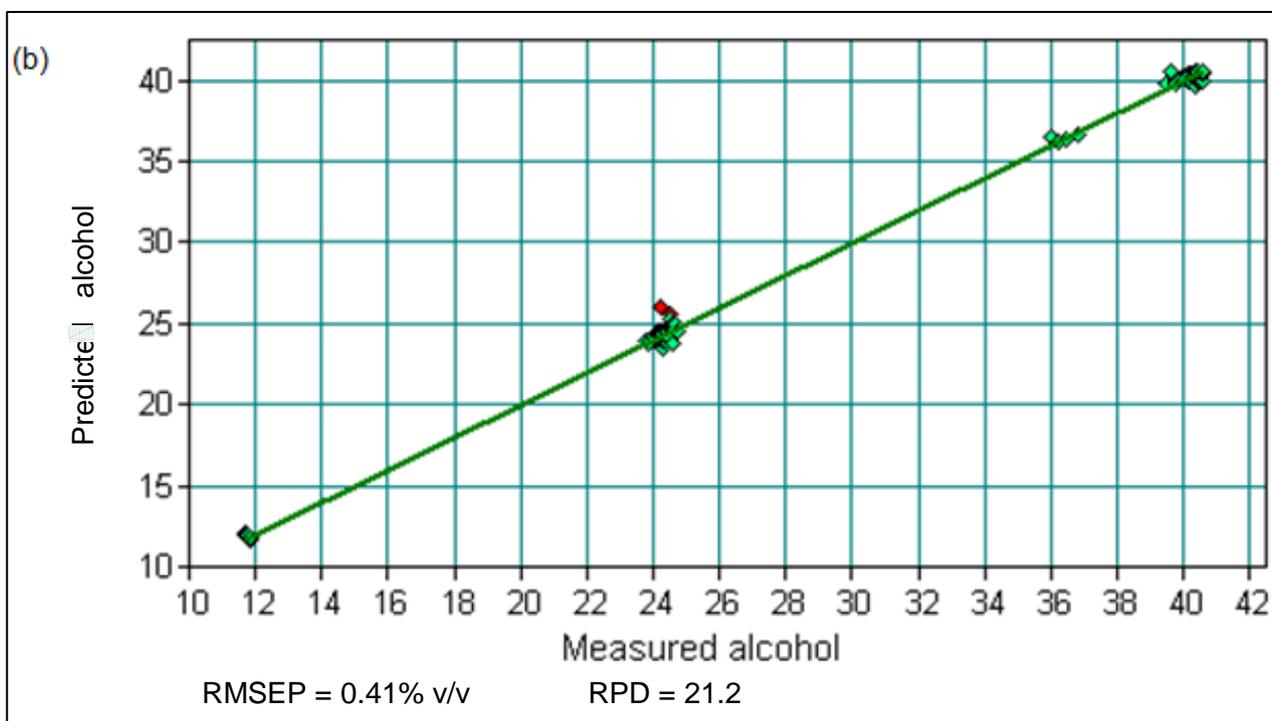
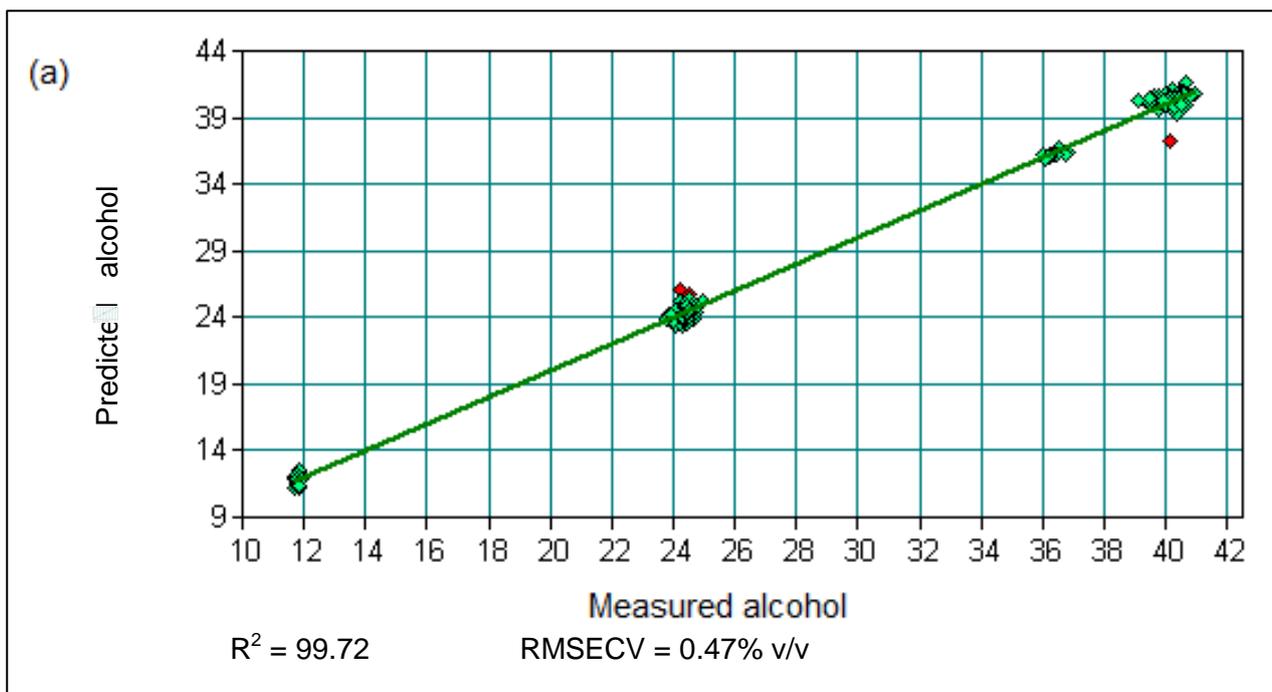
^{a (cal)}N: number of samples calibration set; ^{b (cal)}Min: minimum calibration set; ^{c (cal)}Max: maximum calibration set; ^dR² coefficient of determination; ^eRMSECV: root mean squared error of cross validation; ^{f (val)}N: number of samples validation set; ^{g (val)}Min: minimum validation set; ^{h (val)}Max: maximum validation set; ⁱRMSEP: residual mean standard error of prediction; ^jRPD: residual predictive deviation ratio

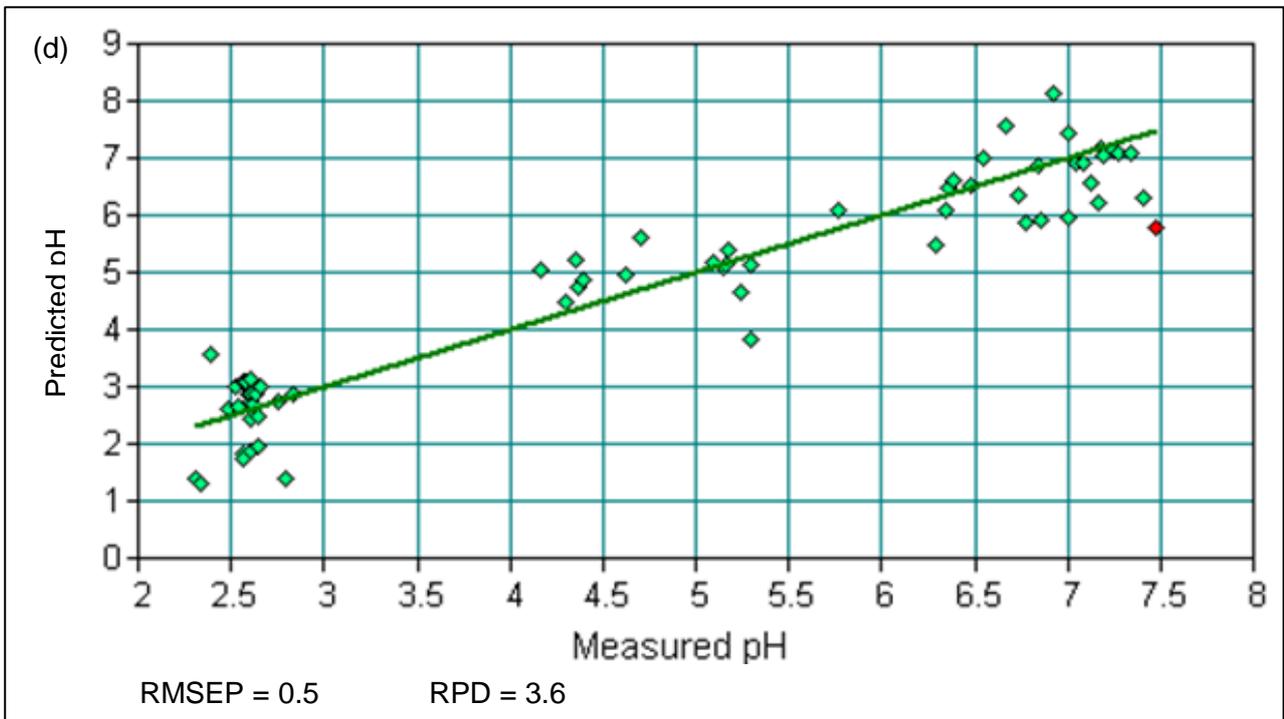
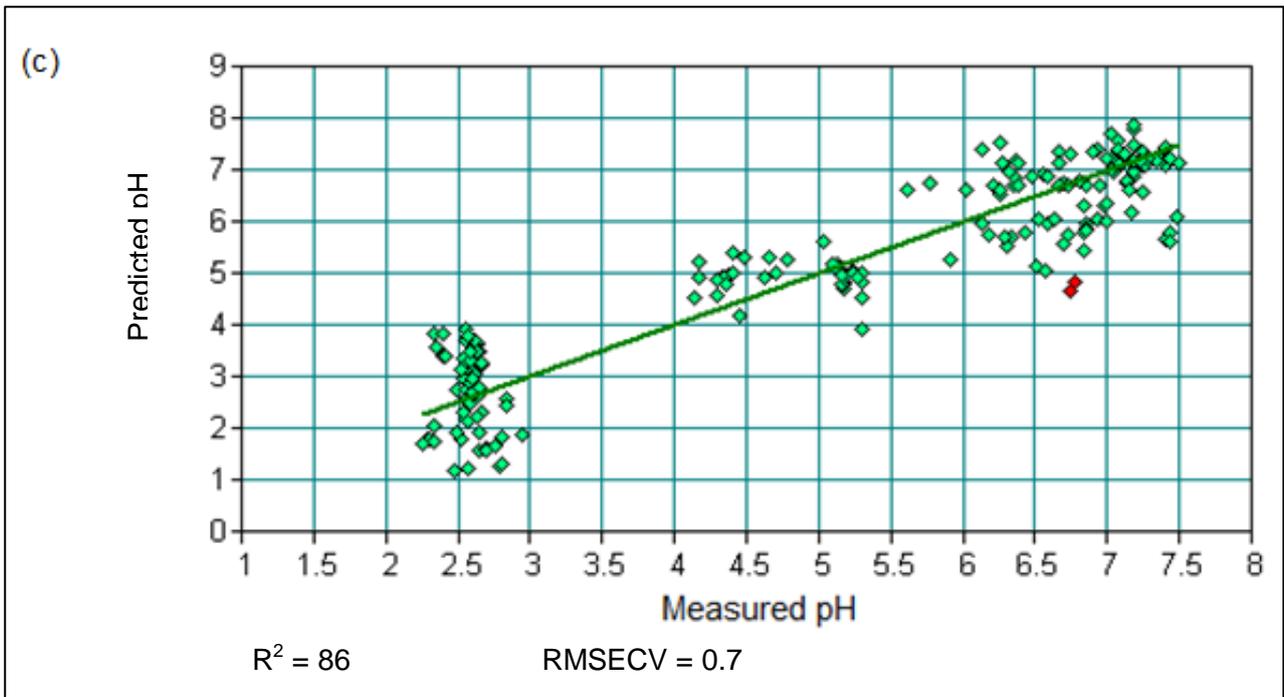
For the development of the alcohol PLS calibration model, 245 spectra obtained in transmission was used. The R² (99.72) indicated an alcohol PLS calibration model with excellent precision. The RMSECV was 0.41% v/v. The reported independent validation set contained 81 samples and the RMSEP of 0.47 % v/v was lower than the tolerance of 0.5% v/v specified by the South African law (South African Liquor Products Act 60 of 1989), but higher than the RMSEP (0.038 to 0.106% v/v) for the spirit calibration models developed by Kleintjes (2013). The RPD value of 21.2 indicated that the PLS model developed were suitable for quantification purposes (Williams & Norris, 2001). In Figure 4.6 (a) and (b), samples concentrated around 11% v/v, 24% v/v, 36% v/v and 43% v/v were expected, due to the alcohol concentration of the industrial samples used for the calibration and validation sets. Samples identified by the software as outliers, were not deselected from the calibration set, as the statistic results were acceptable to the cellar, according to the SEL of 0.39% v/v.

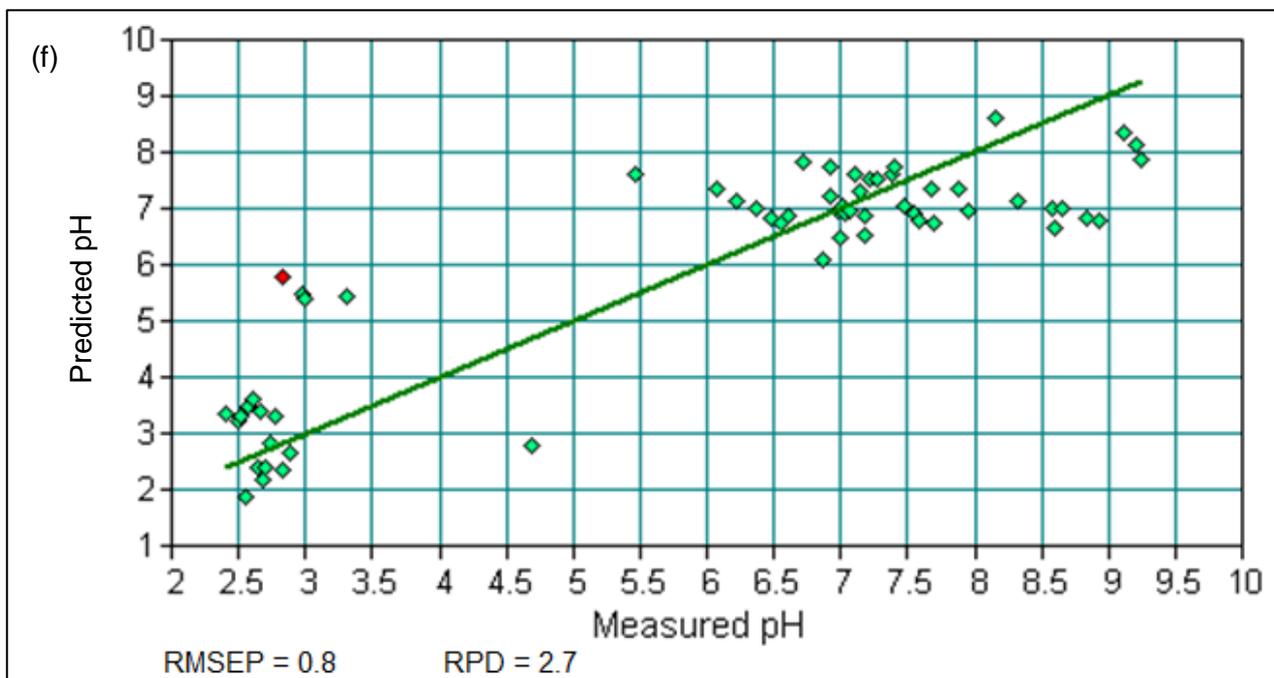
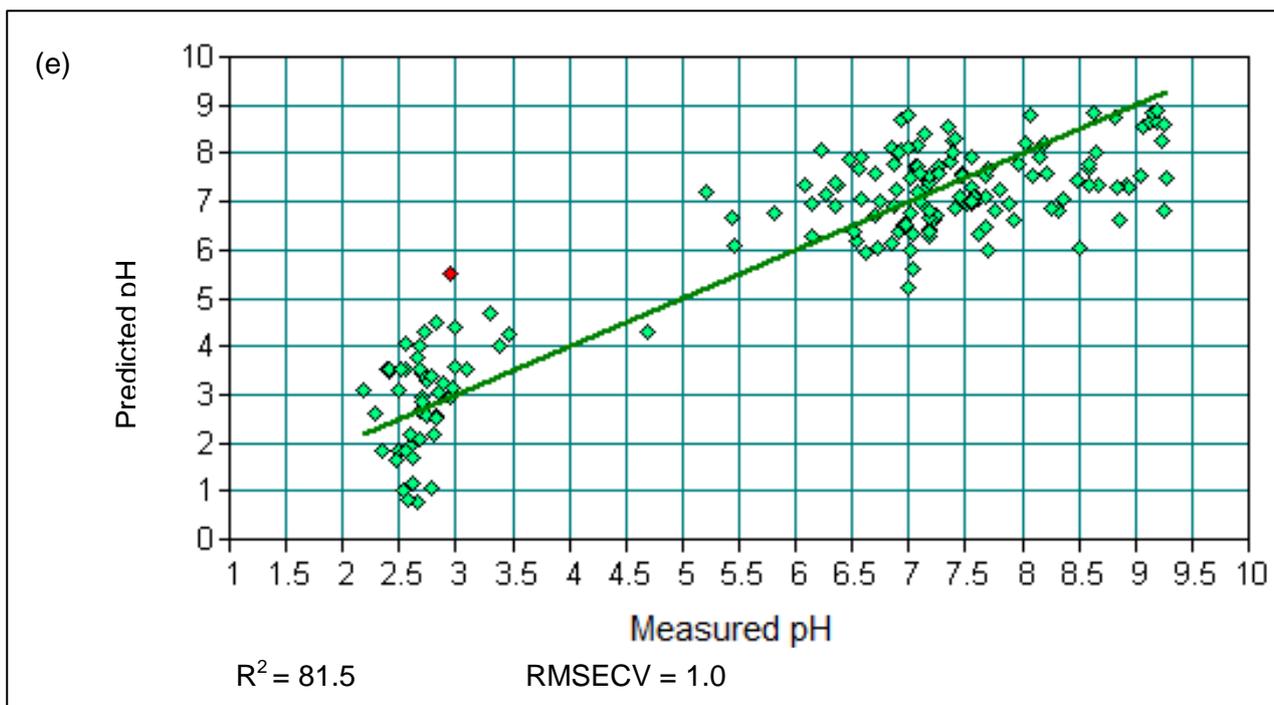
The pH PLS calibration models obtained from 206 spirit-based liqueur spectra in reflection, and 172 in transmission had R² values of 86 and 81.5, indicated good precision. The RMSECV were 0.7 and 1.0, respectively. The reported independent validation sets contained 68 samples for reflection and 56 samples for transmission. The RMSEP for the PLS calibration set obtained in reflection, 0.5, was less than the RMSEP for the PLS calibration set obtained in transmission, 0.8,

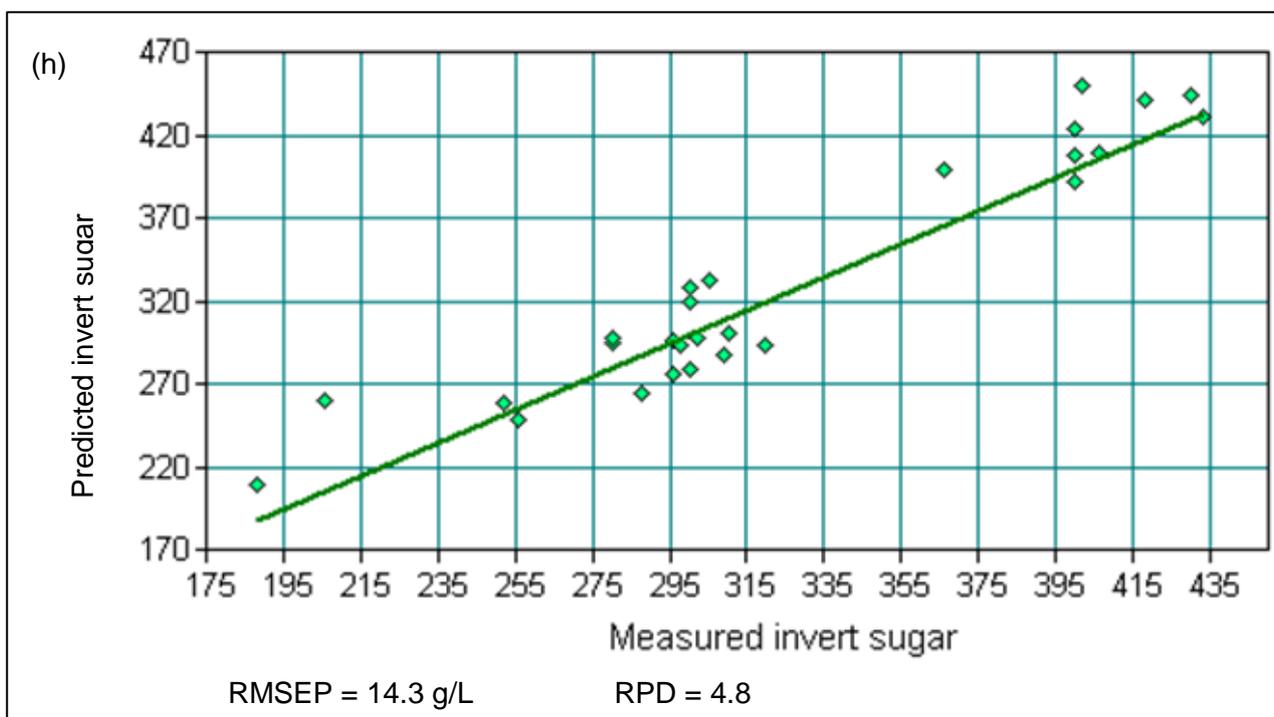
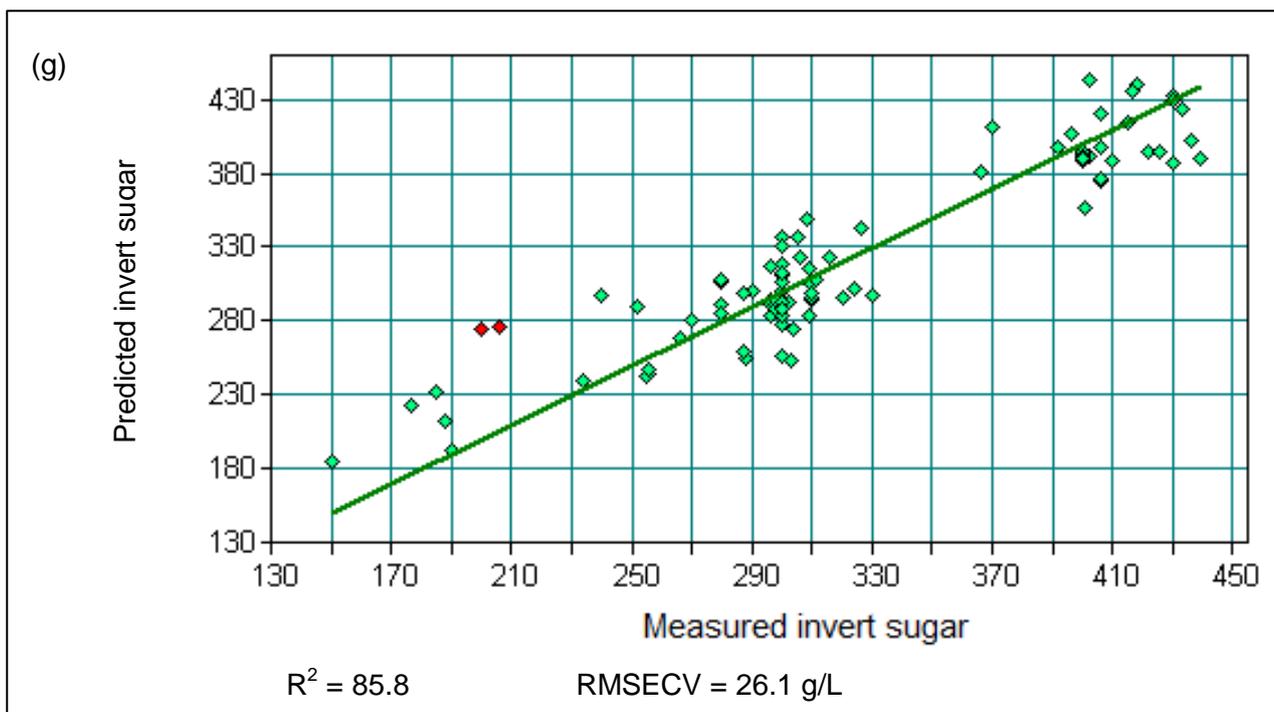
and similar to the SEL (0.5). The RMSEP for the transmission calibration set can be improved by adding more samples to the calibration set. The RPD value of 3.6 indicated that the reflection model developed were suitable for screening purposes. The RPD value of 2.7 indicated that the transmission model developed were suitable for rough screening purposes only and needed further development to increase the predictability of the PLS model. In Figure 4.6 (c) and (d), groups of samples at pH 2.5, 5 and 7 were formed and were a reflection of the reference chemical analysis of the samples in the reflection calibration and validation sets. Figure 4.6 (e) and (f) demonstrates groups of samples around pH 2.5 and 7, with a few samples around 5, due to the absence of Lavoka Caramel and Lavoka Chocolate samples in the transmission calibration and validation sets, which mainly contributed to the pH values of 5.

For the development of the invert sugar PLS calibration models, 87 spectra were obtained in reflection and 73 spectra in transmission. The R^2 value for the invert sugar PLS calibration model obtained in reflection was 85.8 and indicated good precision. The SECV was 26.1 g/L. The R^2 value for the PLS calibration model obtained in transmission was 92.4 and indicated excellent precision. The SECV was 22.4 g/L. The independent validation sets contained 28 and 23 samples for reflection and transmission respectively. The RMSEP of 14.3 g/L for the PLS calibration model obtained in reflection was lower than the RMSEP (16.7 g/L) for the PLS calibration model obtained in transmission. The RPD value for the invert sugar PLS calibration model obtained in reflection was 4.8 and suitable for screening purposes, whereas the PLS calibration model obtained in transmission (RPD = 5.7) was suitable for quality control purposes. Figure 4.6 (g) and 4.6 (h) illustrated clusters of samples around 170 g/L, 290 g/L and 410 g/L and were expected in accordance to the invert sugar range of the samples used for the calibration and validation sets in reflection. The same distribution of samples in Figure (i) and (j) for the calibration and validation sets in transmission were expected. The RMSEP values for invert sugar PLS calibration models were close to the SEL value of 10 g/L and were accepted by the cellar.









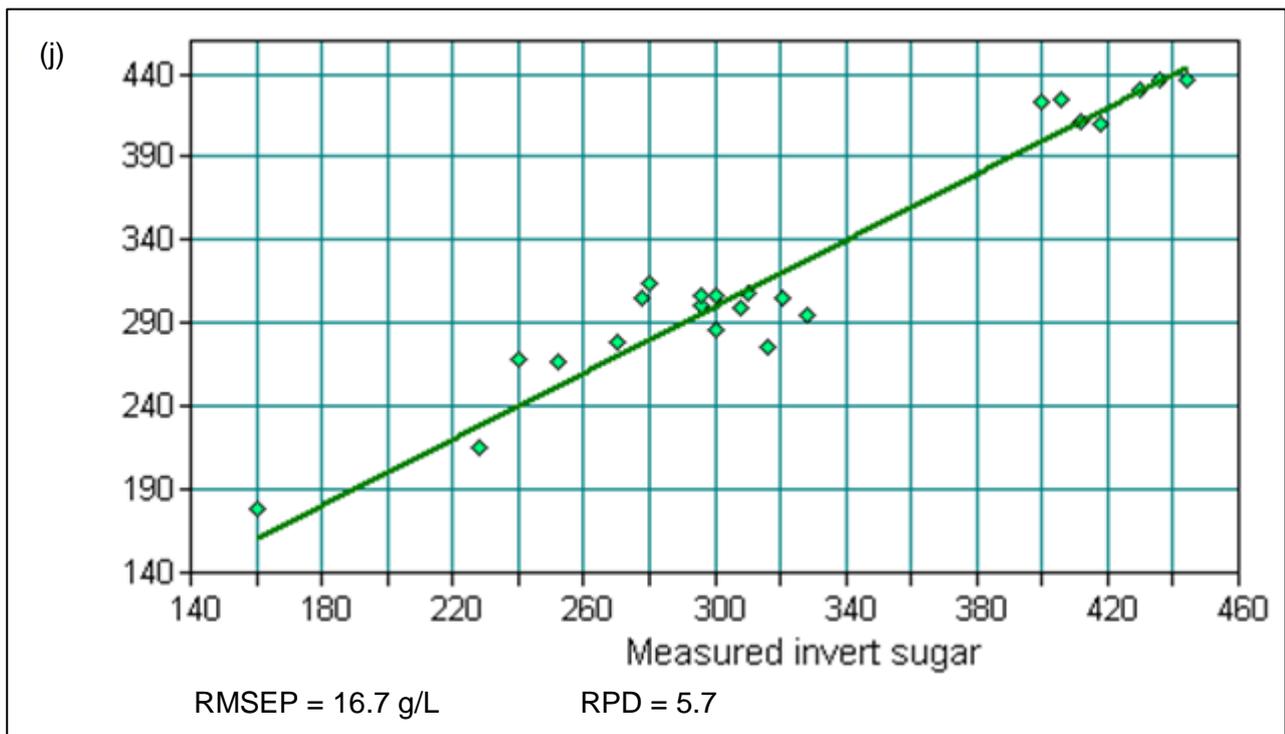
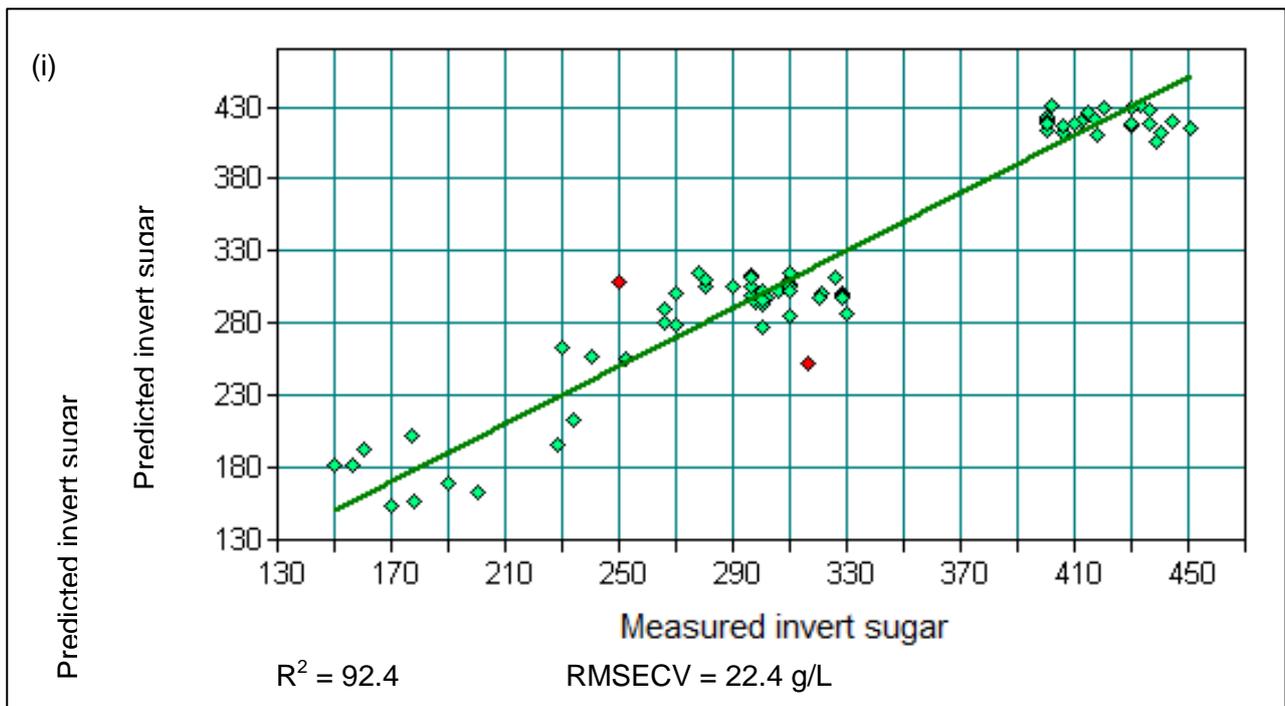


Figure 4.6: Regression plots of predicted versus measured (a) alcohol calibration; (b) alcohol test set validation; (c) pH calibration obtained in reflection; (d) pH test set validation obtained in reflection; (e) pH calibration obtained in transmission; (f) pH test set validation obtained in transmission; (g) invert sugar calibration obtained in reflection; (h) invert sugar test set validation obtained in reflection; (i) invert sugar calibration obtained in transmission; (j) invert sugar test set validation obtained in transmission. Statistics for calibration and test set validation illustrated in Table 4.7 (a) and (b) respectively.

4.4 CONCLUSION

The aim of the study was to evaluate the potential of FT-MIR spectroscopy for the quantification of alcohol, pH and invert sugar in South African spirit-based liqueurs. The samples were obtained in reflection and transmission scanning modes and calibration models were developed using PLS regression software. The calibration models were evaluated using performance criteria for R^2 and RMSECV (Urbano Cuadrado *et al.*, 2004) and test set validation statistics RMSEP, and RPD (Williams & Norris, 2001). The models with cross validation were implemented to include the most variation with the highest amount of samples in the calibration sets. The alcohol PLS calibration model obtained in transmission mode provided excellent precision, R^2 99.72 and RPD 21.2, and was fit for quantification purposes. The pH PLS calibration models obtained in reflection- and transmission mode provided good precision with R^2 values 86 and 81.5, respectively. The RPD value 3.6 indicated that the pH PLS calibration model obtained in reflection mode was suitable for screening purposes, whereas the RPD of 2.7 for the pH PLS calibration model in transmission for rough screening only and needed further development to increase the predictability of the PLS model. The predictive ability of the pH PLS calibration model can be increased by adding more types of spirit-based liqueur samples at different quality control points to the calibration set. The invert sugar PLS calibration models obtained in reflection- and transmission mode provided good precision with R^2 85.8 and excellent precision R^2 92.4, respectively. The invert sugar PLS calibration model obtained in reflection is suitable for screening purposes (RPD 4.8) and the invert sugar PLS calibration model obtained in transmission mode for quality control purposes (RPD 5.7). The results obtained in this study indicate that the models can be transferred to the cellar and implemented for their proposed applications. This study contributes to the foundation on multivariate data analysis and PLS calibration model development of spirit-based liqueurs in an industrial cellar.

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Chapter 5

General discussion and conclusions

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 CONCLUDING REMARKS AND FUTURE WORK

Fourier transform mid-infrared (FT-MIR) spectroscopy in combination with multivariate data analyses techniques is a well-established analytical tool for chemical analysis in the beverage industry (Bauer *et al.*, 2007; Cozzolino *et al.*, 2011). The increasing consumer demands in the local and international markets for low-cost, high-quality products, forced DGB (Pty) to re-evaluate the current status of the analytical quality control measures employed during production and bottling processes of wine and spirit-based liqueurs. The ultimate aim of the company is to reduce the use of wet chemistry methods currently in use in the analytical laboratory, reduce the generation of toxic waste and implement automated measurements where possible. In view of the success already achieved with FT-MIR spectroscopy in the beverage industry, the need to expand and optimise the technology was clear. The task at hand was therefore the development of calibration models for fast, accurate, cost-efficient analyses that contain samples representative of the grapes harvested and spirit-liqueur products produced by the cellar. Two objectives were identified for this study.

The first objective was to develop a FT-MIR calibration model for ammonia in freshly pressed grape juice and to implement the calibration model for quantification control purposes at the cellar. Samples from all the cultivars harvested during 2010 and 2011 at the cellar were included in the calibration set. The main restrictions in the sampling were that during 2010 fewer samples were analysed due to optimisation of the complex reference method and the availability of the borrowed Alpha-P spectrometers. Red cultivars also needed to be decolourised and were time-consuming. As a result of two different spectrometers used to obtain spectra during 2010 and 2011, respectively, a drift in spectra were observed between the two vintages. Thus, the standardisation of analytical equipment at the cellar is important for future calibration development and the transfer of partial least squares (PLS) calibration models between spectrometers.

The second objective was to develop FT-MIR calibration models for the quantification of alcohol, pH and invert sugar in spirit-based liqueurs at the cellar. Due to the availability of the Alpha-P spectrometer from the supplier, the spectra collected for the calibration models sets obtained in attenuated total reflectance (ATR) and transmission were not identical and therefore, the statistics could not be compared. Fewer samples were scanned in transmission due to the full-time use of the spectrometer for the production of wine at the cellar. The reference method for invert sugars is time-consuming and fewer samples were scanned in developing the calibration model. A calibration model for ethanol in ATR could not be developed due to the absence of a

closed flow injection system on the spectrometer's sample plate that restricts evaporation at the time of the project (www.bruker.com).

The PLS calibration models were evaluated using the performance criteria coefficient of determination (R^2) and root mean squared error of cross validation (RMSECV) at calibration stage. The overall precision of the PLS calibration models developed were good or excellent ($R^2 \geq 70$). The predictive performance of the PLS calibration models were evaluated using root mean square error of prediction (RMSEP) and residual predictive deviation ratio (RPD) at validation stage. The RMSEP values for the PLS calibration models were in agreement with the standard error of laboratory (SEL) for all the parameters under study. The alcohol calibration model was implemented for quality- and process control purposes (RPD = 21.2). The ammonia-, invert sugar- and pH PLS calibration models for spectra obtained in ATR were implemented for screening purposes with RPD values of 3.3, 4.8 and 3.6, respectively. The invert sugar PLS calibration model for spectra obtained in transmission was implemented for quality control purposes at the cellar (RPD = 5.7). The pH PLS calibration model for spectra obtained in transmission was implemented for rough screening (RPD = 2.7) and future development was necessary to increase the predictability of the model.

The PLS calibration models developed in this study are accurate, and simple to operate compare to the complex and lengthy reference methods. A fast decision making process is possible that will improve communication between viticulturist, winemakers, cellar master and laboratory staff. The laboratory staff will be able to analyse the large amount of grapes annually harvested by the cellar and routine screening of every tank of juice before inoculation is possible. Little sample preparation is necessary and the methods generate minimum chemical waste. The PLS calibration models contribute to the standardisation of analytical methods at the cellar for grape compositional analysis and forms a basis for quantitative analysis of spirit-based liqueurs.

This study creates the opportunity for various future research projects at the cellar. These projects include the continuously maintaining of PLS calibration models to improve the regression statistics. Updating the calibration sets with grape juice samples from future harvesting seasons and additional spirit-based liqueur product types produced by the cellar, are essential.

Future development of cost-effective methods for determining of other grape juice and spirit-based liqueur parameters that is suitable for practical industry use is possible. Vibrational spectroscopy techniques can be investigated for the potential of on-line or process monitoring. This has a positive impact on the quality and financial aspects of the product and business.

The results obtained in this study made a significant contribution towards validation of FT-MIR as a powerful tool for rapid quantification of quality indicating parameters in wine and spirit-based liqueurs. The contribution is particularly valuable in the context of ongoing research to

improve the quality of products at the cellar to meet consumer demands. The knowledge gained on quantification of quality indication parameters of spirit-based liqueurs is novel and this is one of the first reports on implementation of mid-infrared (MIR) spectroscopy for the quality control of South African spirit-based liqueurs.

5.2 LITERATURE CITED

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