

Chemical characterisation of South African young wines

by

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

December 2007

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DECLARATION

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

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SUMMARY

The rapid expansion of the world wine industry has increased the pressure on wine producers to produce high quality, distinguishable wines. The use of sensory evaluation alone as a tool to distinguish between wines is limited by its subjective nature. Chemical characterisation using analytical methods and data analysis techniques are increasingly being used in conjunction with sensory analysis for comprehensive profiling of wine. Analytical chemistry and chemometric techniques are important and inextricable parts of the chemical characterisation of wine. Through this process insight into the inherent composition of wines, be it in a general sense or related to a particular wine category is gained. Data generated during chemical characterisation are typically compiled into electronic databases. The application of such information towards wine quality control includes the establishment of industry benchmarks and authentication.

The current project is part of The South African Young Wine Aroma Project, a long term research initiative funded by the South African Wine Industry with the ultimate aim to establish a comprehensive, up-to-date, database of the volatile composition of young wines. The data generated during this thesis represent the first contribution towards realising this ambition.

Three clearly defined aims were set for this project, the first of which is the chemical characterisation of South African young wines in terms of selected volatile and non-volatile compounds and Fourier transform infrared spectra, with particular focus on the volatile composition. FTMIR spectra are information rich and non-specific instrumental signals that could provide invaluable information of the inherent composition of the wines. The second aim is the evaluation of the analytical methods used to generate the data and in the last instance, the optimisation of FTMIR spectroscopy for rapid quantification of major wine parameters and volatile compounds.

The concentrations of 27 volatile compounds in South African young wines were determined by gas chromatography coupled to flame ionisation detection (GC-FID) using liquid-liquid extraction of the analytes. Wine samples of the 2005 and 2006 vintages produced from six of the most important cultivars in the South African wine industry, namely Sauvignon blanc, Chardonnay, Pinotage, Cabernet Sauvignon, Merlot and Shiraz were used. The producing cellars were from four major South African wine producing regions, namely Stellenbosch, Paarl, Robertson and Worcester. The data captured made a significant contribution to the establishment of the Aroma Project Database. Univariate statistics showed wide variations in the chemical composition of the wines. Red wines were generally characterised by high levels of higher alcohols and white wines by high levels of esters. Most of the differences between vintages were cultivar dependent and phenological differences between cultivars were suggested as a possible cause. Fusel alcohols, iso-acids and esters of fusel alcohols were particularly responsible for differences between red wines. A combination of fatty acids and higher alcohols were responsible for differences between production regions. However, using univariate statistics alone was limited in identifying characteristic features of the chemical composition of the wines.

In order to explore the correlations between the volatile components, FTMIR spectra and non-volatile components the data were further investigated with multivariate data analysis. Principal component analysis was successfully employed to distinguish between wines of different vintages and cultivars. The role of the volatile composition was more influential in the separation of vintage and red wine cultivar groupings than the non-volatile components or the FTMIR spectra. Almost all the individual volatile components contributed to the separation between the vintages and cultivars, thereby highlighting the multivariate nature required to establish the distinguishing features pertaining to each of these categories. The FTMIR spectra and the non-volatile components were more important than the volatile components to characterise the differences between the white cultivars. It was not surprising that both the volatile components and the FTMIR spectra were needed to distinguish between both red and white cultivars simultaneously. It was of interest the full spectrum, including all wavenumbers were required for a powerful classification model. This finding supports the initial expectation that the non-selective but information rich signal captured in the FTMIR spectra is indispensable. No distinction could be made between the production regions, which was not surprising since the wines used in this study was not of guaranteed origin. Furthermore, no clear correlation could be established between the chemical composition or the FTMIR spectra and the quality ratings of the wines. Limitations in the dataset were pointed out that must be taken into account during further investigations in the future.

The liquid-liquid extraction method used during the analysis of the volatile components was evaluated for precision, accuracy and robustness. Generally good precision and accuracy were observed. There were slight indications of inconsistencies in the recoveries of analytes between the red and white wine matrices. Certain parameters of the protocol, namely sample volume, solvent volume, sonication temperature and sonication time, were identified as factors that had a major influence on quantification. The results obtained in this study made a major contribution towards establishing this technique for routine GC-FID analysis in our environment.

Due to the high sample throughput in wine laboratories, the use of rapid quantitative analytical methods such as FTMIR spectroscopy is becoming increasingly important. Enzymatic-linked spectrophotometric assays and high performance liquid chromatography (HPLC) methods were evaluated for their suitability to serve as reference methods for optimising and establishing FTMIR calibrations for glucose, fructose, malic acid, lactic acid and glycerol. Pigmented and phenolic compounds were identified as sources of interference in the determination of organic acids in red wines with both enzymatic assays and HPLC. The use of fining treatments for the decolourisation of red wine samples was investigated. Activated charcoal was more efficient in terms of colour removal than polyvinyl polypyrrolidone (PVPP), but neither were compatible with the specific enzymatic method used in this study. Solid phase extraction (SPE), a method commonly used during sample clean-up prior to HPLC analysis of organic acids in wine, and PVPP fining were evaluated as sample preparation methods for HPLC analysis to optimise the quantification of organic acids in red wine. Four different types of SPE cartridges were evaluated and the SPE method was optimised in order to recover the maximum amount of organic acids. However, low recoveries, in some instance less than 50%, for the organic acids in wine were reported for the optimised SPE method. In this respect one was the worst. On average, excellent recoveries were

observed for the organic acids using the PVPP method that were in excess of 90%. This method therefore provides a very valuable and simple alternative to SPE for sample-cleanup prior to HPLC analysis. One aspect that still needs to be investigated is the reproducibility of the method that should still be optimised. In general, enzymatic analysis was more suitable for the determination of glucose and fructose, while HPLC analysis were more suitable for the quantification of organic acids. Efficient glycerol quantification was observed with both enzymatic and HPLC analysis, although a lower measurement error was observed during the HPLC analysis.

Apart from reliable reference methods, successful FTMIR calibrations also rely on the variability present in the reference sample set. The reference sample set used to establish FTMIR calibrations must ideally be representative of the samples that will be analysed in the future. Commercial, or so-called global, FTMIR calibrations for the determination of important wine parameters were evaluated for their compatibility to a South African young wine matrix. The prediction pH, titratable acidity, malic acid, glucose, fructose, ethanol and glycerol could be improved by establishing a brand new FTMIR calibration, thereby clearly indicating that the South African young wine matrices were significantly different from the samples of European origin that were used to establish the commercial calibrations. New preliminary calibration models were established for a young wine sample matrix and were validated using independent test sets. On average the prediction errors were considered sufficient for at least screening purposes. The effect of wavenumber selection was evaluated. Relatively successful models could be established for all the compounds except glucose. Wavenumber selection had an influence on the efficiency of the calibration models. Some models were more effective using a small amount of highly correlated wavenumbers, while others were more effective using larger wavenumber regions.

Preliminary FTMIR calibration models for the screening of volatile compound groups in young wines were evaluated. Compound groups were compiled based on chemical similarity and flavour similarity. Good linearity were observed for the "total alcohol", "total fatty acids", "esters" models while an interesting polynomial trend was observed for the "total esters" model. Relatively high prediction errors indicated the possibility of spectral interferences, but the models were nevertheless considered suitable for screening purposes. These findings are a valuable contribution to our environment where fermentation flavour profiles must often be examined.

The important role sound and validated analytical methods to generate high quality analytical data, and the subsequent application of chemometric techniques to model the data for the purpose of wine characterisation has been thoroughly explored in this study. After a critical evaluation of the analytical methods used in this study, various statistical methods were used to uncover the chemical composition of South African young wines. The use of multivariate data analysis has revealed some limitations in the dataset and therefore it must be said that wine characterisation is not just reliant on sophisticated analytical chemistry and advanced data analytical techniques, but also on high quality sample sets.

OPSOMMING

Die geweldige uitbreiding wat die afgelope tyd in die internasionale wynbedryf plaasgevind het plaas geweldige druk op wynprodusente om uitnemende en kenmerkende produkte te lewer. Die gebruik van slegs sensoriese evaluering om tussen wyne te onderskei word beperk deur die subjektiewe aard van die tegniek. Chemiese karakterisering deur die gebruik van analitiese metodes en data analitiese tegnieke word toenemend gebruik in samewerking met sensoriese analise om omvattende profiele vir wyne saam te stel. Analitiese chemie en chemometriese tegnieke, 'n onlosmakende kombinasie, speel 'n belangrike rol in die chemiese karakterisering van wyn. Deur chemiese karakterisering kan nuwe insigte ten opsigte van die natuurlike samestelling van wyn verkry word, hetsy dit oor die algemeen of spesifiek ten opsigte van 'n sekere wyn tipe is. Data wat tydens die chemiese karakterisering van wyn genereer word kan saamgevoeg word in 'n elektroniese databasis. So 'n databasis kan aangewend word om standaarde vir die wynbedryf vas te stel of vir egverklaring.

Die huidige projek is deel van Die Suid-Afrikaanse Jongwyn Aroma Projek, 'n langtermyn navorsingsinisiatief wat deur die Suid-Afrikaanse wynbedryf befonds word. Die uiteindelige doel van die projek is om 'n oorsigtelike, opgedateerde databasis van die vlugtige samestelling van Suid-Afrikaanse jong wyne saam te stel. Die data wat tydens hierdie studie genereer is verteenwoordig die eerste bydrae om hierdie mikpunt te verwesenlik.

Drie duidelik onderskeibare mikpunte is vasgestel vir hierdie projek. Die eerste daarvan is die chemiese karakterisering van Suid Afrikaanse jong wyne ten opsigte van vlugtige verbindings, sekere belangrike vaste verbindings en Fourier transform mid-infrarooi (FTMIR) spektra, met spesifieke fokus op die vlugtige verbindings. Die FTMIR spektra is 'n informasie ryke en nie-selektiewe instrumentele sein wat onontbeurlike inligting ten opsigte van die inherente samestelling van wyn kan verskaf. Die tweede mikpunt is die evaluering van die analitiese metodes wat gebruik is om die bogenoemde data te genereer en die optimisering van FTMIR kalibrasie modelle vir die vinnige bepaling van belangrike wyn parameters sowel as vlugtige verbindings, wat dan ten einde die derde mikpunt is.

Die konsentrasies van 27 vlugtige verbindings in Suid Afrikaanse jongwyne is bepaal met gas chromatografie gekoppel aan vlam-ioniserende deteksie (GC-FID) saam met die gebruik van vloeistof ekstraksie. Wynmonsters van die 2005 en 2006 oesjare, berei van ses van die belangrikste kultivars van die Suid Afrikaanse wynbedryf in naamlik Sauvignon blanc, Chardonnay, Pinotage, Cabernet Sauvignon, Merlot en Shiraz is gebruik. Die wyne was afkomstig van wynkelders uit vier belangrike Suid-Afrikaanse produksie streke naamlik Stellenbosch, Paarl, Robertson en Worcester. Hierdie data is 'n belangrike bydrae tot die samestelling van die Aroma Projek databasis. Enkelveranderlike statistiese metodes het groot variansie in die samestelling van Suid-Afrikaanse jongwyne aangedui. Rooi en witwyne het hoofsaaklik verskil op grond van hoër alkohole en ester inhoud. Meeste van die verskille tussen oesjare was kultivarafhanklik en fenologiese verskille tussen die kultivars is as rede hiervoor aangevoer. Iso-vetsure, fusel alkohole

en iso-esters het 'n noemenswaardige bydrae gemaak tot die verskille tussen kultivars. Daar was ook verskille tussen produksie streke veral ten opsigte van Robertson en Worcester. Hierdie verskille was oorwegend toegeskryf aan verskille in vetsuur en hoër alkohol konsentrasies. Die gebruik van enkelveranderlike statistiek was egter nie voldoende om die kenmerkende eienskappe van die chemiese samestelling van die wyne te identifiseer nie.

Multiveranderlike data analise is aangewend om die verband tussen die vlugtige samestelling, FTMIR spektra en die samestelling van sekere belangrike vaste verbindings verder te ontleed. Verskille in die samestelling van oesjare en kultivars is uitgewys deur hoofkomponent analise. Die rol van die vlugtige verbindings met betrekking tot die onderskeiding tussen oesjare en rooiwijn kultivars was meer invloedryk as die van die FTMIR spektra en die vaste verbindings. Bykans al die individuele verbindings het 'n bydrae gelewer tot die skeiding tussen die oesjare en kultivars, wat die multiveranderlike aard van die datastel bevestig. Die rol van die FTMIR spektra en die vaste verbindings was meer beduidend met betrekking tot die witwijn kultivars. Dit was nie verbasend dat 'n kombinasie van FTMIR spektra en vlugtige verbindings die sleutel was tot 'n suksesvolle klassifikasie model van beide wit en rooi kultivars nie. Intendeel, die beste klassifikasie model is verkry waar FTMIR golflengtes wat normaalweg met geraas geassosieer word ingesluit word in die model. Hierdie bevinding bevestig die onontbeerlike rol van FTMIR spektra as 'n informasie ryke, nie-selektiewe analitiese tegniek. Geen verband kon bevestig word tussen chemiese samestelling en die produksie areas of ten opsigte van wynkwaliteit nie. Sekere beperkings in die datastel is uitgewys wat in ag geneem moet word tydens verdere ondersoek.

Die vloeistofekstraksie metode wat tydens die bepaling van die vlugtige verbindings gebruik is, is evalueer ten opsigte van noukeurigheid, akkuraatheid, en robuustheid. Oor die algemeen is goeie noukeurigheid en akkuraatheid waargeneem. Daar was enkele aanduidings van ongelykhede tussen die herwinnings wat tydens rooi- en witwijn analise waargeneem is. Sekere aspekte van die ekstraksie protokol, naamlik monstervolume, oplosmiddelvolumen asook die temperatuur en duur van sonikasie, is geïdentifiseer as faktore wat 'n wesenlike invloed het op die resultate. Hierdie resultate is 'n beduidende bydrae tot die daarstelling van 'n gevestigde GC-FID tegniek vir roetine analise in ons omgewing.

Weens die groot aantal monsters in wyn laboratoriums geanaliseer word is die gebruik van vinnige analitiese metodes soos FTMIR spektroskopie van groot belang. Die bruikbaarheid van ensiematiese bepalinge en HPLC (hoëdruk vloeistof chromatografie) analises as verwysingsmetodes vir die bepaling van glukose, fruktose, appelsuur, melksuur en gliserol is ondersoek. Gekleurde en fenoliese verbindings is geïdentifiseer as wesenlike bronne van analitiese geraas tydens die bepaling van organiese sure in rooiwijn met beide ensiematiese metodes en HPLC analises. Die gebruik van breimiddels as ontkleurmiddels vir rooiwijn is ondersoek. Geaktiveerde koolstof was 'n meer effektiewe ontkleurmiddel as polivinilpolipyrillodoon (PVPP), maar nie een van die behandelings was versoenbaar met die ensiem metode wat gebruik is nie. Vastestof fase ekstraksie (Solid phase extraction; SPE) word algemeen gebruik om organiese sure van fenoliese komponente te skei voor HPLC analise om sodoende die bepaling van organiese sure te optimaliseer. Verskeie SPE kolomme is ondersoek en die voorgestelde SPE metode is geoptimaliseer om die maksimum herwinning van organiese sure te verkry. Ten einde was lae herwinning vir

organiese sure, soms laer as 50%, met die verbeterde SPE metode gemerk. Die PVPP metode het baie groter hoeveelhede organiese sure herwin, meestal meer as 90%. Hierdie metode is 'n waardevolle en eenvoudige alternatief tot SPE vir monstervoorbereiding voor HPLC analise. Die reprodiseerbaarheid van die metode moet egter geoptimeer word. Oor die algemeen is ensiematiese metodes as meer geskik beskou vir die bepaling van glukose en fruktose terwyl HPLC analise meer geskik was vir die bepaling van appelsuur en melksuur. Beide metodes was geskik vir die bepaling van gliserol, hoewel 'n laer laboratorium fout waargeneem is tydens HPLC analises.

Suksesvolle FTMIR kalibrasie modelle is nie net afhanklik van goeie verwysingsmetodes nie, maar ook van omvattende verwysings monsters. Kommersieële, of globale, kalibrasies vir die bepaling van belangrike wyn parameters is ondersoek ten opsigte van hul geskiktheid in 'n Suid Afrikaanse jongwyn matriks. In sommige gevalle is beduidende matriks effekte opgemerk en die voorspelling van pH, titreerbare suur, appelsuur, glukose, fruktose, etanol en gliserol kon verbeter word met die opstelling van splinternuwe kalibrasie modelle. Voorlopige kalibrasie modelle vir jongwyne is opgestel en die effek van golfgetal seleksie is ondersoek. Redelike suksesvolle kalibrasie modelle is verkry vir die meeste van die wyn parameters, met die uitsondering van glukose. Golfgetal seleksie het beslis 'n rol gespeel. Sommige modelle was meer effektief indien 'n klein aantal, hoogs gekorreleerde golfgetalle gebruik is, terwyl ander modelle meer effektief was wanneer groter dele van die mid-infrarooi spektra gebruik is.

Die bepaling van groepe vlugtige verbindings in wyn met behulp van voorlopige FTMIR kalibrasies is ondersoek. Die vlugtige verbindings is gegroepeer volgens chemiese struktuur en volgens geurbydrae. Liniere kalibrasie modelle vir "totale alkohole", "totale vetsure" en "esters" is verkry terwyl die kalibrasie model vir "total esters" 'n polinomiese tendens gevolg het. Relatief hoë prediksie foute is waargeneem wat moontlik deur inmenging in die spektra veroorsaak is. Ten spyte daarvan is die modelle goedgekeur vir die sifting van vlugtige verbindings in jong wyne. Hierdie resultate is 'n waardevolle bydrae tot ons omgewing waar die fermentasie profiele van wyne gereeld ondersoek moet word.

Die rol van goeie analitiese metodes om hoë gehalte analitiese data te genereer en die daaropvolgende rol chemometriese metodes in wyn karakterisering is deeglik bestudeer in hierdie studie. Na afloop van 'n kritiese ondersoek van die analitiese metodes is verskeie statistiese metodes gebruik om die chemiese samestelling van Suid Afrikaanse jongwyne te ontdek. Die gebruik van meervoudige veranderlike data analise het beperkinge in die datastel uitgewys. Die uiteindelijke afleiding is dat wyn karakterisering nie net afhanklik is van gesofistikeerde analitiese chemie en gevorderde data analise nie, maar ook van hoë gehalte datastelle.

BIOGRAPHICAL SKETCH

Leanie Louw was born in Cape Town, South Africa on the 10th of October 1983. She attended Swartland Primary School and Matriculated at Bloemhof Girl's High School in 2001. Leanie obtained a BScAgric degree in Oenology in 2005 at the Stellenbosch University. In 2006, Leanie enrolled for a MScAgric in Enology at the Department of Viticulture and Oenology, Stellenbosch University.

ACKNOWLEDGEMENTS

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

Dr. Hélène Nieuwoudt, Institute for Wine Biotechnology, Stellenbosch University, for being my mentor. Her support, initiative and critical evaluation were a constant source of encouragement, not only regarding the completion of this work, but also in my development as a scientist.

Prof. Pierre van Rensburg, Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, for his guidance and invaluable contribution to this work.

Karolien Roux and Andreas Tredoux, Chemical Analytical Laboratories, Institute for Wine Biotechnology, Stellenbosch University, for their endless patience in assisting me on my journey to becoming a chromatographer.

Hugh Jumat, Chemical Analytical Laboratories, Institute for Wine Biotechnology, Stellenbosch University, for his assistance in the generation of more than 2000 gas chromatograms.

Dr. André de Villiers, Department of Chemistry, Stellenbosch University, for his valuable guidance on the subject of HPLC and SPE.

Dr. Marietjie Stander, Centre of Analytical Facilities, Stellenbosch University, for the use of her HPLC facilities.

Sulette Malherbe, Institute for Wine Biotechnology, Stellenbosch University, for her time and efforts concerning my HPLC training

Prof. Daan Nel and Dr. Martin Kidd, Department of Statistics, Stellenbosch University, for sharing their knowledge and experience in statistics.

Prof. Tormod Naes, Centre for Biospectroscopy and Data Modelling, Matforsk Oslovegen, Norway, for his input in terms of multivariate data analysis.

Hanlie Swart and Karen Vergeer, Institute of Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, for their efforts in the preparation of this manuscript as well as for their friendly smiles.

Winetech, The National Research Foundation and the **Harry Crossley Foundation** for funding.

My family and friends for their love and support and for putting up with months of neglect.

And above all to my **Creator** for blessing me with courage and patience.

PREFACE

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

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**

Chemical characterisation of wine in perspective

Chapter 3 **Literature Review**

Introductory review of data analysis techniques relating to the chemical characterisation of wine

Chapter 4 **Research Results**

The volatile composition of South African young wines: a global comparison

Chapter 5 **Research Results**

Chemical characterisation of South African young wines using FTMIR spectroscopy, gas chromatography and multivariate data analysis

Chapter 6 **Research Results**

A comparative evaluation of the suitability of enzymatic assays and HPLC as reference methods for the establishment of FTMIR calibrations of important wine constituents

Chapter 7 **Research Results**

Optimisation of the quantification of major wine parameters in South African young wines using Fourier Transform mid-infrared spectroscopy

Chapter 8 **Research Results**

FTMIR calibrations of volatile compounds in wine

Chapter 9 **General Discussion and Conclusions**

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ADDENDUM A: METHOD VALIDATION REPORT – GC-FID METHOD FOR THE DETERMINATION OF ALCOHOLS, FATTY ACIDS AND ESTERS IN WINE

ADDENDUM B: ENZYMATIC ASSAYS – REACTIONS AND CALCULATIONS

Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

INTRODUCTION AND AIMS

1.1 INTRODUCTION

Substantial knowledge on the chemical composition of wine is one of the key factors required to monitor and improve wine quality. Understandably, wine quality can be viewed from many perspectives, but for most it certainly would include aspects related to wholesomeness, authenticity and flavour. The term 'flavour' generally refers to the entirety of sensorial perceptions, including taste, smell and mouth-feel (Francis and Newton, 2005) and in chemical terms both volatile and non-volatile wine components are implicated.

The somewhat loosely defined concept of chemical characterisation of wine goes by many names in the published literature: profiling, fingerprinting and authentication, among others (Bevin *et al.*, 2006; Marini *et al.*, 2006; Setkova *et al.*, 2007). The concept as such remains the same and can be best described by its two-fold purpose. Firstly, the term chemical characterisation refers to the generation of quantitative data on specific chemical compounds, followed by analysis of the data using descriptive statistics such as means, standard deviations and analysis of variance. This result in the description of the wine in terms of the distribution of concentration ranges of the chemical compounds tested and wines are frequently characterised in the context of specific categories such as wine style, grape cultivars, geographical origin, process technology, age and so forth. Data are typically captured in electronic databases to facilitate easy comparison and an example is the European Wine Database project that was recently launched by the European Office for Wine, Alcohol and Spirit Drinks (Wine inspection and quality, n.d). In the second instance wine characterisation refers to the application of multivariate techniques to chemical data and/or instrumental signals of wine samples in order to extract the maximum useful information about their distinguishing or unique features. Information gained in this way is typically used to develop multivariate mathematical models that define the membership of the samples to known classes or groups (Berrueta *et al.*, 2007). New unknown samples are then classified in one of the known classes on the basis of similar instrumental or chemical measurements. This approach was used successfully to determine the geographical origin of wines from four different countries, using the chemical values of 63 wine parameters (Capron *et al.*, 2006).

At present most major wine producing countries have extensive research programs on the chemical characterisation of wine and application of the information to flavour and aroma analysis amongst other fields. Spain is a major contributor to research in this field (Calleja and Falqué, 2005; Díaz *et al.*, 2003; Ferreira *et al.*, 2000; López *et al.*, 1999; Marti *et al.*, 2004). Other countries that have contributed include France and Germany (Danzer *et al.*, 1999; Fischer, *et al.*, 1999; Preys *et al.*, 2005); Australia (Cozzolino *et al.*, 2005); Portugal (Câmara *et al.*, 2007); Italy (Buratti *et al.*, 2004) and Greece (Makris *et al.*, 2006).

Several research projects funded by the South African Wine Industry are focussed on the characterisation of wines. Different approaches, focussing on wholesomeness, authenticity and flavour related issues, have been taken. The determination of the ethyl carbamate, a potential

carcinogenic substance, in South African wines is an important contribution to the characterisation of the wholesomeness of wine (WW-08-20). Authenticity is an important driving force in the South African wine research industry and South Africa has contributed to the establishment of a database of analytical parameters of wines from Third World countries (WW-08-26) as part of the EU Wine Database Project. The authentication of the origin of wine using multi-element analysis has received particular attention (WW-08-28). Other projects focussed on the characterisation of aroma and flavour and/or the related chemical constituents in wine, although several of these studies were limited to small sample sets and selected compounds. The characterisation of the sensory properties of South African wines have resulted in the development of Aroma Wheels for South African brandies (Jolly and Hattingh, 2001) as well as the South African cultivar, Pinotage (Marias and Jolly, 2004). From a chemical point of view, both volatile components and phenolic components have been investigated (Marais *et al.* 1981; Rossouw and Marias, 2004)

South African research groups have made important contributions in development of analytical methods for the analysis of important wine constituents. Examples include stirbar sorptive extraction methods for the determination of wine contaminants (David *et al.*, 2000; Sandra *et al.*, 2001); a solid phase extraction method for the determination of polyphenols, organic acids and sugars in wine (de Villiers *et al.*, 2004); capillary electrophoresis methods for the determination of organic acids in wine (de Villiers *et al.*, 2003) and a headspace sorptive extraction method for the determination of volatile compounds in wine (Weldegergis *et al.*, 2007).

The use of Fourier transform mid-infrared (FTMIR) spectroscopy as a rapid analytical tool for wine chemistry related issues, has recently been introduced in the South African Wine Industry and several projects are currently underway. Reports on these projects have been presented at the 3rd International Viticulture and Oenology Conference of the South African Society for Enology and Viticulture, Somerset West, South Africa, 14-17 November 2006. The identification of wines produced by genetically modified organisms with FMTIR spectroscopy and chemometrics was presented (Osborne *et al.*, 2006a). Other projects include investigations of the use of FMTIR spectroscopy as a rapid quality control method for spirit products (Kleintjies *et al.*, 2006) and fortified wines (Lochner *et al.*, 2006); the identification of problem fermentations (Malherbe *et al.*, 2006) and the authentication of Sauvignon blanc wines (Treurnicht *et al.*, 2006). Furthermore, chemical profiles of South African young wines, based on chemical data generated with FTMIR spectroscopy, and the compositional trends and differences between cultivars, vintages and production region have been presented (Louw *et al.*, 2006). In addition, the usefulness of ATR-FTMIR spectroscopy for the discrimination between untransformed and genetically modified wine yeasts and the discrimination between wine spoilage organisms (Osborne *et al.*, 2006b) have been reported.

The Winetech Aroma Project (WW-08-31, 2006) which includes this current project, is a recent initiative to characterise South African young wines in terms of volatile components. Young wines are defined as unwooded single-varietal wines that have not been bottled and are therefore not yet commercially available, are of specific interest as complexity caused by blending, ageing and oak maturation are not included in the sample matrix. Four research groups are involved in the project namely the ARC-Nietvoorbij, the Department of Chemistry of the University of Cape Town,

the Department of Chemistry of Stellenbosch University and the Institute for Wine Biotechnology/Department of Viticulture and Oenology of Stellenbosch University. The ultimate aim of the Winetech Aroma project is the construction of a comprehensive up-to-date database containing the chemical profiles of perceived aroma compounds of SA wine cultivars and styles originating from the various wine producing areas in SA. The information captured in the database will serve as a benchmark for studies focussed on specific authenticity issues and for the industry. The outcomes of the Winetech Aroma Database project include the development of several methods (Weldegergis *et al.*, 2007) for the analysis of volatile components, one of which forms part of this current study.

1.2 PROJECT AIMS

Three clearly defined goals were identified for this project. The main aim of this project was to generate analytical data and Fourier transform mid-infrared spectra of South African young wines in order to describe and characterise the wines based on their chemical composition. The particular focus was on the determination of volatile compounds and selected non-volatile parameters namely pH, titratable acidity, organic acids, sugars, ethanol and glycerol.

The data were captured in an electronic database and analysed by univariate and multivariate statistical techniques to identify trends, similarities and differences in the chemical profiles related to vintage, cultivar and origin. Furthermore it was attempted to classify the wines into varietal and geographic origin classes based on chemical and FTMIR spectral characteristics using multivariate data analysis. Multivariate data analysis was also used to investigate the possibility of using chemical data to predict the quality of South African young wines as allocated by judges at the South African Young Wine Show.

The secondary aims of this project were to evaluate and optimise the analytical methods used to generate the data. These methods include the liquid-liquid extraction method used for the determination of the volatile compounds as well as the enzymatic methods and HPLC methods used as reference methods for the FTMIR calibrations of the mentioned non-volatile components. In addition, some sample preparation procedures used during the enzymatic, HPLC and FTMIR analyses were evaluated.

An additional aim of this project was to evaluate FTMIR calibration models for the prediction of chemical data from spectroscopic data in terms of their performance in a young wine matrix. In addition the usefulness of FTMIR spectroscopy as a screening method for the general volatile composition of wines was investigated.

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Chapter 2

LITERATURE REVIEW

**Chemical characterisation of wine in
perspective**

LITERATURE REVIEW

2.1 INTRODUCTION

During the last two decades the world wine industry grew substantially and has become increasingly competitive. The modern day consumer is confronted with a vast selection of wines from across the globe. Apart from the European Old World wines, younger wine producing countries, such as the United States of America, Australia, Argentina, Chile and South Africa also compete in the international wine market with New World wines. On a global scale it has become more and more important for wine industries to produce distinguishable wines.

Traditionally, wines were compared by sensorial evaluation. However it was obvious that this method's subjective nature was a major pitfall. A need was established for a more objective way of characterising wine, especially in terms of varietal and geographic origin. The 1950's and 1960's saw the introduction of advanced analytical methods, including gas chromatography and high pressure liquid chromatography, that allowed the quantification of several analytes at the same time (Reneinicus, 1998; Rounds and Gregory, 1998) . The successful application of these methods to wine analysis increased the potential for their use in the objective characterisation of wine (Kwan *et al.*, 1979; Noble *et al.*, 1980).

Another driving force behind the chemical characterisation of wine was the fact that wine composition and the role of some wine constituents were still largely unclear. With the advances made in analytical chemistry it became possible to accumulate large amounts of data per wine sample and thereby getting a very necessary overview of the chemical composition of wine in the form of a chemical "profile".

The masses of data generated with the newly developed technology still needed to be interpreted and explained. It soon became clear that investigating wine properties in terms of individual analytes was not sufficient. The complexity of the wine matrix and the various viticultural and oenological factors that influence it could be better explained by taking the interaction between variables into account. The application of chemometric techniques, or multivariate data analysis, in food science complied with this need. Multivariate data analysis provided the means to contract datasets with multiple variables in order to present the data in a way that could be easily interpreted without compromising the inherent variability in the dataset. Pattern recognition techniques could be used to correlate specific chemical constituents to wine characteristics that could not be directly characterised with analytical methods. The work of Kwan and Kowalski in the late 1970's was a groundbreaking and well-acclaimed contribution to the application of pattern recognition techniques to distinguish between wines based on their chemical properties (Kwan and Kowalski, 1978; Kwan *et al.*, 1979).

The combination of pattern recognition techniques with analytical techniques had a further application. The advances made in chromatographic analysis enabled the separation of many unknown compounds that needed identification. Chemometric techniques could be used to

determine the weight or influence of the unidentified compounds on the discrimination between different classes of wines. Compounds that did not contribute to the wine characteristics could be eliminated. In this way, only the compounds that were highly significant could be analysed with mass spectrometry for identification, thereby avoiding redundant efforts (Kwan and Kowalski, 1980).

2.2 WINE CHARACTERISATION

Chemical profiles can be constructed for many wine classes, the most important being geographical origin and grape variety. Other properties to consider is wine style, for instance dry table wines, ice wines, brandies etc. Wines could also be profiled according to the relative age of the wine. The characterisation of wine has an important role in wine quality control and could be used to identify adulterated products.

When the possibilities of chemical analysis as a more objective way to characterise wine were identified, the question of which analytes could be best linked to specific wine properties immediately followed. The complex nature of wine and the influence of viticultural and winemaking practices on its composition had to be considered. Several types of compounds have already been identified as important constituents in wine. These include phenolic compounds, macro and trace elements, amino acids, classic wine quality parameters such as ethanol, glucose, organic acids and SO₂, sensory data, volatile components and isotopic compounds. These compounds are all influenced by viticultural and oenological practices to a certain extent, but their influence on the inherent characteristics of specific cultivars or wines of origin was yet to be determined.

2.2.1 GRAPE CULTIVAR

The most obvious transition from sensory evaluation to chemical characterisation is the analysis of the volatile compounds responsible for the aroma of wine. The main analytical method used to quantify volatile compounds in wines is gas chromatography. These instruments can be coupled to various detectors, of which the flame ionisation detector (GC-FID)¹ is the most common. This detector responds well to organic compounds, has a wide linear range and a high level of sensitivity, but its major limitation is the need of references to identify substances (Reineccius, 1998). By coupling a gas chromatograph to a mass spectrometer (GC-MS) the mass spectra generated can be used to identify the compounds that were chromatographically separated. Analysis with these methods allows insight into the composition of a wine, but does not give information on sensory properties. The sensory attributes of the individual compounds can be determined by capturing and evaluating the chromatographic effluent of each compound through a sniff port. This technology, gas chromatography olfactometry, or GC-O, were used in combination with GC-FID and GC-MS in one of the first studies to distinguish between wines made from different grape varieties (Noble *et al.*, 1980). In this study, sixty compounds were identified,

¹ A complete list of abbreviations used in this review is presented in section 3.

including one never previously reported in wine. Separated groups of samples could be observed on PCA score plots, each associated with a different cultivar. The compounds that corresponded to the Riesling wines were associated with spicy and floral aromas, which are consistent with typical Riesling characteristics.

Although the role of compound groups like methoxypyrazines and norisoprenoids in specific cultivars have been identified (Lacey *et al.*, 1991; Sefton *et al.*, 1993) it seems that the backbone of the volatile composition of all wines are based on alcohols, esters and fatty acids (Schreier, 1979). Most of these compounds are by-products of alcoholic fermentation although some can be grape derived or formed by microbes other than yeasts. Examples are hexanol that can be grape-derived and acetic acid that can be formed by acetic acid bacteria (Schreier, 1979). Strong correlations have been found between grape variety and the main groups of by-products from yeast amino acid metabolism, namely isoacids and higher alcohols, ethyl esters of isoacids and acetate esters of higher alcohols. (Ferreira *et al.*, 2000) The authors suggested that the amino acids profiles of grapes greatly contribute to the aromatic differences between cultivar wines (Ferreira *et al.*, 2000). This statement can be supported by studies in which strong correlations have been found between grape variety and amino acid composition (Soufleros *et al.*, 2003; Vasconcelos and Chaves das Neves, 1989) Other studies support the influence of higher alcohols and short-chain ethyl esters in varietal differentiation. (Falqué *et al.*, 2001; Lopéz *et al.*, 1999) Principal component analysis showed that higher alcohols, especially 2-phenylethanol, butanol and hexanol were influential in distinguishing between Portuguese cultivars, Boal, Malvasia, Sercial and Verdelho. The same was noted for propionic acid, hexanoic acid and octanoic acid and ethyl esters (Câmara *et al.*, 2006). German cultivars, Riesling, Silvaner and Mueller Thurgau, could successfully be classified by a combination of volatile components including terpenes, hexanol, phenylethyl alcohol, diethyl succinate and hexanoic acids (Danzer *et al.*, 1999). Marti (Marti *et al.*, 2004) noted from the mass spectrometry analysis of different Catalonian wines that ion fragments associated with medium chain fatty acids differed significantly between Cabernet Sauvignon and Merlot wines (Marti *et al.*, 2004). In South Africa, a successful discrimination between Pinotage and Cabernet Sauvignon wines could be made based on their hexanol and amyl alcohol content using stepwise discriminant analysis (Marias *et al.*, 1981a).

The development of electronic nose and tongue technology provided an innovative way to directly link sensory data to chemical data. Very few studies have been conducted to classify cultivar wines with data generated by electronic sensors and different opinions exist on the matter. Roussel *et al.* (2003) declared that electronic nose sensors were not suitable for classification of grape musts into cultivar classes as nearly 50% of the samples were incorrectly classified with PLS-D. Cozzolino *et al.* (2005) was able to use data from aroma sensors to successfully classify Riesling and unwooded Chardonnay with 90% accuracy. The authors of this study also claimed that the combination of electronic nose technology with mass spectrometry contributed to the success of their results as interferences normally caused by ethanol in aroma sensor studies were thereby excluded (Cozzolino *et al.*, 2005).

The role of phenolic compounds in wine flavour and quality has been very well established. Phenolic compounds form during grape ripening and contribute to the visual, flavour and mouth

feel characteristics of wine (Castillo-Sanchez *et al.*, 2006; Péres-Magariño and Gonzáles-SanJosé, 2006). Although the phenolic composition of a wine can be altered during winemaking processes (Castillo-Sanchez *et al.*, 2006) the grape variety could still play an influential role in the final phenolic composition of a wine. Cyanidin, procyanidin B2, coumaric acid, epicatechin and delphinine were identified as the main discriminant factors between Shiraz, Cabernet Sauvignon and Merlot wines produced in Greece (Makris *et al.*, 2006). Similar results were observed in a study done on the phenolic composition of South African wines (Rossouw and Marias, 2004). It was observed that the monomeric flavan-3-ols, catechin and epicatechin were much more influential in the discrimination between red cultivars than the polymeric phenols.

An interesting link was found between the phenolic characterisation of varietal wines and wine classification based on spectroscopy. The first study in which the UV-vis and MIR spectra of wines and their phenolic extracts were investigated with multivariate data analysis in order to classify cultivar wines showed promising results (Edelmann *et al.*, 2001). Although the UV-vis spectra (250-600 nm) of the phenolic extracts could only distinguish the Pinot noir wines from the other varieties, namely Cabernet Sauvignon, Merlot, Blaufränkisch, St. Laurent and Zweigelt, the MIR (940-1760 cm^{-1})² spectra of the phenolic extracts allowed the classification of nearly all the varieties into separate groups using hierarchical cluster analysis. Some overlaps between varieties of close genetic similarity, (Blaufränkisch and Zweigelt) were observed. Where SIMCA was applied to the MIR spectra of the phenolic extracts of the wines, 97% of the wines could be correctly classified into their varietal classes. However, poor classification results were obtained when the MIR spectra of the directly analysed wines were compared, and it was concluded that for successful classification, interfering carbohydrates and organic acids should be removed with SPE prior to analysis. The reason given for this statement was that major wine constituents like sugars, ethanol and organic acids, that absorb strongly in the MIR spectral region, are present at higher concentrations compared to phenolic compounds and therefore causes difficulties in the analysis of phenolic compounds with MIR spectroscopy (Edelmann *et al.*, 2001). The use of a combination of UV and FTIR spectra for the classification of grape musts according to grape variety has also been investigated. Roussel *et al.* (2003) processed fused UV and FTIR spectra with genetic algorithms, but could not achieve the same classification success rate than the rates achieved with FTIR spectra alone. It was found that using FTIR spectra pre-processed with genetic algorithms, grape musts could be classified according to their grape variety with a prediction error of 9.6%. This was achieved using selected infrared wavenumbers (Roussel *et al.*, 2003). In both these studies it was found that mid-infrared spectroscopy could distinguish better between grape varieties than UV spectroscopy.

Near infrared spectroscopy (NIR) (800 nm – 2500 nm) has also been successfully applied in the classification of wine. Arana *et al.* (2005) obtained a 97% correct classification between two white grape varieties, Viura and Chardonnay, using discriminant analysis and the NIR spectra of

² Electromagnetic waves can be referred to by their wavelength (in nm) or by their wavenumber (in cm^{-1}). Wavenumbers indicates the number of waves per centimeter. In other words, electromagnetic waves with longer wavelengths like infrared waves will have smaller wavenumbers. Mid-infrared waves are generally referred to by their wavenumber while near infrared waves are referred to by their wavelength.

wines. The value of NIR spectroscopy for the classification of grape varieties is emphasised when these results are compared to the 86.1% correct classification that was reached using classical ripening parameters, berry weight and total soluble solids in the same study (Arana *et al.*, 2005).

2.2.2 GEOGRAPHICAL ORIGIN

The geographical origin of wines is economically very important. The influence of climate, topography and soil composition of wine quality means that wines produced in different areas are often distinctly different. This phenomenon is also visible in wine prices as wines from certain regions are often considered to be of higher quality than others. Most European wine producing countries have strict origin control systems in place that ranks wines from different production areas according to quality. Origin quality control systems typically dictate the viticultural and oenological practices to be used in each region. Due to the economical implications of the origin control systems, the authentication of the origin of wine in European countries is of major importance. Although New World wine producing countries like South Africa do not have rigidly applied origin control systems, the geographical origin their wines still have major market related implications, both locally and internationally.

There are several reasons why macro and trace elements could be useful for the characterisation of wines of origin. The mineral content of wine grapes is mainly due to the uptake of nutritional elements from the soil (Kwan *et al.*, 1979). The differences in the mineral composition in the variety of soil types used for the cultivation of wine grapes could be reflected in the mineral composition of the resulting wines. Furthermore, the mineral concentration of wines remained relatively stable during the course of wine production, compared to other wine constituents (Etiévant *et al.*, 1988). This implicates that the information about the soil on which the vines were cultivated would not be lost due to changes in the mineral composition that occur during wine production.

In a preliminary study, the mineral composition of 40 Pinot noir wines was used to discriminate between the origins of the wines. The levels of 17 elements was determined with atomic emission spectrometry and indicated that barium significantly contributed to the differentiation between French and American Pinot noir wines. The aluminium content of the wines was identified as an important distinguishing factor between Pinot noir from California and the Pacific North West (Kwan *et al.*, 1979). The elements rubidium and lithium, measured with flame emission spectrophotometry were found to be important in the characterisation of French red wines from the Narbonne, Bordeaux and Angers production areas based on results obtained with PCA and SDA (Etiévant *et al.*, 1988). These two elements were also highly significant in the discrimination of Galician (Spanish production region, where the Ribeira Sacra sub-region is of high economic importance) wines based on abovementioned chemometric techniques as well as the classification procedures, KNN, LDA and SIMCA (Latorre *et al.*, 1994; Rebolo *et al.*, 2000).

Technological development in analytical chemistry as well as chemometric methods introduced new ways to optimise the use trace elements for the classification of the origin of wine. The development of inductively coupled plasma spectrometry methods (ICP) for the analysis of

elements expanded the numbers and concentration range of elements that can be analysed simultaneously (Günzler and Williams, 2001). In 1997 the combination of ICP-OES (inductively coupled plasma optical emission spectrometry) analysis and pattern recognition techniques were evaluated for its suitability in the classification of wines from different German wine research institutes. Based on the high classification success rates achieved with a variety of pattern recognition techniques, the use of ICP-OES for the characterisation of wine origin was found highly feasible. The use of the chemometric technique, artificial neural networks gave the best classification results, but was time-consuming to compute. Results obtained with Bayes stepwise discriminant analyses and Fischer discrimination were also satisfactory and quicker to determine (Sun *et al.*, 1997). ICP-OES analysis also indicated that Al, Ba, Ca, Co, K, Li, Mg, Mn, Mo, Rb, Sr and V were highly significant in the discrimination between wines from the four most important Bohemian wine regions (Czech Republic) (Sperkova and Suchánek, 2005). Using these elements a 100% correct classification for all the red wines were accomplished with discriminant analysis. Similar efficient classifications were obtained with the elemental analysis, using ICP coupled to mass spectrometry, of South African wines of origin. Swartland, Robertson and Stellenbosch wines could be completely classified with stepwise and pair wise discriminant analysis. The minerals Li, B, Al, Sc, Mn, Ni, Se, Rb, Sr, Cs, Ba, W and Tl were found to be the most influential (Coetzee *et al.*, 2005).

At first, the role of phenolic compounds in the distinction between wines from different geographic origin seemed unimportant (Gambelli and Santaroni, 2004; Kallithraka *et al.*, 2001). No correlation could be observed between phenolic composition, although some distinction could be made in terms of the anthocyanin content of wines from Northern and Southern Greece (Gambelli and Santaroni, 2004; Kallithraka *et al.*, 2001). These results were obtained using univariate data analysis and PCA. When more powerful data mining techniques like discriminant analysis and SIMCA were used in later studies, correlations between wine origin and phenolic composition could be clearly observed (Makris *et al.*, 2006; Marini *et al.*, 2006). Flavanols, the major anthocyanins and caftaric acid had a strong discriminant influence on the geographical origin of Grecian wine (Makris *et al.*, 2006). Furthermore, procyanidin B1 and B2, total polyphenols and quercetin and vanillic acid were important for the discrimination of wines from different Italian denominations (Marini *et al.*, 2006).

One of the first studies that successfully used volatile composition and pattern recognition techniques to characterise wines from different production areas was published by Kwan and Kowalski in 1980. Using gas chromatographic data and pattern recognition techniques they were up to 98 % successful in classifying Pinot noir wines from France and USA and between 77 and 92% between wines from Pacific Northwest and California. Hexanol and 2-phenylethanol were identified as important compounds (Kwan and Kowalski, 1980). These two alcohols were also reported as the most influential in the differentiation between South African Chenin blanc wines from respectively the Stellenbosch, Robertson and Lutzville regions (Marais *et al.*, 1981b). Hexanol can be found in grape skins and Marais proposed that the different winemaking procedures performed in different regions might influence the amount of hexanol extracted from the grape skin into the wine. Alternatively, the differences observed in the hexanol content of wines production

regions might be due to the different concentrations of the precursors present in the grapes (Marias *et al.*, 1981b). In the same study, which was based on gas chromatographic data and discriminant analysis, isoamyl acetate was identified as an important factor in the discrimination between Colombar wine from Robertson and Lutzville (Marais *et al.*, 1981b). Both isoamyl acetate and 2-phenylethanol is formed by yeast from amino acid precursors during alcoholic fermentation (Lambrechts and Pretorius, 2000). The efforts of Marais *et al.* was extended to the geographical characterisation of South African red wines and again amino acid derived compounds were found influential in the discrimination between the origin of the wines (Marais *et al.*, 1981a). In this case, Cabernet Sauvignon wines from the Stellenbosch and McGregor production areas could successfully be separated using iso-valeric acid, isoamyl acetate and ethyl butyrate as discriminant factors. The proposed role of amino acid profiles in the discrimination between wines of origin is supported by the relative success with which amino acids were used to classify wines of origin (Soufleros *et al.*, 2003). More recently, gas chromatography was used to distinguish between Spanish wines from Ribeira Sacra and Monterrei (Calleja and Falque, 2005). In this case, volatile compounds with chain lengths of four and six carbons respectively were found especially influential.

In addition to the use of gas chromatography, other advanced analytical methods were investigated for its feasibility to distinguish between the volatile composition of wine origin classes. Head space mass spectrometry have also been used to characterise wines and it was observed that ion fragments associated with fatty acids such as isobutyric acid, butyric acid, hexanoic acid and octanoic acid contributed to differences between wines from Priorat and Terra Alta in Spain (Marti *et al.*, 2004). The use of HS-SPME-GC-TOF-MS in combination with sophisticated chemometric technique, Kohonen self organising maps, were successfully used to discriminate between Canadian and Czech ice wines (Giraudel *et al.*, 2007). As with the characterisation of wine cultivars, electronic sensors have been used to classify wines of origin. Data captured by the electronic nose sensors represents the volatile composition of the sample, while the electronic tongue represents the non-volatile flavour related constituents. Italian Barbera wines from in various production areas could be 100% correctly classified with electronic nose and tongue data using LDA (Buratti *et al.*, 2004).

Several studies that have used quantitative data, like ethanol and sugar concentrations, that were determined through infrared spectroscopy were unsuccessful in classifying wines by regions (Arana *et al.*, 2005; Minnaar and Booyse, 2004). However, by using spectral data as variables in stead of quantitative data, Arana *et al.* were able to increase the classification rate of Chardonnay grapes from two Spanish sub-regions from 59.0% to 79.2%. However, when discriminant analysis was performed on data collected near the end of harvesting, the classification rate was 100% (Arana *et al.*, 2005).

Ultraviolet-visible spectroscopy proved to be more effective for the classification of Spanish wines according to origin, where more than 89% of the samples were classified correctly, than according to grape cultivar compared (75%). These results were obtained with SIMCA (Urbano *et al.*, 2006). Another Spanish study indicated that UV-vis data could be applied more effectively towards discriminating between wines of origin with the partitioning based classification methods

SVM and ANN (Acevedo *et al.*, 2007). Vis-NIR spectroscopy (400-2500 nm) have also been applied towards the classification between wines from different geographical origin (Liu *et al.*, 2007) Riesling wines from Australia, New Zealand and Europe were classified with stepwise-LDA with a overall correct classification of 78%. Better results were obtained using PLS-D where the percentage correct classifications were between 70-98%. Considering the small sample set used these results shows great promise for spectroscopy and wine origin classification (Liu *et al.*, 2007).

As previously mentioned, amino acids have been used with relative success to classify wines of origin (Soufleros *et al.*, 2003). However, the characterisation of wines by their geographical origin using the amino acid derivatives, biogenic amines, was less successful (García-Villar *et al.*, 2007).

2.2.3 WINE STYLE

The chemical characterisation of wines has also been applied to distinguish between broader wine categories than cultivars and origin of table wines. Several studies have also focussed on the volatile compositional differences between red, white and rosé table wines. The aim of these studies were focussed on the establishment of chemical profiles and the identification of compounds of interest rather than objectively distinguishing between these wine classes, which can of course easily be done without advanced technology. Furthermore, studies on wine characterisation are not limited to dry table wines, also includes research on other wine styles such as ice wines and fortified wines.

The concentration ranges and variations of higher alcohols, esters and fatty acids were found to differ significantly between red white and rose wines. Isoamylc acids, higher major alcohols, ethyl hexanoate, acetates, ethyl octanoate and decanoic acid were found to be most significant in the differentiation between these wine styles from the Spanish Denomination of Origin "Vinos de Madrid" (Gil *et al.* 2006). Similar results, where the higher major alcohols were significantly higher in red wines than white and rosé wines, were found in wines from the Canary Islands, another Spanish Denomination of Origin (Díaz *et al.*, 2003). These results are in accordance with the literature on the formation of these compounds by yeasts during the production of the respective wine styles (Lambrechts and Pretorius, 2001).

Canadian ice wines have been successfully distinguished from late harvest wines from the same winery, vintage and variety using principle component analysis. In this case 2-phenyl ethyl acetate, the unsaturated 2-propenol and ethyl-9-decenoate as well as pentadecyl-2-furancarboxylic acid was mostly responsible for the variation in the data model (Setkova *et al.*, 2007).

Different styles of fortified wines and their spectroscopic attributes were investigated by Palma and Barroso (2002). It was found that wavelenghts 3626-5000cm⁻¹ were effective in the classification of armagnacs, cognacs and brandies according to beverage type, and the classification of Spanish, South African and French brandies according to their geographical origin. (Palma and Barroso, 2002)

2.2.4 AGEING

Several wine production countries apply categorical labels to wines that have been subjected to a specified ageing regime. These categories have a major influence on the market value of the wines. Such is the case of the Spanish red wine classes Crianza, Reserva and Gran Reserva³. From an authentication viewpoint it can be very valuable to chemically distinguish between such wine classes.

Wine age classes are largely administrative and a large degree of variance within each wine class can be expected. This was observed when the mass spectrometry data of the abovementioned Spanish wine classes were compared to young Spanish wines in order to distinguish between the age groups (Martí *et al.*, 2004). In many cases the interclass difference as determined with SIMCA was quite small, making it difficult to discriminate between the groups. The PCA scores of these groups showed some sample groupings, but large degree of overlapping occurred between groups. Although the largest percentage of wines was classified correctly, a quarter of the wines could not be classified at all due to the small interclass differences. In a separate study, some distinction between young Spanish wines and Crianza and Reserva wines could be made based on biogenic amine content. Differences between Crianza and Reserva classes were less pronounced (García-Villar *et al.*, 2007).

An alternative to distinguishing between specific wine age classes would be to simply distinguish between aged and non-aged wines. In terms of spectroscopy the ultraviolet and visible light regions seems especially promising for this purpose as it would indirectly capture information on the changes in phenolic composition and colour properties that occur during ageing of red wines. In an effort to discriminate between aged and non-aged wines based on UV-vis spectroscopy it was indeed found that the most significant information was present in the ultraviolet region (Urbano *et al.*, 2006). Unfortunately only 75% of the samples used in that specific study were classified correctly.

Palma and Barroso (2002) achieved a 99.5% correlation between the FTIR spectra and the age of Fino sherry wines by subjecting selected wavelength regions to partial least square regression. The same wavelengths were considered efficient to classify Jerez brandies according to their relative age (Palma and Barosso, 2002).

2.2.5 QUALITY CONTROL AND AUTHENTICATION

Traceability is an important part of quality control procedures. Recently, FTIR have been applied to fingerprint wines from different cellars in order to trace the wines after transportation (Bevin *et al.*, 2006). The wines were scanned with similar instruments at its departure location and at its arrival location. Between instrument noise could be eliminated by excluding the wavenumbers 1543-1717

³ Young Spanish red wines have been aged for less than 12 months in oak barrels. Crianza wines have to be aged in barrels for at least 12 months. Reserva wines have to be matured for at least 36 months of which a minimum of 12 months must occur in barrels. Gran Reserva wines must be aged for more than 60 months: 24 months in barrels followed by 36 months in bottles (Martí *et al.*, 2004)

cm^{-1} and $2971\text{-}3627\text{ cm}^{-1}$. These wavenumbers are generally associated with water absorbance. By comparing the infrared spectra all the wines could be traced back to their original cellars. The only exceptions were the wines undergoing malolactic fermentation in which case high levels of CO_2 gas caused some spectral interference.

The demand for a link between sensory evaluation and objective analytical techniques has also been created in terms of wine quality assessment. During the sensory analysis of wine, a panel of judges has to be trained to assess wines in terms of specific characteristics. Outlier judges, who experience the sensory characteristics of wines differently compared to the other judges, can significantly influence the results from a sensory evaluation. By applying principle component analysis to data from sensory evaluation, outlier judges could be identified (Scaman *et al.*, 2001).

Colour measurements have already been connected to wine quality control and Spanish red wines could be classified according to colour acceptability with a prediction error of 4.6% (Ortiz *et al.*, 1995). The combination of colour measurements and powerful classification techniques such as SIMCA also showed potential to detect blends of red and white wines that have been presented as rosé wines (Meléndez *et al.*, 2001).

The detection of illegal additives in wine is highly relevant to the current situation in the wine industry. The use of analytical techniques and chemometrics to detect adulterated wines has been widely investigated. Studies have shown that wines containing added glycerol, beetroot sugar, ethanol and methanol can easily be identified with chemometric techniques (Dixit *et al.*, 2005; Kosir *et al.*, 2001; Penza and Cassano, 2004).

The rising number of incidents involving adulterated wines has contributed to value of large databases of wine compositional data. The Australian Wine Research Institute was established in 1955 and, by providing analytical services to more than 3000 clients from the Australian wine industry since then, they have managed to compile a large database of wine composition. In a publication by Godden and Gishen, data from 1984 to 2004 have been investigated and several compositional trends could be observed during this period. The authors suggested that, apart from the data's historical and academical significance, the varietal and regional averages could also be used to by industry members as a standard to compare their own wines by. (Godden and Gishen, 2005). The European office for wine, alcoholic and spirit beverages (BEVABS) launched a database project containing isotopic data of wines in 1993 as an answer to the increase in fraudulent winemaking practices in Europe. Each year the deuterium content of more than 1000 wines from all the European wine producing regions are analysed with NMR (nuclear magnetic resonance) and added to the database. In addition several other parameters like origin, year, cultivar, vinification, chemical analysis, earth quality, climatic conditions, etc. After the development of IRMS (isotope ratio mass spectroscopy) techniques, the analysis of isotopes like carbon 13 and oxygen 18 were included in the project. This project allows authorities to compare wines to reliable references in order to identify adulterated samples (Wine inspection and quality, n.d.)

A thorough knowledge of the composition and inherent attributes of wines is vital for wine quality control. The role of advanced analytical and chemometric techniques for the objective assessment of wines is evident from the discussed studies. Based on the results from the research

studies discussed in this review, the chemical characterisation of wine can be successfully applied to uphold industry standards.

2.3 ABBREVIATIONS USED

ANN: Artificial neural networks

Al: Aluminium

B: Boron

Ba: Barium

Cs: Caesium

FTIR: Fourier transform infrared

GC-FID: Gas chromatography coupled to a flame ionisation detector

GC-MS: Gas chromatography coupled to mass spectrometry

GC-O: Gas chromatography olfactometry

HS-SPME-GC-TOF-MS: Head space solid phase microextraction gas chromatography time of flight mass spectrometry

ICP: Inductively coupled plasma spectrometry methods

ICP-OES: Inductively coupled plasma optical emission spectrometry

IRMS: Isotope ratio mass spectrometry

KNN: K-nearest neighbour

LDA: Linear discriminant analysis

Li: Lithium

MIR: Mid-infrared electromagnetic region

Mn: Manganese

NIR: Near infrared electromagnetic region

Ni: Nickel

NMR: Nuclear magnetic resonance

PCA: Principal component analysis

PLS-D: Partial least square regression – discriminant analysis

Rb: Rubidium

Sc: Scandium

Se: Selenium

SIMCA: Soft independent modelling of class analogies

SPE: Solid phase extraction

Sr: Strontium

SVM: Support vector machines

Tl: Thallium

UV: Ultraviolet electromagnetic region

UV-vis: Ultraviolet and visible electromagnetic regions

Vis-NIR: Visible and near infrared electromagnetic regions

W: Tungsten

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Chapter 3

**Introductory review of data
analysis techniques relating to
the chemical characterisation of
wine**

LITERATURE REVIEW

3.1 INTRODUCTION

Data generation and data analysis are two inextricable parts of the scientific process. The past fifty years have seen great advances in analytical chemistry. With the sophistication of analytical techniques such as chromatography and spectroscopy, and its application in the wine laboratory, a myriad of chemical data has become accessible. However, due the inextricability of data generation and data analysis, the wide variety of available data necessitates an extensive range of data analytical techniques (Kaufmann, 1997).

As is the case in any applied science, the choice of data analytical techniques is dependent on the type of problem at hand. One of the first issues to address during the compositional characterisation of wine is to determine the accuracy of the analytical method and therefore the reliability of the data. When this has been established, the characterisation of wine can be approached in a stepwise manner. Firstly, the general structure of the data can be described in terms of the number of samples and variables, the value range of each variable, the distribution of the value ranges and so forth (Gil *et al.*, 2006). A further step would be to identify trends and patterns in the data set. One could attempt to find significant differences between groups of samples, i.e. cultivars or vintages, based on a specific characteristic such as ethanol content or pH (Gil *et al.*, 2006). Furthermore it could be established whether or not it is possible to distinguish between groups of samples based on their chemical qualities (Liu *et al.*, 2007). This could lead to the classification of samples into specified groups or classes (Liu *et al.*, 2007).

Several statistical techniques are available to investigate these issues. Univariate statistics deal with one variable at a time. It can provide very useful information on the properties of the data set and relationships between samples in terms of single variables. However, in most cases there are also interesting interactions between variables which can be best explained with multivariate data analysis (Kaufmann, 1997). The univariate and multivariate statistics that are discussed in this chapter have been described in standard statistical textbooks and software packages (Esbensen, 2002; Otto, 1999; Statsoft. Inc., 2003).

3.2 UNIVARIATE STATISTICS

3.2.1 ERROR MEASUREMENTS

The precision of an analytical method can be evaluated based on several parameters. Standard error of laboratory (SEL) is commonly used in literature to determine the measuring error of the analytical method based on two measurements of the same sample (Nieuwoudt *et al.*, 2004; Urbano-Cuadrado *et al.*, 2004). An alternative to SEL is the standard deviation of difference (SDD) (Esbensen, 2002). While SEL (Eq. 1) is based on the difference between the two measurements in

terms of the size of the sample set, SDD (Eq. 2) is based on the difference between two measurements of a sample in terms of the average difference between measurements.

$$SEL = \sqrt{\frac{\sum (y_1 - y_2)^2}{2n}} \quad (1)$$

Where y_1 and y_2 are duplicate measurements of a sample and n is the number of samples (Fern, 1996).

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

Where d_i is the difference between duplicate measurements of a sample, d_m is the average difference between duplicate measurements and n is the number of samples.

3.2.2 DESCRIPTIVE STATISTICS

3.2.2.1 Data distribution

Any given data range have a minimum and a maximum value, and the way the individual data points are spread in between is called the data distribution. Most statistical tests are based on the assumption that data is distributed in a specific way. Therefore, the distribution of the dataset must be determined to ensure that the statistical tests that are used are valid. The easiest way to determine the distribution of measurement values in a dataset is to plot a histogram, where value intervals are plotted against the number of observations in each interval (Figure 1). Techniques that rely on the assumption that the data follow a normal distribution, like the example in Figure 1a, are called parametric tests. However, in complex matrices such as wine, data sets often do not follow a normal distribution (Figure 1b). In such a case, parametric tests can be substituted by non-parametric tests that are not influenced by the distribution of the data set. Normal-probability plots are another way of determining whether a data set follows a normal distribution. Data that follows a normal distribution will be represented in a straight line on a normal probability plot. The normal-probability plot in Figure 3a shows data that follows a normal distribution with a slight deviation at the lower end of the data range. In Figure 3b, the data do not follow a normal distribution at all. Statistical tests, such as the Shapiro-Wilk test, can be used to support observations made from histograms or normal-probability plots.

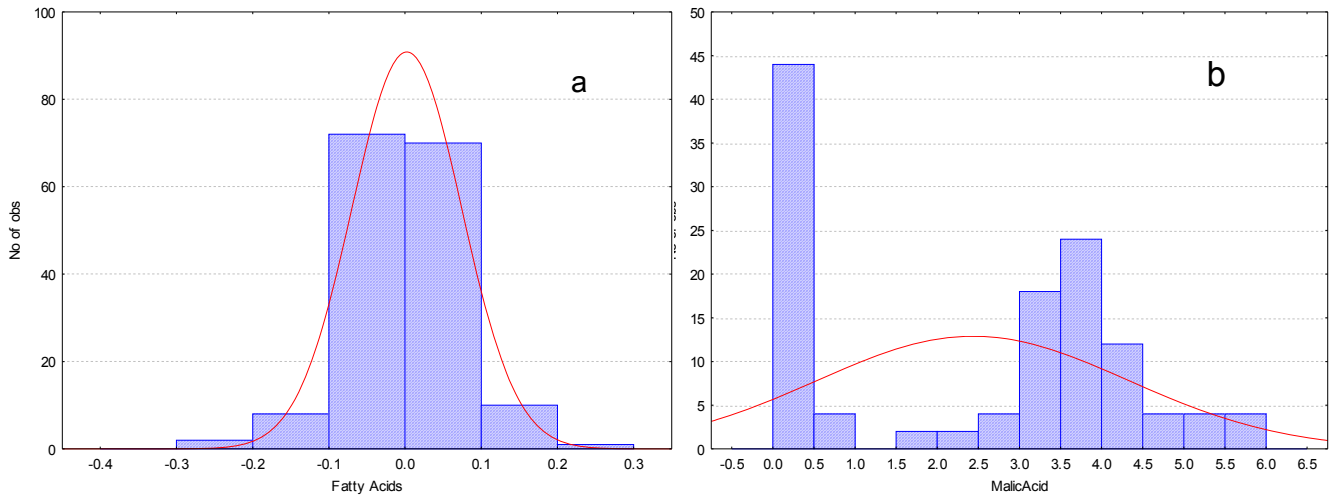


Figure 1. Example of a histogram where the data follow a normal distribution (a) and where the data do not follow a normal distribution (b) (Own data).

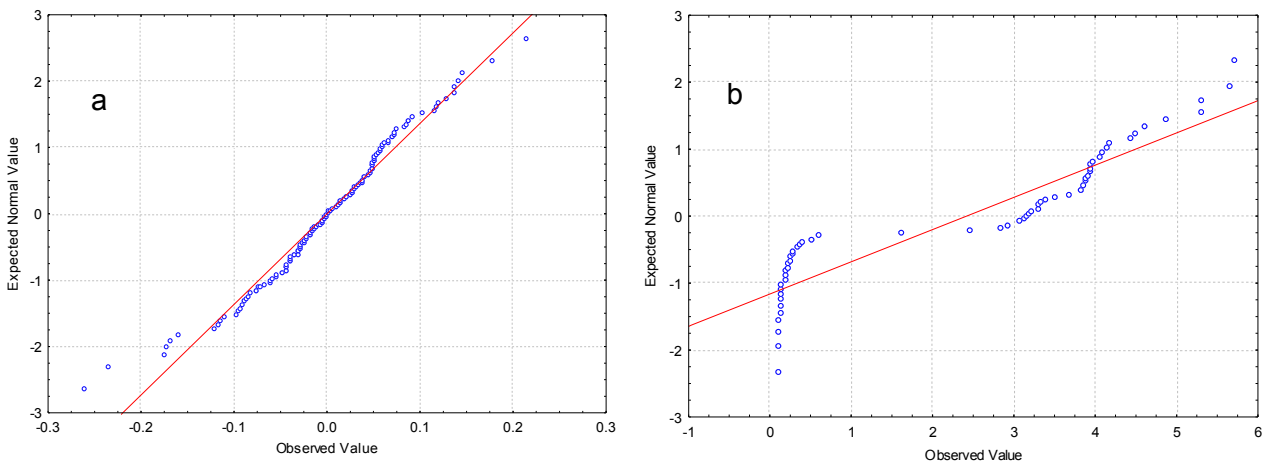


Figure 2. Example of a normal-probability plot where the data follow a normal distribution with a slight deviation at the lower end of the data range (a) and where the data do not follow a normal distribution at all (b)(Own data).

3.2.2.2 Location parameters

Datasets can be quantitatively described by certain location parameters. These parameters indicate the position of majorities of samples in the data set and can easily be graphically displayed by means of a box plot (Fig. 3). Box plots also can be used for data sets that do not follow a normal distribution as they are calculated by rank order statistics, in other words the values in the data set are ranked from lowest to highest. Box plots consist of several components, namely the median or middle quartile (Q_2), the lower quartile (Q_1), the upper quartile (Q_3) and the whiskers. The median is a very robust measurement and represents the value where 50% of ranked samples are smaller than the median and 50% are larger than the median. The median is generally calculated as the value of the ranked sample in position $(n+1)/2$. The lower quartile represents the point where 25% of the data sets have lower values and the upper quartile where 75% of the dataset has lower values. The whiskers normally add 1.5 times the interquartile range ($Q_3 - Q_1$) to the top and the

bottom of the box respectively and indicate the non-outlier value range of the data set. The upper and lower whiskers are calculated as follows:

$$\text{Upper whisker} = Q_3 + 1.5 (Q_3 - Q_1) \quad (5)$$

$$\text{Lower whisker} = Q_1 - 1.5 (Q_3 - Q_1) \quad (6)$$

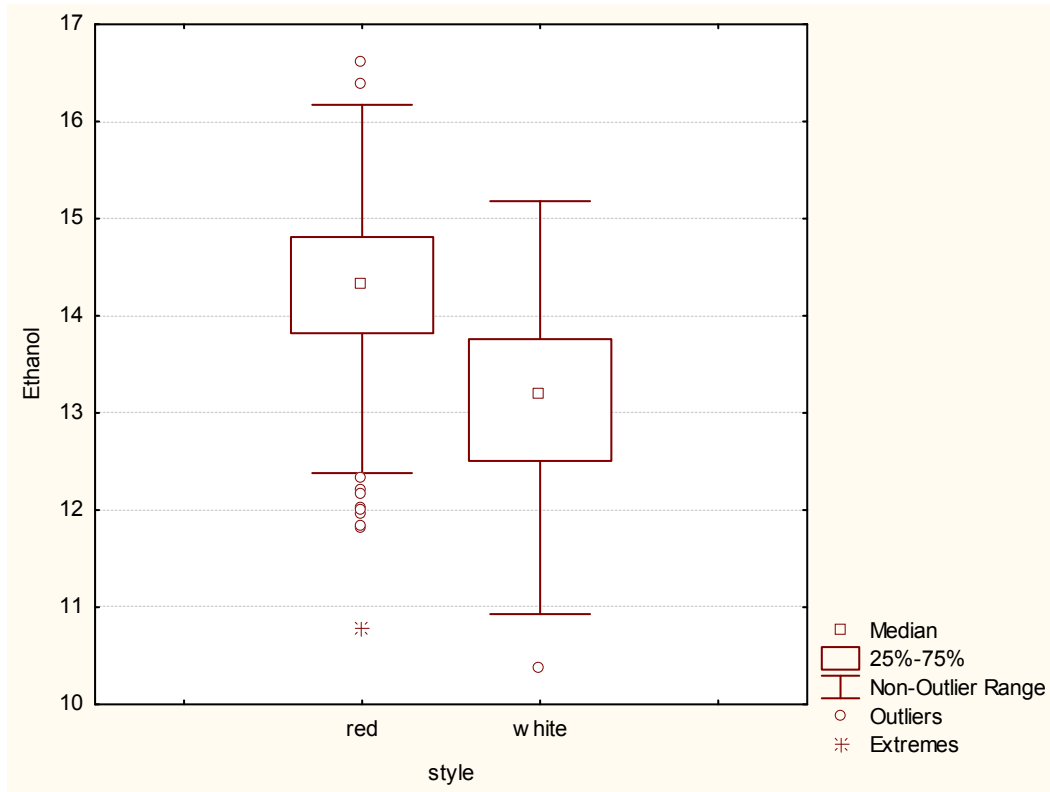


Figure 3. An example of box plots. In this case the ethanol ranges in red and white wines are compared. Some outliers can be observed (Own data).

3.2.2.3 Variation

The two most common descriptive statistics used to describe variation in a data range is the standard deviation and the coefficient of variation (CV). The standard deviation describes the variance in the data set in terms of the typical value with which a given sample will deviate from the sample average (Eq. 3). CV is the ratio of the standard deviation (s) and the sample average and is expressed as a percentage (Eq. 4).

$$s = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n-1}} \quad (3)$$

Where s is the standard deviation, x_i is sample i , \bar{x} is the sample mean and n is the number of samples.

$$CV (\%) = \frac{s}{\bar{x}} \times 100 \quad (4)$$

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

The coefficient of variance can also be used to express the variation between replicate measurements of the same sample, thereby giving an indication of the precision of the measurements.

3.2.3 ANOVA

An important issue during the chemical characterisation of food and beverage products are whether or not a specific group of samples is significantly different from another in terms of certain characteristics. In the event of a dataset following a normal distribution, analysis of variance (ANOVA) is used to determine significant differences. The total variance in a dataset is defined as the sum of squares of the deviation of each sample from the grand mean. In ANOVA, the total variance (SS^2_{total}) is partitioned into variance between groups (SS^2_{groups}) and variance within groups or the residual variance (SS^2_R) (Eq. 7-11).

$$SS^2_{total} = SS^2_{groups} + SS^2_R \quad (7)$$

$$\text{With } SS^2_{total} = \sum_{j=1}^q \sum_{i=1}^{n_j} (y_{ij} - \bar{y}_{total})^2 \quad (8)$$

$$SS^2_{groups} = \sum_{j=1}^q n_j (\bar{y}_j - \bar{y}_{total})^2 \text{ and } \bar{y}_j = \frac{1}{n_j} \sum_{i=1}^{n_j} y_{ij} \quad (9)$$

$$SS^2_R = \sum_{j=1}^q \sum_{i=1}^{n_j} (y_{ij} - \bar{y}_j)^2 \quad (10)$$

$$\bar{y}_{total} = \frac{1}{n} \sum_{j=1}^q \sum_{i=1}^{n_j} y_{ij} \quad (11)$$

Where q is the number of groups, n_j the number of replicate determinations per group j , and n is the total number of measurements.

To determine whether there is a significant difference between groups, an F-test is performed:

$$F = \frac{\frac{SS_{fact}^2}{(q-1)}}{\frac{SS_R^2}{(n-q)}} \quad (12)$$

The groups are significantly different if the calculated F value is higher than the predetermined critical F value (F_{crit}). The F test is usually accompanied by a probability test, where the p value indicates the probability the F test is false. Probabilities are normally determined at a 95% confidence interval. In other words, if the probability that the F test is false is more than 5%, the results of the F test are rejected.

Post-hoc tests can be performed after ANOVA analysis to determine the extent of the differences between groups. Several post-hoc tests are available and each is calculated based on a different set of assumptions. If the assumptions are very strict or conservative, it is likely that differences between specific groups will not be regarded as significant. On the other hand, if a post-hoc test is too lenient, significant differences may be falsely indicated. Two examples are the Bonferonni test and the Tukey test. The Bonferonni test is very conservative, especially if there are many groups included in the analysis, while the Tukey test is more lenient. Therefore, if there are only two or three groups that are compared, a Bonferonni test will give reliable results but if there are a large number of groups, the less conservative Tukey test will be more realistic. There are many different tests available, and the choice between post-hoc tests should be guided by the type of problem.

ANOVA tests can also be performed using more than one set of groups. In the case of factorial ANOVA tests, the between group variance is analysed for each set of groups individually. This is called the main effects. Additionally, the interaction between the main effects is also investigated. In the event where the interaction between main effects is significant, it means that the differences between groups based on one main effect are dependent on the value of the other effect. In the example in Table 1, the p values, marked in red, indicate that there are significant differences between the samples in the various cultivar groups and between the samples from the various region groups. It also indicates that there is a significant interaction between the cultivar and region categorical predictors, in other words, the differences between cultivars in one region is not the same as in another region and *vice versa*.

Table 1. Example of a factorial ANOVA (Own data).

	SS ^a	dF ^b	MS ^c	F	p
Intercept	718332.7	1	718332.7	2629.213	0.000000
Cultivar	226781.2	4	56695.3	207.514	0.000000
Region	11158.8	3	3719.6	13.614	0.000000
Cultivar*Region	6750.7	12	562.6	2.059	0.018647
Error	110377.7	404	273.2		

^aSum of squares; ^bDegrees of freedom; ^cMean sum of squares

The use of ANOVA as a statistical tool to chemically characterise wine have been widely reported (Ferreira *et al.*, 2000; Gil *et al.*, 2006)

In the event where the data do not follow a normal distribution, ANOVA can be substituted with non-parametric tests such as the Kruskal-Wallis test and the median test. Non-parametric tests transform numerical data into rank order data. The advantage of this is that these tests do not rely on conservative assumptions regarding the distribution of the data. However, the disadvantage is that some possibly relevant information may be lost during the transformation from numerical to rank order data.

3.3. MULTIVARIATE DATA ANALYSIS

In more complex data sets containing multiple variables, it becomes difficult to visually represent patterns and trends. A very useful way of showing trends based on multiple variables is graphical representations like radar plots. For these types of graphs the values of the variables must be standardised. The use of radar plots (Figure 4) is an efficient method to represent profiles of a specific object in terms of certain variables. Radar plots are frequently used in wine characterisation studies to present compositional profiles of different wine types (Ferreira *et al.*, 2000; Kim *et al.*, 1996)

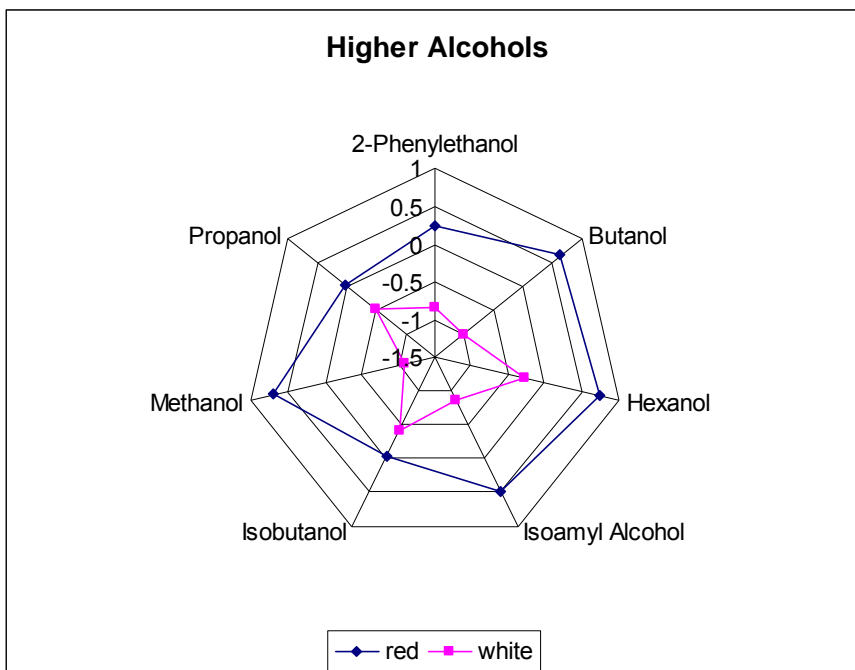


Figure 4. An example of a radar plot, indicating different profiles for the average higher alcohol content of red and white wines (Own data).

In wine characterisation, one would expect a certain wine characteristic to be dependant on several factors. Moreover, it is very likely that the factors are influenced by each other factors. In fact, interaction between factors was already evident from the factorial ANOVA example. The complexity of the variation in such data sets is best explained with multivariate data analysis. Multivariate data analysis can be roughly divided into two groups (Lavine, 2006). Unsupervised classification techniques are used to identify structures in the data set, such as distinct groups of

samples and the variables that influences the sample groupings as well as groups of highly correlated variables. Supervised classification techniques are used to classify samples into a known number of classes based on the composition of the samples. Classification models can be established to classify unknown wines with techniques such as linear discriminant analysis (LDA) or soft independent modelling of class analogies (SIMCA).

3.3.1 UNSUPERVISED CLASSIFICATION

3.3.1.1 Principal component analysis

The use of principal component analysis (PCA) in the chemical characterisation of wine have been widely reported (Garcia-Villar *et al.*, 2007; Liu *et al.*, 2007). The purpose of PCA is to compress the dataset without compromising the information within it. This is done by transforming the variables in the dataset to a reduced number of new variables called principal components (PC's). Principal components are linear functions of the original variables and therefore contain all the information that was present in the original variables.

The first step in PCA is to plot the data, consisting of n samples and p variables into a p -dimensional plot. There will be n number of points in the plot. The first PC, or PC 0, is in fact the $n+1^{th}$ sample and is characterised by the mean value for each variable. There will now be $n+1$ points on the plot. If the variables in the dataset are in some way correlated to each other, the points in the data space will appear to have a linear trend to some degree. This trend is defined as the direction of the largest variance in the dataset. PC 1 is a straight line through PC 0 in the direction of this largest variance. If a point in the data matrix is projected onto the plane of PC 1, the distance between the projected point and PC 1 is called the score (t). The distance between the original point and the projected point is called the residual. The residual is also equal to the perpendicular distance between each point in the data space and a given PC. The exact location of PC 1 in the data space is where the sum of all the squared residuals is the smallest. The subsequent PC's are calculated in a similar way. PC 2 is defined as the PC orthogonal to PC 1 and in the direction of the second largest variance in the dataset. The number of PC's needed to explain the variation in the dataset depends on the level of correlation between the original variables.

The scores, as described earlier, is a projection of the samples in the data matrix onto a PC. If the dataset is transposed (in other words, the variables are seen as the "samples" so that the data matrix contains p "samples" and n "variables") the variables can now be projected on the PC plane. These projected variables are called loadings (p^l). Therefore, PCA decomposes the original data matrix (X) into a score matrix (T) a loadings matrix (P^T) for each PC.

The score and loading matrices for two PC's can be plotted in comprehensible two-dimensional plots called scores plots and loadings plots. The position of the scores and loadings on these plots gives an indication of the structure in the data. Samples that share similar properties will have scores close to one another on the score plot. Loadings that are grouped together indicate that the corresponding variables are closely related. Figure 6 shows an example of a

scores plot. In the scores plot, two groups of scores can be seen. The red group on the left hand side belongs to white wines and the blue group on the right hand side belongs to red wines. One outlier red wine can be seen in the top right hand corner. It is clear that the red and white wines forms two groups based on variation in PC 1. Some interesting correlations can also be observed between the variables on the PC 1 axis on the loading plot (Figure 7). Malic acid can be observed on the negative end of the PC 1 axis. It is said that “malic acid has a negative high loading on PC 1”. Lactic acid and pH can be observed on the positive end of PC 1 and therefore “lactic acid and pH have high positive loadings on PC 1”. The high loadings of these three variables indicate that they contribute to the variation between the red and white wines on PC 1 that was seen on the scores plot. In fact, the malic and lactic acid concentration of white and red wines are very different due to the process of malolactic fermentation that is commonly applied to red wines but not to white wines. The relation between the positions of pH, lactic acid and malic acid on the loadings plot indicated that pH and lactic acid are positively correlated to each other and negatively correlated to malic acid. These correlations make sense in the context of the separation between red and white wines based on malolactic fermentation. Malolactic fermentation is a de-acidification process and therefore a high pH and high lactic acid concentration are both products of malolactic fermentation while malic acid is the substrate.

On the loading plot volatile acidity has “a high positive loading on the PC 2 axis”. Interestingly, the outlier on the scores plot has a corresponding high positive score on PC 2. Upon closer investigation, the outlier red wine sample has a lot higher volatile acidity compared to the other red wines

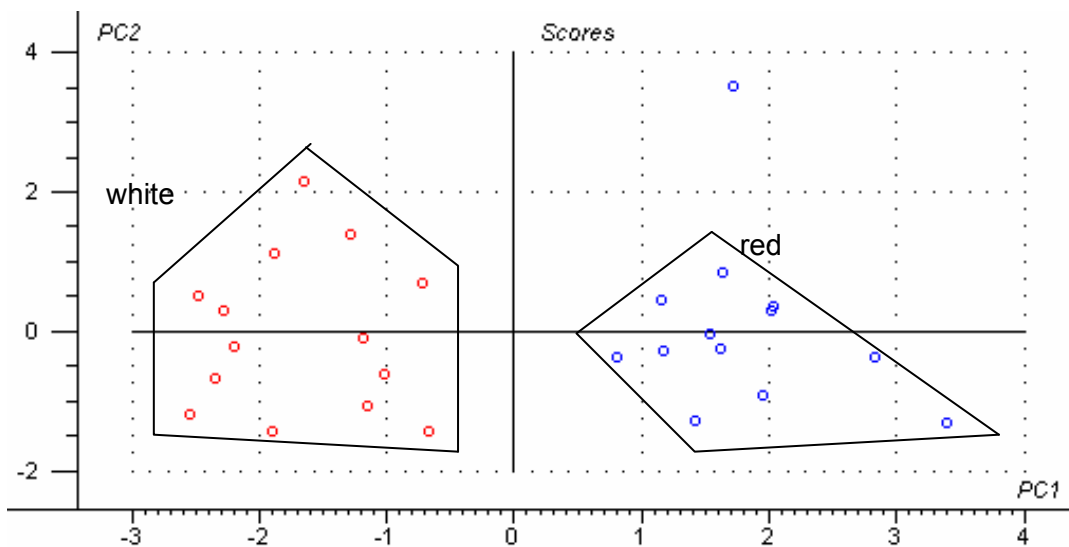


Figure 6. Example of a PCA scores plot. Each marker represents the PCA scores of a specific wine on PC 1 and PC 2. White and red wine samples form two groups on the left and right hand side of the plot respectively. An outlier red wine sample can be observed in the top right hand corner (Own data).

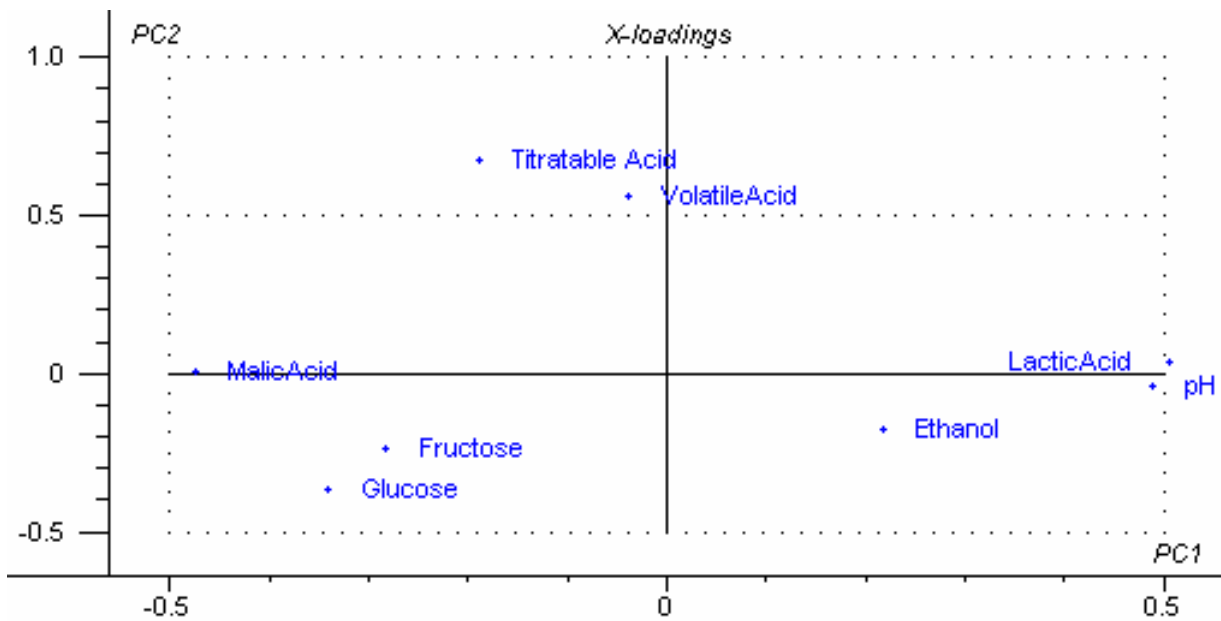


Figure 7. An example of a PCA loadings plot (right). Each marker represents the PCA loadings of a specific variable on PC 1 and PC 2. Lactic acid and pH have high positive loadings on PC1 and are negatively correlated to malic acid that has high negative loadings on PC 1. Volatile acidity has a high positive loading on PC 2 (Own data).

3.3.1.2 Partial least square regression

A method that is very similar to PCA is partial least square regression (PLS). This method finds a correlation between one set of variables called *x*-variables and another set of variables called *y*-variables. The *x*-variables can be instrumental measurements like spectra or chemical data whereas the *y*-variables can also be chemical measurements or it can be sensory observations or varietal classes for example. Depending on the degree of correlation between the *x*-variables and *y*-variables, PLS-regression models are used to predict the values of the *y*-variables from the values of the *x*-variables of unknown samples. The PLS algorithm decomposes the *X*-matrix in the same way as PCA, but based on information from the *Y*-matrix. This is done according to the NIPALS (nonlinear iterative partial least squares) algorithm as explained in the book: *Multivariate data analysis in practice* (Esbensen, 2002).

PLS regression models need to be validated to test their efficiency and some examples of validation methods include test set validation and cross-validation. The most reliable validation method is test set validation where an independent sample set is fitted onto the calibration. In the case of cross validation, the sample set used to build the PLS model is also used to validate it. The calibration sample set is randomly divided into segments after which the set is fitted onto the algorithm leaving out one segment at a time until each segment has been left out once. Since cross validation is based on the original calibration sample set, the results can sometimes be too optimistic.

The performance of the PLS models can be evaluated in terms of bias, coefficient of determination (R^2) and the relationship between the precision of the reference method and the calibration. The bias (Eq. 13) 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.

$$\text{Bias} = \frac{\sum_{i=1}^n (\hat{y}_i - y_i)}{n} \quad (13)$$

The precision of a calibration with R^2 values higher than 0.9 can be considered excellent, between 0.9 and 0.7 is good enough for quantification and between 0.7 and 0.5 is suitable for screening between low, medium and high values but not for quantification.

The standard error of cross validation (SECV) is an indication of the prediction error of a calibration model as determined with cross-validation. It is suggested that SECV values lower than $1.5 \times \text{SEL}$ indicate excellent precision, while values between $2 \times \text{SEL}$ and $3 \times \text{SEL}$ indicate good precision. The standard error of prediction (SEP) represents the prediction performance as determined with test set validation. SEP and SECV are calculated as follows:

$$\text{SEP or SECV} = \sqrt{\frac{\sum_{i=1}^n (\hat{y}_i - y_i - \text{bias})^2}{n - 2}} \quad (14)$$

The ratio of the standard deviation in the dataset to the SEP of the dataset is referred to as the residual prediction deviation (Williams, 1995). The guidelines for the interpretation of this parameter state that calibrations with RPD values exceeding 5 are suitable for quantification while calibrations with RPD values between 3 and 5 are suitable for screening purposes. The major drawback of the RPD criterion is that standard deviation, which forms part of the calculation, is influenced by the concentration range of the sample set. The standard deviation provides information on the variance within the sample set as opposed to the variance between measurements. An alternative criterion is the relationship between SEP and SDD where the SEP should be smaller than $2 \times \text{SDD}$ (Esbensen, 2002). A summary of the abovementioned performance criteria is given in Table 2.

Table 2. Summary of performance criteria for the precision of infrared calibrations

Performance ^a parameter	Fit for quantification	Fit for quantification	Fit for screening	Unsuitable
R^2 ^b	>0.9	0.7 - 0.9	0.5 - 0.7	> 0.5
SECV:SEL ^b	<1.5	2 - 3	n/a	n/a
SEP:SDD ^c	<2	<2	n/a	n/a
RPD ^d	>5	>5	3-5	<3

^a Abbreviations: R^2 = Coefficient of determination; SECV = Standard error of cross validation; SEL = Standard error of laboratory; SEP = Standard error of prediction; SDD = Standard deviation of difference; RPD = Residual prediction deviation

^b (Shenk and Westerhaus)

^c (Esbensen, 2002)

^d (Williams, 1995)

In terms of wine characterisation, PLS regression have been used to determine the correlation between the chemical composition of wine and wine classes, such as cultivar or vintage (Garcia-Villar *et al.*, 2007; Palma and Barosso, 2002)). Alternatively, PLS have been used to quantify chemical compounds from spectroscopic data, which was consequently used to investigate the chemical properties of wine (Minaar and Booyse, 2004).

3.3.1.3 Cluster analysis

Cluster analysis is an unsupervised classification technique that is used to re-organise a data set to reveal structural information within it. Hierarchical cluster analysis is the most popular of these types of algorithms. The basic principle of this, and other, clustering methods is the assumption that the closer two points in a multi-dimensional space are to each other, the more similar they are. The distance between each two points in the data matrix is calculated and represented as a distance matrix. The two closest points in the distance matrix are then combined to form a new point. Using this new point, a new distance matrix is calculated. The two closest points in this new matrix are again combined to form a new point and the process is repeated until each point has been linked. The results are presented in the form of a dendogram that shows the relationship between the samples in the dataset. Cluster analysis is especially useful if the similarities between samples are slightly unclear and have been used to discriminate between the phenolic extracts of different cultivar wines (Edelmann *et al.*, 2001) .

3.3.2 SUPERVISED CLASSIFICATION

3.3.2.1 Linear discriminant analysis

Linear discriminant analysis (LDA), like PCA and PLS, compresses a given data set into a smaller, more meaningful, data matrix consisting of discriminant functions. The discriminant functions are a linear function of the original x-variables and eigenvectors, which will not be discussed at present. A score for each object in the data set is calculated for each discriminant function. The scores of two discriminant functions can be plotted against each other in order to observe groupings of objects. For each group a centroid, an object defined by the mean value of all the x-variables for an object group, is plotted. The distance between a group centroid and any given object on the discriminant function plot is called the Mahalanobis distance. The Mahalanobis distances between each sample and each group centroid are calculated. A sample is classified into the group with the nearest centroid. The performance of a LDA model is evaluated by the classification of another, independent, known sample set. The results are often given in the form of a table indicating the number of correctly classified samples per group (Table 3).

Table 3. Example of LDA results, indicating the percentage correct classification per class, the number of samples in each class and the classes they were assigned to.

	Percent	Chardonnay	Cabernet	Pinotage	Sauvignon blanc	Shiraz	Merlot
Chardonnay	59	17	0	0	12	0	0
Cabernet	63	0	22	0	0	11	2
Pinotage	92	0	0	23	0	2	0
Sauvignon blanc	94	2	0	0	31	0	0
Shiraz	70	0	3	2	0	21	4
Merlot	88	0	1	1	0	1	21
Total	77	19	26	26	43	35	27

Palma and Barosso (2002) have used LDA to discriminate between brandies and sherries of different ages. Discriminant analysis can also be applied to identify variables that contribute the most to the distinction between classes. With step-wise discriminant analysis (SDA), a selection of variables is kept out of calculation in a step-wise fashion until all possible combinations of variables have been kept out. The most important variables will be the selection that is able to classify the samples the most accurately. This technique to identify important discriminant variables are often used during the chemical characterisation of wine (Coetzee *et al.*, 2005). Sometimes SDA is referred to as Bayes SDA. This prefix refers to the Bayes's theorem which is used to determine the optimal classification during SDA if the samples in all the classes obey a multivariate normal distribution. Bayes SDA have been used by Sun *et al.* to discriminate between German wines from different producers (Sun *et al.*, 1997). Discriminant analysis can also be performed in a pair-wise fashion where samples are classified into pairs of classes i.e. Class A and not Class A as opposed to Class A, Class B and Class C. Such a pair-wise discriminant analysis was used by Coetzee *et al.* to discriminate between South African wines from different production regions based on their mineral composition (Coetzee *et al.*, 2005).

3.3.2.2 Soft independent modelling of class analogies

SIMCA (soft independent modelling of class analogies) is based on similarities between class members. This method uses PCA models of samples in known classes to describe a box (model) around each class in the data space and to calculate specific statistical criteria for each box. The SIMCA models for each class are calculated as follows:

$$x_{ij}^q = \bar{x}_j^{-q} + \sum_{a=1}^{A_q} t_{ia}^q l_{ja}^q + e_{ij}^q \quad (15)$$

Where

\bar{x}_j^{-q} = mean of variable j in class q

A_q = number of significant principal components in class q

t_{ia}^q = score of object i on component a in class q

l_{ja}^q = loading of variable j on principal component a in class q

e_{ij}^q = residual error of object i and variable j

The classification of samples is based on the residual variance of a specific sample (s_i^2) and the total residual variance (s_0^2) of each class:

$$s_0^2 = \sum_{i=1}^n \sum_{j=1}^p \frac{e_{ij}^2}{(n - A_q - 1)(p - A_q)} \quad (16)$$

$$s_i^2 = \sum_{j=1}^p \frac{e_{ij}^2}{p - A_q} \quad (17)$$

Where n is the number of objects and p is the number of variables

If the residual variance of the sample exceeds the total residual variance of the class, it is rejected as a member of the class. One very important difference between SIMCA and LDA is that SIMCA can assign unknown samples to more than one class. SIMCA is commonly used to classify wine samples during the chemical characterisation of wine (Edelmann *et al.*, 2001).

3.3.2.3 K-nearest neighbour

K-nearest neighbour (K-NN) is a supervised classification technique based on the similarities between samples in much the same way as LDA. When samples are plotted in a multidimensional space, as defined by multiple independent variables, the geometrical distance between the samples is called the Euclidean difference. For K-NN analysis, the Euclidean distance between each sample in the data set and all the other samples are calculated. For each sample, the other samples are listed from the nearest to the most distant. The sample is classified into the class to which the majority of the k closest samples belong, where the odd number k was chosen based on specific attributes of the dataset. K-NN was used in some of the first studies on the chemical characterisation of wine (Kwan and Kowalski, 1978)

3.3.2.4 Artificial neural networks and support vector machines

As LDA and K-NN are based on the similarity between samples, artificial neural networks and support vector machines are based on partitioning of the data space. These types of methods divide the data space into a number of divisions. Samples that share similar properties will occur in the same data space division. Ideally these samples would belong to the same known class.

Artificial neural networks are based on the principles of biological neurons. In very simple terms, the method collects input data (x -variables) for each sample, mathematically transforms it through a series of neuron layers to a certain output which is then used to allocate samples to a section or class in the data space (Figure 8). This technique is very powerful, but relies on large training sets to avoid chance classifications. Support vector machines are a very recently developed method based on the partitioning of the data space and always act as a binary classifier. Both artificial neural networks and support vector machines deal well with noisy data. Compared to artificial neural networks, support vector machines are much more simple and

requires a smaller training data set. Artificial neural networks have been successfully used to classify wines from different origins (Sun *et al.*, 1997). The application of support vector machines to the chemical characterisation of wine was investigated by Acevedo *et al.* (2007) and promising results were obtained.

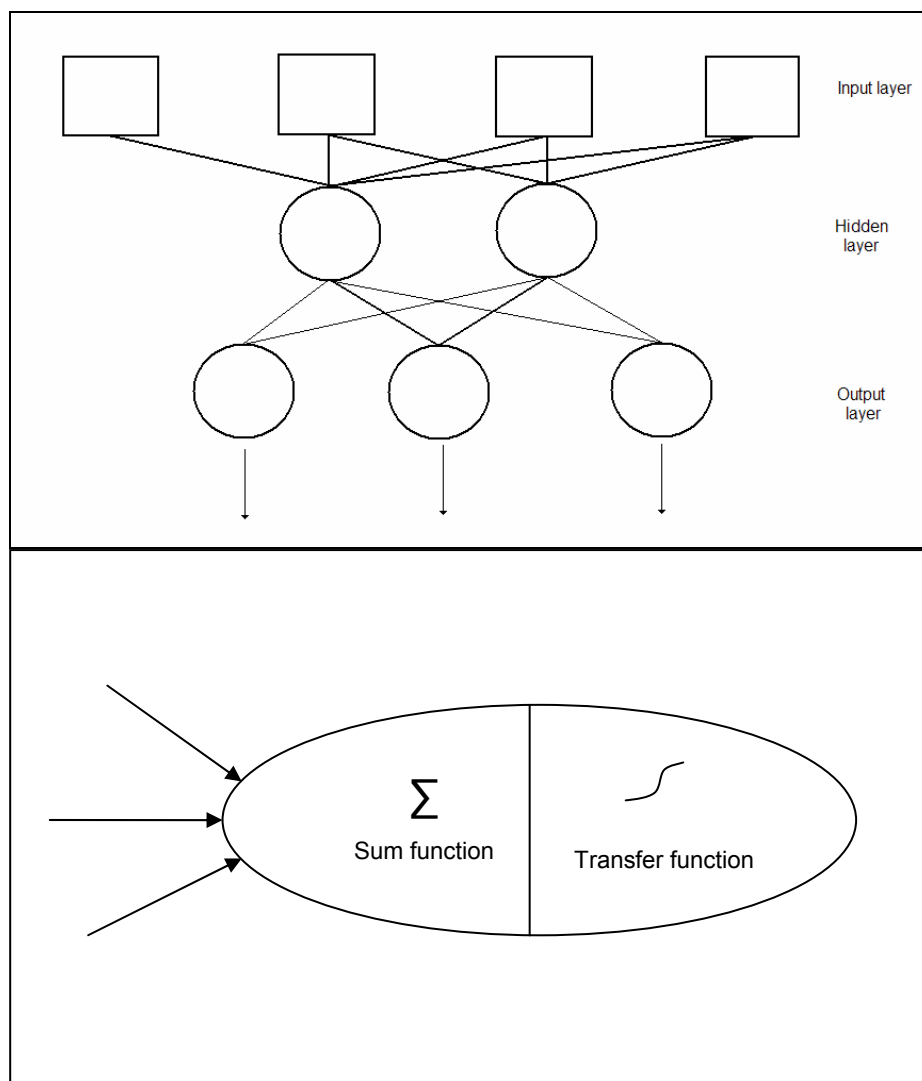


Figure 8. Structure of an artificial neural network (top) and the operation of a single neuron (bottom) (Adapted from: Otto, 1999)

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Chapter 4

RESEARCH RESULTS

Volatile composition of young South African wines: a global comparison

RESEARCH RESULTS

ABSTRACT

Higher alcohols, esters and fatty acids have been identified as the backbone of the volatile composition of wine and have a important influence on wine quality. The objective of this study was to present an overall view of the volatile composition of South African wines, specifically regarding higher alcohols, esters and fatty acids. The first part of the study concerned the validation of a liquid-liquid extraction method to be used for the analysis of 27 volatile compounds in wine with gas chromatography. The method was validated in terms of accuracy, precision and robustness. The method performed well in terms of accuracy and precision and some parts of the protocol were identified where deviations caused a significant influence on the results. The second part of the study involved the statistical investigation of trends and patterns in the volatile composition of South African young wines made from six cultivars in four important production regions over two vintages. Wines were compared in terms of style, cultivar, vintage and geographic origin using descriptive statistics, ANOVA and radar plots. Significant differences were observed between the chemical composition of red and white wines, different vintages, cultivars and production regions. Differences between vintages were cultivar dependant. Important findings were made in terms of the volatile composition of Pinotage wines in relation to the other red and white wine cultivars.

4.1 INTRODUCTION

The continuous growth of the world wine industry, now spread over all six continents, has made it increasingly important to produce high quality wines. Although wine quality is largely influenced by viticultural and winemaking practices, knowledge of the inherent natural composition of wine can give valuable additional insight that could be used to maintain a competitive edge.

The volatile composition of wine is directly linked to aroma, and therefore the quality, of wine. Although the role of compound groups like methoxypyrazines and norisoprenoids in specific varietal characteristics have been identified (Lacey *et al.*, 1991; Sefton *et al.*, 1993) it seems that the backbone of the volatile composition of all wines are based on alcohols, esters and fatty acids. The most abundant alcohols in wine, apart from ethanol, are 1-propanol, isobutanol, isoamyl-alcohol and 2-phenylethanol. Higher alcohols have a pungent smell at high concentrations, but at less than 0.3 g/L they add to the complexity of wine (Lambrechts and Pretorius, 2000). The alcohol, 2-phenylethanol can contribute to the honey, spicy, rose-like aromas in wine (Francis and Newton, 2005). The most important esters present in wine are ethyl esters of saturated carboxylic acids, such as hexanoic acid, and acetate esters of higher alcohols, of which isoamyl acetate is an example. Esters are generally associated with pleasant, fruity, floral aromas (Lambrechts and Pretorius, 2000). Aliphatic saturated fatty acids are the most common fatty acids found in wine and chain lengths of up to 14 carbon atoms have been reported (Schreier, 1979). Acetic, hexanoic,

octanoic and decanoic acids are some of the most important fatty acids in wine. At high concentrations, these compounds are associated with rancid, cheesy and vinegar-like aromas, but are usually present below their detection threshold in healthy wines (Lambrechts and Pretorius, 2000; Schreier, 1979).

Most of these compounds are by-products of alcoholic fermentation although some can be grape derived or formed by microbes other than yeasts (Schreier, 1979). Strong correlations have been found between grape variety and the main groups of by-products from yeast amino acid metabolism, namely isoacids and higher alcohols, ethyl esters of isoacids and acetate esters of higher alcohols (Ferreira *et al.*, 2000). It was suggested that the amino acids profiles of grapes greatly contributes to the aromatic differences between cultivar wines (Ferreira *et al.*, 2000). Several studies support the influence of higher alcohols, esters and fatty acids in varietal differentiation (Camara *et al.*, 2006; Danzer *et al.*, 1999; Falqué *et al.*, 2001; Lopéz *et al.*, 1999). It has been established that the differences between the volatile compositions of wine cultivars are quantitative rather than qualitative (Ferreira *et al.*, 2000). In several studies it was possible to distinguish between wines from different wine producing areas based on their composition of higher alcohols, esters and fatty acids, confirming the underlying importance of these wine constituents (Calleja and Falqué 2005; Marais *et al.*, 1981 a and b).

Gas chromatography coupled to flame ionisation detectors (GC-FID) is a common work horse in volatile analysis. This detector responds well to organic compounds, has a wide linear range and a high level of sensitivity which makes it very suitable for volatile analysis. The major limitation of GC-FID is the need of references to identify substances (Gil *et al.* 2006; Reineccius, 1998).

One of the main problems with quantification of volatile compounds in wine is the wide range of concentration at which these compounds are present. Concentration ranges of volatile compounds include values from ng/L (3-isobutyl-2-methoxypyrazine at levels of 9-42 ng/L) to several mg/L (acetic occurring at levels exceeding 300 mg/L) (Francis and Newton, 2005). It has therefore become essential to extract and concentrate the compounds of interest prior to analysis. Several extraction methods have been used during the sample preparation step of volatile analysis methods. These include headspace sampling methods, solid phase micro extraction, simultaneous distillation/extraction and liquid-liquid extraction methods (Reineccius, 1998). Liquid-liquid extractions are often the preferred method of extraction for analysis of volatile compounds in wine. Depending on the solvent used, it has higher sensitivity for trace components than headspace analysis and is less prone to contamination than head space analysis and distillation processes (Reineccius, 1998).

The sample sets used in quantitative volatile research studies are mostly small and do not provide a general overview of wine composition. Data sets often consist of less than 100 wines, the exceptions containing up to 200 wines, and usually less than 20 wines per cultivar (Ferreira *et al.*, 2000; Gil *et al.*, 2006). There are no recent data available on the basic volatile composition of South African wines as most studies have focused on specific cultivars and the unique compounds that characterizes them (Marais and Swart, 1999; Van Wyk *et al.*, 1979).

The aim of this study is to present a global perspective on the volatile composition of six of the most important South African grape varieties from the four major wine production areas. This

project forms part of the Winetech Aroma Project, which involves the establishment of a database of the volatile composition of South African young wines as determined with a variety of analytical methods. This paper also includes information on the validation of the liquid-liquid extraction method used for the gas chromatographic analysis of the volatile compounds in this study.

4.2 MATERIALS AND METHODS

4.2.1 WINES

A total of 496 single varietal young wines from the 2005 and 2006 South African Young Wine Shows were analyzed. The sample set contained wines from cellars located in four wine producing regions, namely Paarl, Stellenbosch, Robertson and Worcester and were made from either Sauvignon blanc, Chardonnay, Pinotage, Merlot, Cabernet Sauvignon or Shiraz grapes. Table 1 shows a detailed distribution of the sample set.

Table 1. Distribution of samples between cultivar, origin and vintage

Cultivar	2005				2006			
	Paarl	Stellenbosch	Robertson	Worcester	Paarl	Stellenbosch	Robertson	Worcester
Sauvignon blanc	9	13	20	14	13	14	10	10
Chardonnay	5	1	20	18	5	1	10	10
Pinotage	10	7	4	14	9	3	5	10
Shiraz	14	9	12	17	13	4	10	10
Cabernet Sauvignon	16	13	15	13	5	8	9	10
Merlot	10	10	12	17	7	7	10	10

4.2.2 CHEMICALS, STANDARDS AND WINE SIMULANT

4.2.2.1 Chemicals and standards

Ethyl Acetate and isoamyl acetate was purchased from Riedel de Haën (Seelze, Germany). Methanol, hexanol, acetic acid and 2-phenylethanol standards were from Merck (Darmstadt, Germany). Ethyl butyrate, propanol, isobutanol, butanol, hexyl acetate, ethyl lactate, propionic acid, iso-butyric acid butyric acid, iso-valeric acid, diethyl succinate, valeric acid, 2-phenylethyl acetate, 4-methyl-2-pentanol and hexane were from Fluka (Buchs, Switzerland). Hexanoic acid, octanoic acid, isoamyl alcohol, ethyl caprylate, ethyl caprate were from Aldrich (Steinheim, Germany). Decanoic acid and ethyl hexanoate were purchased from Sigma (St. Louis, USA). Diethyl ether, ethanol and NaSO₄ were also purchased from Merck (Darmstadt, Germany).

4.2.2.2 Wine simulant

The internal standard and volatile standards were dissolved in a wine simulant consisting of 12% v/v ethanol and 2.5 g/L tartaric acid (Merck, Darmstadt, Germany) in de-ionised water from a MilliQ

water purifying system (from Millipore, Billerica, MA, USA) with the pH adjusted to 3.5 with 0.1 M NaOH (Merck, Darmstadt, Germany).

4.2.3 EXTRACTION PROCEDURE

Five milliliters of wine with internal standard, 4-methyl-2-pentanol, (100 μ l of 0.5 mg/l solution in wine simulant) were extracted with 1 mL of diethyl ether by sonicating the ether/wine mixture for five minutes. The wine/ether mixture was then centrifuged at 3600 g for 3 minutes. The ether layer was removed and dried on NaSO₄. Each extract was injected into the GC-FID instrument in triplicate.

4.2.4 GAS CHROMATOGRAPHY CONDITIONS

Instrumentation: A J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m Length \times 0.32 mm i. d. \times 0.5 μ m f.t was used. The initial oven temperature was 33°C for 17 minutes after which the temperature was increased by 12°C /min to 240°C, at which it was held for 5 minutes. 3 μ l of the dietyl extract was injected at 200°C. The split ratio was 15:1 and the split flow rate 49.5 ml/min. The column flow rate was 3.3 ml/min and the total run time was 50 minutes. The detector temperature was 250°C. After each sample run, a post run of 5 minutes at oven temperature 240°C, with a column flow of 6 ml/min cleaned the column from high boiling contaminants. After every 30 samples the column was thermally cleaned by injecting hexane several times isothermally, holding it for 10 minutes per injection at an oven temperature of 220°C.

4.2.5 METHOD VALIDATION PROCEDURE

The selectivity of the procedure was tested by injecting a 9% dilution mixture of all the standards, the matrices (red and white wines), and the spiked matrices (red and white wines spiked with a 6.25% dilution of the mixture of standards) in consecutive runs. The concentration ranges for the calibration curves and evaluation of linearity was based on results from Distell Ltd. (South Africa) for the same analysis. The limit of detection and limit of quantitation were determined with a signal to noise ratio of 3 and 10 respectively. Recovery experiments were performed on a red and white wine by injecting an extract of the wine and of the same wine spiked with a mixture of standards for all the compounds analysed. The difference between the concentration of the analytes in the spiked wine and the non-spiked wine were calculated as a percentage of the amount with which it was spiked. Several factors were identified that could possibly be influence the efficiency of the extraction procedure in the event of deviations from the protocol: the amounts of salt, ether and wine, the length of sonication, the pH of the wine, the temperature of the water in the ultrasonic bath, the ethanol concentration of the wine as well as the wine matrix (red or white). The influence of the variation of these parameters was evaluated comparing the concentrations obtained for the different analytes in question. The effect that differences in the matrix for red and white wines can have on analyte concentration was evaluated using the results from the recovery experiment. The

effect of natural variations in wine pH was investigated by adjusting the pH of the same sample to 3, 3.5 and 4 respectively. The effect of variations in ethanol concentration on extraction efficiency were determined by preparing synthetic wine samples containing 16%, 14%, 12% or 10% ethanol. Sample volumes of 4.5, 5.0 and 5.5 ml and ether volumes of 0.75, 1.0 and 1.25 ml were compared. The sonication lengths examined were 4.5, 5.0 and 5.5 minutes and the temperature of the sonication bath were 14°C, 28°C and 41°C. A workable amount of NaSO₄ was chosen as 0.15 g, and different amounts of salt varying between 0.05 and 0.25 g were used to determine whether this influences the concentrations obtained for the analytes. The repeatability of the extraction procedure were determined by injecting seven individual extracts of the same wine and the same extract five times consecutively and five extracts of the same synthetic wines on five different days.

4.2.6 STATISTICS

One way ANOVA and factorial ANOVA were performed with Statistica 7. Box plots were drawn with the same software to determine non-outlier concentration ranges. The data that was used for the radar plots, which were plotted in Excel 2002 (Microsoft Corporation, www.microsoft.com) were standardized in Statistica 7.0 (Statsoft Inc., www.statsoft.com)

4.3 RESULTS AND DISCUSSION

4.3.1 EVALUATION OF EXTRACTION PROCEDURE

Selected highlights of the validation procedure will be discussed in this section. Refer to Addendum A for a full validation report.

4.3.1.1 Selectivity, linearity, limit of detection, limit of quantitation and recovery

Good separation between analytes were observed and peaks were identified by the retention times of authentic standards. The corrected peak areas gave linear responses over the concentration intervals tested. Correlation coefficients, R^2 , were above 0.990 for all analytes (data not shown). The limits of detection and quantitation for each analyte are given in Table 2.

Table 2. Selected results from validation of the liquid-liquid extraction method

Compound	LOD	LOQ	White wine	Red wine
			% Recovery	% Recovery
Ethyl Acetate	0.10	0.35	59.11	47.21
Methanol	10.98	36.59	74.50	54.17
Ethyl Butyrate	0.02	0.06	62.41	65.89
Propanol	0.25	0.82	44.39	35.70
Isobutanol	0.05	0.16	70.55	69.22
Isoamyl Acetate	0.01	0.05	86.38	62.62
Butanol	0.06	0.20	42.68	51.24
Isoamyl Alcohol	0.02	0.06	63.75	50.96
Ethyl Hexanoate	0.02	0.07	68.13	63.98
Hexyl Acetate	0.02	0.07	64.28	67.36
Ethyl Lactate	0.52	1.72	55.49	34.43
Hexanol	0.02	0.05	82.69	75.62
Ethyl Caprylate	0.02	0.06	132.69	74.63
Acetic Acid	1.21	4.04	50.58	42.42
Propionic Acid	0.22	0.73	43.17	31.83
Iso-Butyric Acid	0.06	0.20	62.23	64.17
Ethyl Caprate	0.07	0.23	59.01	84.52
Butyric Acid	0.02	0.07	76.91	83.37
Iso-Valeric Acid	0.03	0.10	92.52	91.95
Diethyl Succinate	0.03	0.09	61.08	60.42
Valeric Acid	0.03	0.10	71.03	72.32
2-Phenylethyl Acetate	0.01	0.04	82.97	88.65
Hexanoic Acid	0.02	0.05	96.58	86.46
2-Phenylethanol	0.06	0.20	63.10	38.25
Octanoic Acid	0.04	0.12	107.46	97.66
Decanoic Acid	0.04	0.12	105.18	107.75

Most recoveries were in the interval 60-110% with the exceptions of ethyl acetate, propanol, butanol, isoamyl alcohol, ethyl lactate, acetic acid and propionic acid. There is also a slight difference in recovery between the white wine matrix and the red wine matrix for methanol, isoamyl acetate, ethyl lactate, ethyl caprylate butyric acid and 2-phenyl ethanol. The recoveries for each analyte in red and white wines are shown in Table 2.

4.3.1.2 Robustness

The influence of the factors described in section 2.1.4 was determined by comparing the percentage standard deviation between analyte concentrations determined with each variation on

the procedure. Percentage standard deviations larger than 10% were considered unacceptable and indicated that variation of that specific factor should be minimized. From the recovery experiment (Table 2) it was seen that the wine matrix influences the extraction process for the analytes, methanol, isoamyl acetate, ethyl lactate, ethyl caprylate butyric acid and 2-phenyl ethanol. All the other analytes are extracted in similar amounts from white and red wine matrices. Unacceptably large percentage standard deviations indicated that the following factors in the protocol should be closely adhered to: amount of diethyl ether, sample volume, length of sonication and temperature of sonication water bath. The amount of NaSO₄ salt, concentration ethanol and pH of the sample did not influence the extraction efficiency significantly (data not shown).

4.3.1.3 Repeatability

The repeatability of the extraction procedure was evaluated in the same way as the robustness. Good repeatability was observed in the consecutive injections of the same extract and the extracts of the same sample. Generally good results were observed for the repeatability over five days, except for methanol concentration which varied more than 20% on day four from the other days (data not shown).

4.3.2 COMPARISON OF WINES

4.3.2.1 Red and white wines

The differences between red and white wines and cultivar wines were investigated with ANOVA. In order to exclude the variance caused by wine colour and cultivar, the differences between wine producing areas were investigated separately within each cultivar group. Box plots were drawn in order to determine the non-outlier concentration ranges for the groups of samples. These concentration ranges of the measured analytes in red and white wines are shown in Table 3.

High standard errors (data not shown) were observed for acetic acid, isoamyl alcohol, methanol and propanol. These compounds have a relatively high polarity. Diethyl ether is relatively non-polar in comparison and therefore the polarity of the compounds would lead to less efficient extraction with ether.

The red wines contained more higher alcohols compared to the white wines. In turn, the white wines contained more esters. Both higher alcohols and esters are produced by yeast during alcoholic fermentation. The production of higher alcohols is favoured by higher fermentation temperatures while esters are formed at higher concentrations at lower fermentation temperatures (Jackson, 1994). In South Africa, red wines are generally fermented at 28°C and white wines at 15°C, thus explaining the differences between these compounds. Diethyl succinate and ethyl lactate, although esters, were found in higher concentrations in red wines than white wines. These esters are linked to lactic acid bacteria activities and are formed during malolactic fermentation, a process that is mainly used during red wine production (Ugliano and Moio, 2005). The red and white wines contained similar amounts of 2-phenylethyl acetate. The white wines contained the most butyric, octanoic and decanoic acids while the red wines had higher concentrations acetic,

hexanoic, isobutyric, iso-valeric, and propionic acids. These results were consistent with previous results in literature (Gil *et al.*, 2006).

Table 3. Concentration ranges in mg/L of analytes in red and white wines

Analyte	Red		White	
	Range	Std Dev	Range	Std Dev
2-Phenylethanol	7.76 - 126.09	± 27.50	5.84 - 17.62	± 5.02
2-Phenylethyl Acetate	nd - 0.54	± 0.18	nd - 0.39	± 0.13
Acetic Acid	234.57 - 845.61	± 135.86	101.52 - 764.73	± 198.46
Butanol	0.85 - 3.15	± 0.64	0.33 - 1.90	± 0.42
Butyric Acid	0.38 - 1.85	± 0.37	0.78 - 3.01	± 0.61
Decanoic Acid	nd - 1.69	± 0.54	0.41 - 2.38	± 0.54
Diethyl Succinate	1.03 - 19.15	± 4.03	nd - 1.50	± 0.60
Ethyl Acetate	20.19 - 119.26	± 20.64	30.22 - 158.29	± 35.54
Ethyl Butyrate	nd - 0.52	± 0.55	0.17 - 0.89	± 0.37
Ethyl Caprate	nd - 0.30	± 0.09	nd - 0.43	± 0.12
Ethyl Caprylate	nd - 0.57	± 0.25	nd - 1.01	± 0.49
Ethyl Hexanoate	nd - 0.88	± 0.39	0.27 - 1.41	± 0.38
Ethyl Lactate	19.64 - 194.70	± 35.82	nd - 29.65	± 15.74
Hexanoic Acid	0.52 - 2.57	± 0.52	3.25 - 7.36	± 1.53
Hexanol	0.18 - 3.19	± 0.72	nd - 2.14	± 0.54
Hexyl Acetate	nd	± 0.09	nd - 0.57	± 0.24
Isoamyl Acetate	nd - 3.34	± 1.17	0.51 - 9.12	± 2.54
Isoamyl Alcohol	119.55 - 543.75	± 88.19	103.69 - 219.23	± 44.16
Isobutanol	2.34 - 97.88	± 24.75	2.26 - 35.29	± 8.30
Isobutyric Acid	0.35 - 3.54	± 0.84	nd - 1.83	± 0.41
Iso valeric Acid	0.37 - 4.58	± 0.99	0.13 - 2.00	± 0.41
Methanol	70.65 - 389.25	± 71.33	nd - 164.15	± 44.34
Octanoic Acid	0.28 - 2.98	± 0.63	1.15 - 10.35	± 1.90
Propanol	2.62 - 114.38	± 34.98	19.20 - 86.80	± 22.35
Propionic Acid	0.76 - 7.23	± 34.35	nd - 43.85	± 13.79
Valeric Acid	nd - 0.56	± 0.24	nd	± 0.09

4.3.2.2 Vintage

The 2005 vintage was overall characterized by much higher levels of valeric acid, propionic acid, methanol, iso-valeric acid, isobutyric acid, butyric acid and butanol than the 2006 vintage. The white wines of this vintage also contained significantly more hexyl acetate, hexanol, ethyl lactate, ethyl butyrate, ethyl acetate and diethyl succinate. The 2005 red wines had statistically higher concentrations of hexanoic acid, ethyl hexanoate, ethyl caprylate, decanoic acid and 2-phenylethyl acetate than those of 2006. The 2006 wines showed significantly higher amounts of octanoic acid and isobutanol and the white wines also contained more isoamyl acetate and ethyl caprate than the previous vintage. The concentration ranges of the compounds that differed significantly between vintages are given in Table 4.

Factorial ANOVAs were performed to determine the influence of vintage, cultivar and origin on the concentration of the volatile compounds, as well as the interaction between vintage and cultivar, vintage and origin and cultivar and origin. The significance of these factors is shown in Table 5.

More than half of the compounds were influenced by the vintage, but interestingly, only five compounds were not influenced by the interaction between vintage and cultivar. This means that the changes that occur over time in the concentration of most of the compounds are not consistent for each cultivar. This could be due to the fact that the different cultivars follow a different ripening schedule and that the effect of climatic conditions, such as the heavy precipitation in October 2004 (Boom, 2005) would be different depending on the stage of ripening. However, only two vintages were examined in this study, and it would therefore be of great value to examine further vintages to see if this phenomenon persists. Only three compounds, 2-phenylethyl acetate, butyric acid and hexanoic acid were significantly influenced by the interaction between vintage and region.

Table 4. Concentration ranges in mg/L of compounds that differ significantly between vintages as calculated with ANOVA's

Compound	All wines			
	2005		2006	
	Range	Std Dev	Range	Std Dev
Butanol	nd - 3.39	± 0.68	0.33 - 3.29	± 0.81
Butyric acid	nd - 3.01	± 0.67	0.38 - 2.60	± 0.50
Isobutyric acid	nd - 3.88	± 0.89	0.13 - 2.43	± 0.56
Iso valeric acid	nd - 4.58	± 1.05	0.13 - 3.87	± 0.98
Propionic acid	nd - 69.41	± 35.63	1.42 - 4.73	± 0.98
Valeric acid	nd - 0.59	± 0.25	nd	nd
Compound	White wines			
	2005		2006	
	Range	Std Dev	Range	Std Dev
Diethyl Succinate	0.28 - 1.50	± 0.63	nd - 0.72	± 0.31
Ethyl acetate	51.53 - 171.29	± 38.49	30.22 - 112.82	± 20.77
Ethyl Butyrate	nd - 1.91	± 0.43	nd - 1.91	± 0.12
Ethyl Caprate	nd - 0.42	± 0.13	0.09 - 0.35	± 0.07
Ethyl Lactate	8.17 - 23.26	± 15.34	nd - 12.24	± 14.31
Hexanol	0.64 - 2.23	± 0.56	0.13 - 1.53	± 0.34
Hexyl Acetate	nd - 0.57	± 0.29	nd - 0.35	± 0.10
Isoamyl Acetate	0.51 - 7.16	± 2.15	1.12 - 11.26	± 2.81
Compound	Red Wines			
	2005		2006	
	Range	Std Dev	Range	Std Dev
2-Phenylethyl acetate	nd - 0.46	± 0.14	nd	± 0.22
Decanoic acid	nd - 1.69	± 0.47	nd	± 0.13
Ethyl Caprylate	nd - 0.75	± 0.31	nd - 0.33	± 0.09
Ethyl Hexanoate	nd - 0.88	± 0.43	nd - 0.37	± 0.12
Hexanoic Acid	0.84 - 2.46	± 0.40	0.52 - 2.71	± 0.64

Table 5. The p-values at a significance level of 5%. Degrees of freedom for each factor indicated by dF. Significant p-values are highlighted.

Compound	Vintage (dF=1)	Cultivar (dF=5)	Origin (dF=3)	Vintage*Cultivar (dF=5)	Origin*Cultivar (dF=15)	Vintage*Origin (dF=3)
2-Phenylethanol	0.6829	0.0000	0.0000	0.1678	0.0018	0.6517
2-Phenylethyl Acetate	0.0000	0.0001	0.3454	0.0000	0.9199	0.0443
Acetic Acid	0.1832	0.0000	0.0179	0.6302	0.2803	0.9745
Butanol	0.0000	0.0000	0.3029	0.0441	0.3672	0.6407
Butyric Acid	0.0000	0.0000	0.3973	0.0000	0.0039	0.0045
Decanoic Acid	0.0000	0.0000	0.5513	0.0000	0.9513	0.1370
Diethyl Succinate	0.5146	0.0000	0.8688	0.0142	0.9165	0.1753
Ethyl Acetate	0.0000	0.0000	0.0684	0.0000	0.0028	0.0784
Ethyl Butyrate	0.4660	0.0000	0.1355	0.0000	0.7026	0.0932
Ethyl Caprate	0.0040	0.0000	0.0974	0.0000	0.8905	0.0728
Ethyl Caprylate	0.0455	0.0000	0.9046	0.0000	0.5863	0.7559
Ethyl Hexanoate	0.0000	0.0000	0.0384	0.0000	0.2236	0.6066
Ethyl Lactate	0.9715	0.0000	0.5874	0.0003	0.1408	0.0924
Hexanoic Acid	0.1711	0.0000	0.4710	0.0052	0.0043	0.0509
Hexanol	0.0000	0.0000	0.0000	0.0001	0.1304	0.5339
Hexyl Acetate	0.1628	0.0000	0.6614	0.0000	0.4216	0.8816
Isoamyl Acetate	0.0000	0.0000	0.1900	0.0000	0.8420	0.9522
Isoamyl Alcohol	0.6106	0.0000	0.0004	0.0968	0.0548	0.2739
Isobutanol	0.0000	0.0000	0.3856	0.0000	0.8041	0.7925
Isobutyric Acid	0.0000	0.0000	0.0000	0.0125	0.2108	0.7744
Iso valeric Acid	0.0000	0.0000	0.0005	0.5828	0.0257	0.5884
Methanol	0.0017	0.0000	0.0511	0.0000	0.3824	0.0838
Octanoic Acid	0.0000	0.0000	0.8301	0.0000	0.6107	0.1171
Propanol	0.7243	0.0000	0.0435	0.0814	0.0235	0.5755
Propionic Acid	0.0000	0.0000	0.4221	0.0000	0.5893	0.5553
Valeric Acid	0.0000	0.0000	0.5535	0.0000	0.9929	0.6687

4.3.2.3 Cultivars

All the compounds were significantly different between cultivars. Pinotage were more comparable to the white cultivars than the red cultivars in the case of several volatile compounds, namely 2-phenylethanol, butyric acid, ethyl acetate, isoamyl acetate, isoamyl alcohol, isobutyric acid and propionic acid (Figure 2). Three of these compounds were subject to a matrix effect between red and white wines, emphasising the similarity between Pinotage and the white wines. In some cases Merlot also stood apart from the other red cultivars, having much lower amounts of 2-phenyl ethyl acetate and much higher amounts of isobutanol, propionic acid and valeric acid. Shiraz differed significantly from the other red cultivars in terms of 2-phenylethanol, isoamyl alcohol and iso-valeric acid. Cabernet Sauvignon were comparable to at least one red cultivar except in terms of ethyl hexanoate, of which it contained significantly lower amounts. The differences between the volatile profiles of the red cultivars are shown in Figure 2 and 3. There were no significant differences between the red cultivars in the case of butanol or hexyl acetate. The differences observed between Pinotage wines and Cabernet Sauvignon wines are in accordance with results from a previous study conducted on South African wines, except in the case of ethyl lactate and 2-

phenylethyl acetate (Marias *et al.*, 1981a). Furthermore, in a previous study, the fusel alcohol acetates, isoacids and fusel alcohols listed above were found to contribute significantly to the differences between grape varieties, confirming the results of this study (Ferreira *et al.*, 2000). These compounds are linked to the amino acid metabolism of yeast cells and, as Ferreira *et al.* (Ferreira *et al.*, 2000) suggested, the differences in the concentration of these yeast metabolites could be due to the unique amino acid profiles of the cultivars. The fact that many of the compounds discussed above have common amino acid precursors supports this statement.

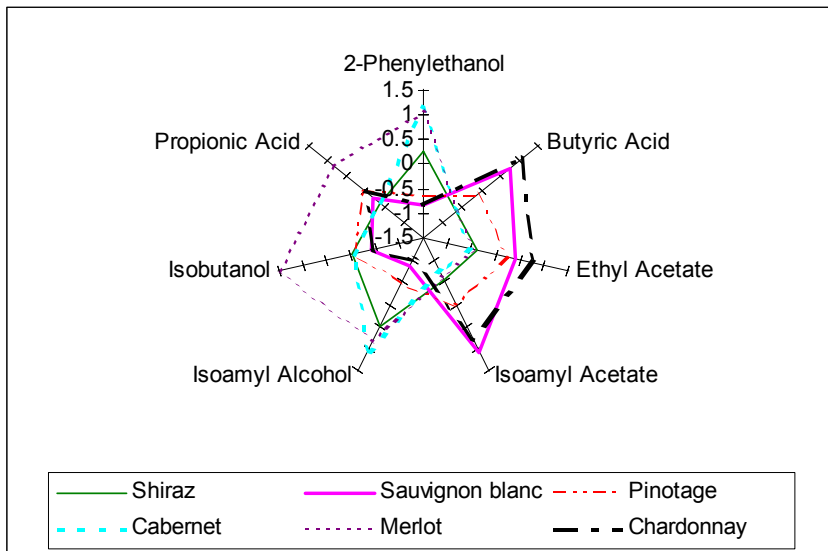


Figure 2. Volatile profiles of the six wine cultivars. The Pinotage profile is more comparable with the white wines than the other red wines.

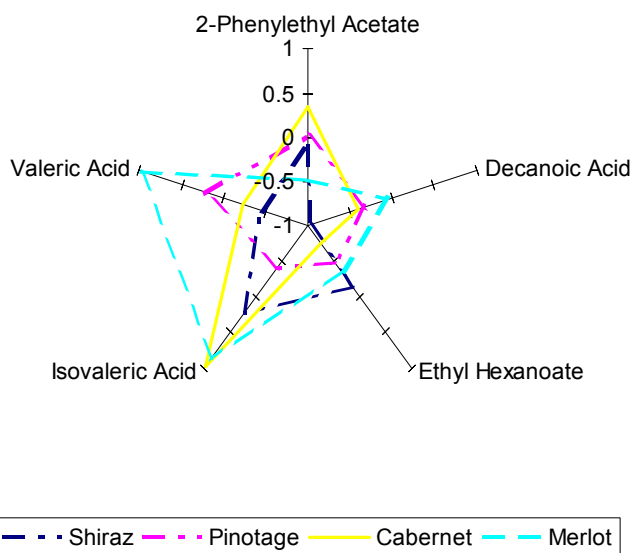


Figure 3. Volatile profile of the four red wine cultivars for some significant compounds

Sauvignon blanc contained significantly more decanoic acid, hexyl acetate and octanoic acid compared to Chardonnay. In turn, Chardonnay contained significantly higher amounts of ethyl hexanoate. The differences between Sauvignon blanc and Chardonnay in terms of these compounds are illustrated in Figure 4. The fatty acids mentioned here are derived from acetyl-CoA,

which is formed from pyruvic acid, an important by-product of alcoholic fermentation. Hexyl acetate is derived from hexanol, which can be a grape constituent or formed from hexanoic acid. Hexanoic acid is also the precursor of ethyl hexanoate. It is clear that the fermentation compounds responsible for the differences between Chardonnay and Sauvignon blanc are metabolically linked. No statistical differences were observed between the white cultivars based on the concentrations of the other volatile compounds.

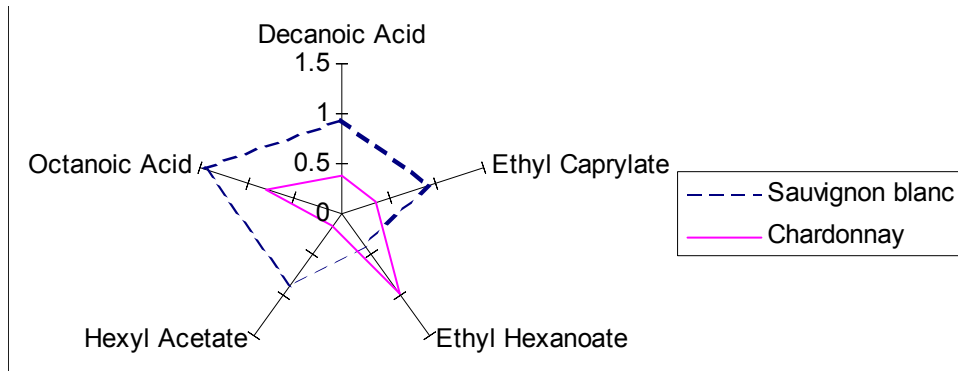


Figure 4. Volatile profiles of Chardonnay and Sauvignon blanc wines for some significant compounds.

4.3.2.4 Geographic origin

Some significant differences between regions could be observed, although the compounds that differed were not the same within each cultivar, as predicted by factorial ANOVA. Within the Shiraz group, the only difference that could be observed was between the hexanol content of the Paarl wines and the Worcester and Robertson wines. Among the other cultivars, the Worcester wines were most different from the other areas based on higher alcohol content, and differed especially from Stellenbosch wines. Fatty acid concentrations were mostly responsible for the differences between Worcester and Robertson wines. Paarl wines differed more from Robertson wines than wines from the other areas. Paarl wines and Stellenbosch wines were the most similar. In fact, the only statistical differences that could be observed were between the Pinotage wines from these regions and then only based on the concentration of ethyl acetate and isobutyric acid. This similarity can possibly be linked to the fact that the two regions are situated next to each other. The Robertson and Worcester wine growing regions are both very large and a range of climatic conditions can be observed within each region. It also needs to be mentioned that the information of the origin of the wines used in this study is based on the geographic location of the cellar. South African wine cellars are allowed to purchase grapes from other wine producing areas and it can therefore not be guaranteed that the grapes used to produce the wines are actually from the same region. The trends that were observed in this study need to be supported in a study where the geographical origins of the wines are guaranteed.

The results of this study gave an overview of the volatile composition of South African young wines. The study showed that there were significant differences between the composition of the 2005 and 2006 vintage wines and that these differences were cultivar dependant. It would be beneficial to confirm these results by analysis of another vintage. There were also significant

differences between the red and white wines which were in accordance to findings in previous studies. The compounds responsible for varietal difference were consistent with literature. An interesting observation was made in terms of the composition of Pinotage wines, where these red wines were more comparable to the white cultivar wines than the other red cultivar wines. There were significant differences between wines from the different regions and it was observed that higher alcohols and fatty acids were the most important compounds in this regards. Wines from Paarl and Stellenbosch very similar and wines from Robertson and Worcester were in many cases different from each other and from the other regions.

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Chapter 5

RESEARCH RESULTS

Chemical characterisation of South African young wines using FTMIR spectroscopy, gas chromatography and multivariate data analysis

RESEARCH RESULTS

ABSTRACT

The powerful combination of analytical chemistry and chemometrics and its application to wine analysis provided a way to gain knowledge and insight into the inherent chemical composition of wine and to objectively distinguish between wines. Extensive research programs are focussed on the chemical characterisation of wine in order to establish industry benchmarks and authentication systems. The aim of this study was to investigate the chemical composition of South African young wines with chemometrics in order to identify compositional trends and to distinguish between different wine classes. Data was generated by gas chromatography and FTMIR spectroscopy and investigated using principal component analysis (PCA), partial least square (PLS) regression and linear discriminant analysis (LDA). Differences between wines were investigated in terms of style, vintage, cultivar, geographic origin and quality. The volatile composition were the most influential in discriminating between wine classes using PCA, although the FTMIR spectra contributed to the discrimination between cultivars using LDA. Distinctions could be made between wine styles, vintages and cultivars but not between geographic origins. There was no correlation between chemical composition and the wine quality indicators used in this study. The characterisation of geographic origin and wine quality could be optimised by using data sets with guaranteed origin and a higher number of very high and very low quality wines.

5.1 INTRODUCTION

During the last two decades the world wine industry grew substantially and has become increasingly competitive. On a global scale it is becoming more and more important to be able to produce distinguishable wines. Traditionally the only way to distinguish wines was by sensorial evaluation, but, due to the subjective nature thereof, chemical analysis were introduced as an more objective alternative to compare wines. In the past, analysts were limited to the number of compounds they were able to analyse, due to costly and time consuming analytical methods, making it hard to identify compounds that are significant in the wine matrix. Recent technological advances have enabled analysts to quantify a multitude of components within a relatively short amount of time, effectively enlarging the possibilities of the chemical characterisation of wine. Parallel to the development of analytical techniques, advances were also made in the field of data analysis. The use of multivariate data analysis or chemometrics has empowered the analyst to gain more insight into complex data sets and to comprehensively represent multi-dimensional variability. Several studies investigating the chemical composition of wine were launched in order to distinguish between wines from different varietal and geographical origin. Multi-element analysis were done to compare different wines of origin as a wine's mineral composition could possibly be related to the soil the grapes were cultivated on (Coetzee *et al.*, 2005; Rebolo *et al.*, 2000).

Phenolic compounds are influenced by the grape variety, ripening conditions, winemaking practices and maturation and their impact on the differentiation between wines have been extensively studied (Makris *et al.*, 2000; Rossouw and Marias, 2004). The volatile composition of wine, alongside phenolic composition, can possibly be linked the strongest to the traditional sensorial analysis, as these compounds are primarily responsible for the distinct flavour of wine. Several types of volatile components have been identified in wine. Of these, higher alcohols, esters and volatile fatty acids are probably the most useful in chemical profiling as they appear to be generic to most wine cultivars. However, the quantitative composition of these compounds are very different between cultivar wines (Ferreira *et al.*, 2000). Most of these volatile compounds are formed by yeast metabolism, but their precursors are often grape derived and could therefore be linked to varietal differences. In addition to the chemical analysis of flavour compounds, electronic nose and tongue detectors have also been coupled to analytical instruments in order to combine sensory and chemical data (Cozzolino *et al.*, 2005; Buratti *et al.*, 2004). A further technological advancement that allowed additional insight into wine composition is spectroscopy. By measuring the wine's absorbance of light at different wavelengths, a global image of the wine can be collected in the form of a spectrum. Wavelengths from the entire light range have been employed in these studies, ranging from UV light, visible light, near infrared and mid infrared as well as fluorescent spectroscopy (Cozzolino *et al.*, 2003; Edelman *et al.*, 2001; Roussel *et al.*, 2006; Urbano *et al.*, 2006). By correlating the absorbance values at a specific wavelength to specific compounds with chemometric algorithms, spectroscopy could successfully be applied towards chemical quantification (Kupina and Shrikhande, 2003).

The knowledge of the chemical composition of wine can be applied in many different ways. The ability to generate large amounts of data in a relatively short period of time with advanced technology allows the compilation of chemical databases. These can serve as a benchmark to which producers can compare their wines. The combination of chemical data and pattern recognition techniques can be applied to the authentication of wine, whether it is to determine wrongful labeling in order to protect denominations of origin or to detect fraudulent vinification activities. If wine composition can be linked to consumer preference or wine quality, compositional information can also play a very important role in market related issues.

Unfortunately, very few attempts have been made to characterize South African wines based on chemical composition. In 1981 Marais described the volatile composition of some SA red and white wines and used the data to distinguish between wines from different production areas (Marias *et al.*, 1981). More recently, Coetzee *et al.* (2005) classified some SA wines according to their geographical origin based on their elemental composition, while Rossouw and Marias investigated the phenolic composition of South African red wine cultivars (Rossouw and Marias, 2004). An unsuccessful attempt have been made to classify South African red wines according to their geographical origin with chemical data quantified with FTIR spectroscopy, but the spectra were not included during the data analysis (Minnaar and Booysse, 2004).

The aim of this study is to firstly contribute to a large scale database of the volatile composition of South African young wines as part of a project launched by Winetech. Secondly the study aims to identify trends and inherent compositional differences between wines of different varieties,

vintage and origin. A third aim is to classify wines into abovementioned groupings by means of chemometric techniques and lastly it will be attempted to predict the quality of the wines based on their chemical or spectral attributes.

5.2 MATERIALS AND METHODS

5.2.1 WINES

A total of 496 single varietal young wines from the 2005 and 2006 South African Young Wine Shows were analyzed. The sample set contained wines from cellars located in four wine producing regions, namely Paarl, Stellenbosch, Robertson and Worcester and were made from either Sauvignon blanc, Chardonnay, Pinotage, Merlot, Cabernet Sauvignon or Shiraz grapes. Table 1 shows a detailed distribution of the sample set.

Table 1. Distribution of samples between cultivar, origin and vintage

Cultivar	2005				2006			
	Paarl	Stellenbosch	Robertson	Worcester	Paarl	Stellenbosch	Robertson	Worcester
Sauvignon blanc	9	13	20	14	13	14	10	10
Chardonnay	5	1	20	18	5	1	10	10
Pinotage	10	7	4	14	9	3	5	10
Shiraz	14	9	12	17	13	4	10	10
Cabernet Sauvignon	16	13	15	13	5	8	9	10
Merlot	10	10	12	17	7	7	10	10

5.2.2 CHEMICALS, STANDARDS AND WINE SIMULANT

5.2.2.1 Chemicals and standards

Ethyl Acetate and isoamyl acetate was purchased from Riedel de Haën (Seelze, Germany). Methanol, hexanol, acetic acid and 2-phenylethanol standards were from Merck (Darmstadt, Germany). Ethyl butyrate, propanol, isobutanol, butanol, hexyl acetate, ethyl lactate, propionic acid, iso-butyric acid butyric acid, iso-valeric acid, diethyl succinate, valeric acid, 2-phenylethyl acetate, 4-methyl-2-pentanol and hexane were from Fluka (Buchs, Switzerland). Hexanoic acid, octanoic acid, isoamyl alcohol, ethyl caprylate, ethyl caprate were from Aldrich (Steinheim, Germany). Decanoic acid and ethyl hexanoate were purchased from Sigma (St. Louis, USA). Diethyl ether, ethanol and NaSO₄ were also purchased from Merck (Darmstadt, Germany).

5.2.2.2 Wine simulant

The internal standard and volatile standards were dissolved in a wine simulant consisting of 12 %v/v ethanol and 2.5 g/L tartaric acid (Merck, Darmstadt, Germany) in de-ionised water from a

MilliQ system water purifying system (from Millipore, Billerica, MA, USA), pH adjusted to 3.5 with 0.1M NaOH (Merck, Darmstadt, Germany).

5.2.3 EXTRACTION PROCEDURE

Five millilitres of wine with internal standard, 4-Methyl-2-Pentanol, (100 μ l of 0.5 mg/l solution in wine simulant) were extracted with 1 millilitres of diethyl ether by sonicating the ether/wine mixture for five minutes. The wine/ether mixture was then centrifuged at 3600 g for 3 minutes. The ether layer was removed and dried on NaSO₄. Each extract was injected into the GC-FID in triplicate.

5.2.4 GAS CHROMATOGRAPHY CONDITIONS

Instrumentation: A J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m Length \times 0.32 mm i. d. \times 0.5 μ m f.t was used. The initial oven temperature was 33°C for 17 minutes after which the temperature was increased by 12°C/min to 240°C, at which it was held for 5 minutes. 3 μ l of the diethyl extract was injected at 200°C. The split ratio was 15:1 and the split flow rate 49.5 ml/min. The column flow rate was 3.3ml/min and the total run time was 50 minutes. The detector temperature was 250°C. After each sample run, a post run of 5 minutes at oven temperature 240°C, with a column flow of 6ml/min cleaned the column from high boiling contaminants. After every 30 samples the column was thermally cleaned by injecting hexane several times isothermally, holding it for 10 minutes per injection at an oven temperature of 220°C.

5.2.5 FTMIR SPECTROSCOPY

The samples were degassed using vacuum filtration. Red wines were filtered twice and white wine samples were filtered three times. A WineScan FT 120 spectrometer equipped with a Michelson interferometer (Foss Analytical, Denmark; <http://www.foss.dk>) were used to generate spectra in the wavenumber region 5011-929 cm⁻¹. Commercial calibrations were used to quantify glucose, fructose, pH, total acidity, volatile acidity, malic acid, lactic acid, ethanol and glycerol. The wavenumbers 5011-2970 cm⁻¹ and 1543-1716 cm⁻¹, which are associated with the absorption by water molecules, were excluded in the data analysis unless stated otherwise.

5.2.6 STATISTICS

Principle component analysis (PCA) were performed in The Unscrambler 9.2 (CAMO Process AS, Oslo, Norway) in order to observe underlying trends in the data. Partial least square regression (PLS) were used to evaluate the correlation between wine quality (the score out of 20 that the wine received at the Young Wine Show) and chemical composition. These tests were also done in The Unscrambler 9.2. Linear discriminant analysis (LDA) were performed in Statistica 7.0 (Statsoft Inc., www.statsoft.com) to classify the wines into their respective cultivar or origin groupings. LDA with spectral data were preceded by principle component analysis.

5.3 RESULTS AND DISCUSSION

A summary of all the analysed compounds and their concentration ranges in the wines are given in Table 2.

Table 2. The concentration ranges of all the analytes in red and white wines.

Volatile compounds (mg/L)				
Compound	Red		White	
	Range	Std Dev ^a	Range	Std Dev
2-Phenylethanol	7.76 - 126.09	27.50	5.84 - 17.62	5.02
2-Phenylethyl Acetate	nd - 0.54	0.18	nd - 0.39	0.13
Acetic Acid	234.57 - 845.61	135.86	101.52 - 764.73	198.46
Butanol	0.85 - 3.15	0.64	0.33 - 1.90	0.42
Butyric Acid	0.38 - 1.85	0.37	0.78 - 3.01	0.61
Decanoic Acid	nd - 1.69	0.54	0.41 - 2.38	0.54
Diethyl Succinate	1.03 - 19.15	4.03	nd - 1.50	0.60
Ethyl Acetate	20.19 - 119.26	20.64	30.22 - 158.29	35.54
Ethyl Butyrate	nd - 0.52	0.55	0.17 - 0.89	0.37
Ethyl Caprate	nd - 0.30	0.09	nd - 0.43	0.12
Ethyl Caprylate	nd - 0.57	0.25	nd - 1.01	0.49
Ethyl Hexanoate	nd - 0.88	0.39	0.27 - 1.41	0.38
Ethyl Lactate	19.64 - 194.70	35.82	nd - 29.65	15.74
Hexanoic Acid	0.52 - 2.57	0.52	3.25 - 7.36	1.53
Hexanol	0.18 - 3.19	0.72	nd - 2.14	0.54
Hexyl Acetate	nd	± 0.09	nd - 0.57	0.24
Isoamyl Acetate	nd - 3.34	± 1.17	0.51 - 9.12	2.54
Isoamyl Alcohol	119.55 - 543.75	88.19	103.69 - 219.23	44.16
Isobutanol	2.34 - 97.88	24.75	2.26 - 35.29	8.30
Isobutyric Acid	0.35 - 3.54	0.84	0.13 - 1.83	0.41
Isovaleric Acid	0.37 - 4.58	0.99	0.13 - 2.00	0.41
Methanol	70.65 - 389.25	71.33	21.54 - 164.15	44.34
Octanoic Acid	0.28 - 2.98	0.63	1.15 - 10.35	1.90
Propanol	2.62 - 114.38	34.98	19.20 - 86.80	22.35
Propionic Acid	0.76 - 7.23	34.35	nd - 43.85	13.79
Valeric Acid	nd - 0.56	0.24	nd	0.09
Major chemical compounds (g/L)				
Compound	Red		White	
	Range	Std Dev	Range	Std Dev
pH	3.21-4.55	0.1833	3.19-4.18	0.1944
Volatile Acidity	0.14-0.84	0.1239	0.24-0.76	0.1030
Total Acidity	4.19-7.20	0.3823	4.33-7.55	0.5761
Malic Acid	nd - 2.28	0.2553	0.39-5.8	0.7418
Lactic Acid	nd-2.58	0.3749	nd-1.19	0.1884
Glucose	nd-2.91	0.4691	nd-4.14	0.5948
Fructose	nd-6.39	0.7788	0.35-4.31	0.8594
Ethanol (%v/v)	10.79-16.61	0.8270	10.38-15.18	0.8887
Glycerol	8.64-16.29	1.0166	4.55-11.68	1.0205

^a Standard deviation

5.3.1 PRINCIPLE COMPONENT ANALYSIS

The largest variation in the dataset as a whole was due to wine style and various degrees of separation between red and white wines could be observed using any combination of volatile components, major chemical compounds or spectra. The best separation could be obtained with PC 1 and PC 2 using the volatile components as variables (Figure 1). The negative end of PC 1, relating to the white wines, on the loadings plot were dominated by hexanoic acid, octanoic acid and some esters, while the positive end, relating to the red wines, were dominated by higher alcohols. This corresponds to results from previous studies (Gil *et al.*, 2006). Good separation could also be obtained with the classical wine parameters and or spectra when looking at PC 1 and PC 3 (data not shown). The most influential classical wine parameters were malic acid, pH, titratable acidity, lactic acid. The influence of malolactic fermentation, a process mainly used in red wine production, on these parameters is well established. Glycerol also contributed to the differences. This contribution can be confirmed by previous findings (Nieuwoudt *et al.*, 2002) Glucose and fructose did not contribute to the distinction between red and white wines at all.

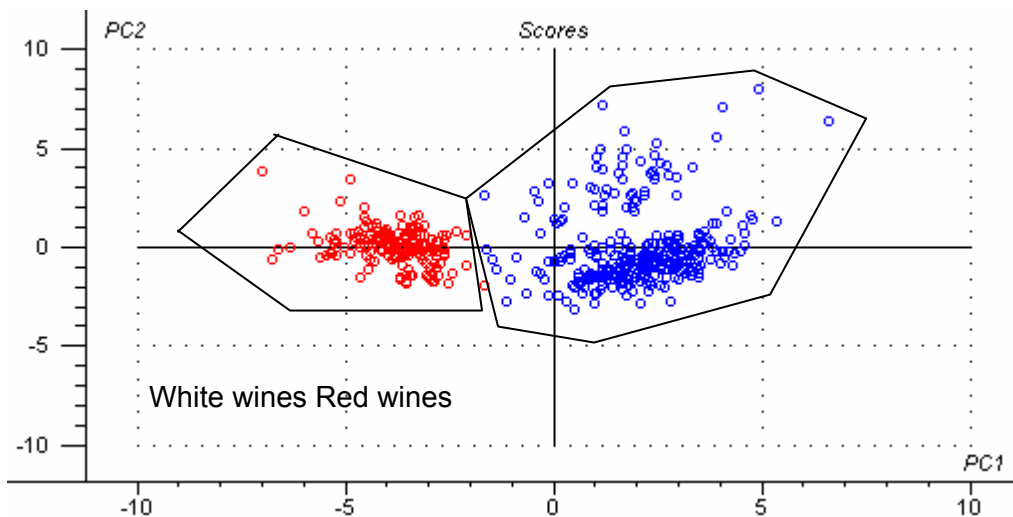


Figure 1. The PCA score plot indicates the separation between red and white wines along the first PC. The volatile compounds were used as variables for the PCA. Of the total variance in the dataset, 36% is explained by PC 1 and 10% by PC 2.

When the dataset was divided into subsections, better separation could be observed between vintages and cultivars. In the subsets containing only white or red wines, a clear distinction between vintages could be made only when using the volatile components as variables (Fig. 2). Isobutanol, butanol and decanoic acid were influential in the separation of the 2005 and 2006 red wines while diethyl succinate and isoamyl acetate were important for differences between the 2005 and 2006 white wines. The spectral data or classical parameters did not contribute to separations between the vintages.

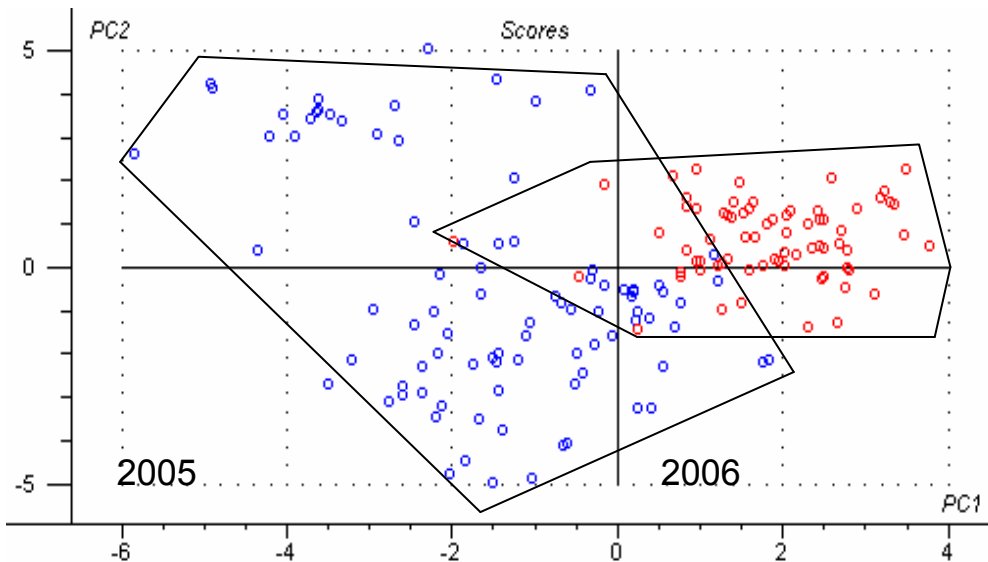


Figure 2. The 2005 and 2006 white wines separated along PC 1 in the PCA done with the volatile compounds as variables. PC 1 and PC 2 explains 18% and 17% of the variance in the dataset respectively.

Good separation could be observed between the Chardonnay and Sauvignon blanc wines when the spectra (Figure 3) or classical wine parameters were used, but the separation worsened when volatile compounds were included in the variable set. Ethanol, pH and lactic acid were strongly associated with Chardonnay wines while titratable acid and malic acid were associated with Sauvignon blanc.

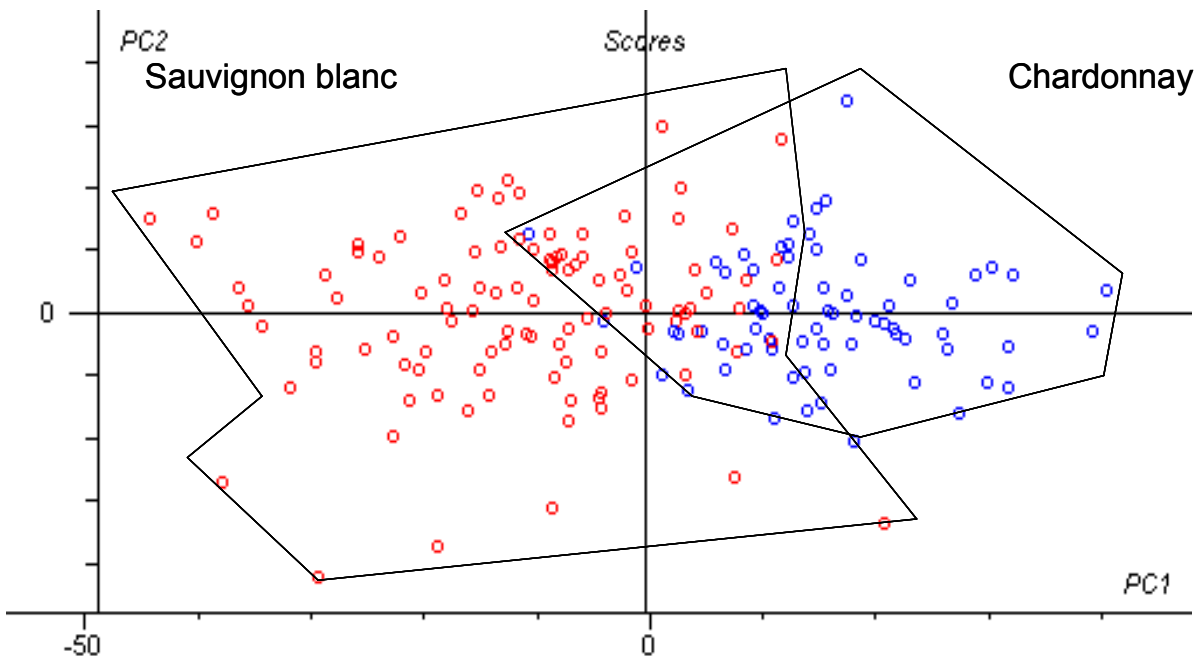


Figure 3. The two white cultivars separated along the first PC axis when the selected spectral wavenumbers were used as variables. PC 1 and PC 2 explains 62% and 28% of the total variance in the data set respectively.

The volatile components contributed the most to the differences between red wines. Very good separation could be observed between vintages in PC1 and PC3, as well as in PC2 and PC3. Separation between cultivar groups were not as clear. Pinotage separated the most from the rest and, as shown in Fig 4, generally correlated well with the PC's where isoamyl acetate had high positive loadings and the isoacids and isoamyl alcohol had high negative loadings. It has been established that isoamyl acetate plays an important role in the varietal characteristics of Pinotage wines (Van Wyk *et al.*, 1979). The high negative correlation between isoamyl alcohol and the isoacids and Pinotage is consistent with the results from Chapter 4. The Merlot wines formed a clear grouping in the 2005 vintage, based on high positive loadings of propionic and decanoic acids and high negative loadings of 2-phenylethyl acetate. However, the Merlot wines could not be separated from the Shiraz or Cabernet wines in the 2006 vintage. The Shiraz and Cabernet wines were difficult to separate when both vintages were used but better results were obtained when the vintages were split up. The separation was mainly due to high loadings of 2-phenylethanol, isovaleric acid and isoamyl alcohol which were negatively correlated with Shiraz. These results are also consistent with the findings in Chapter 4.

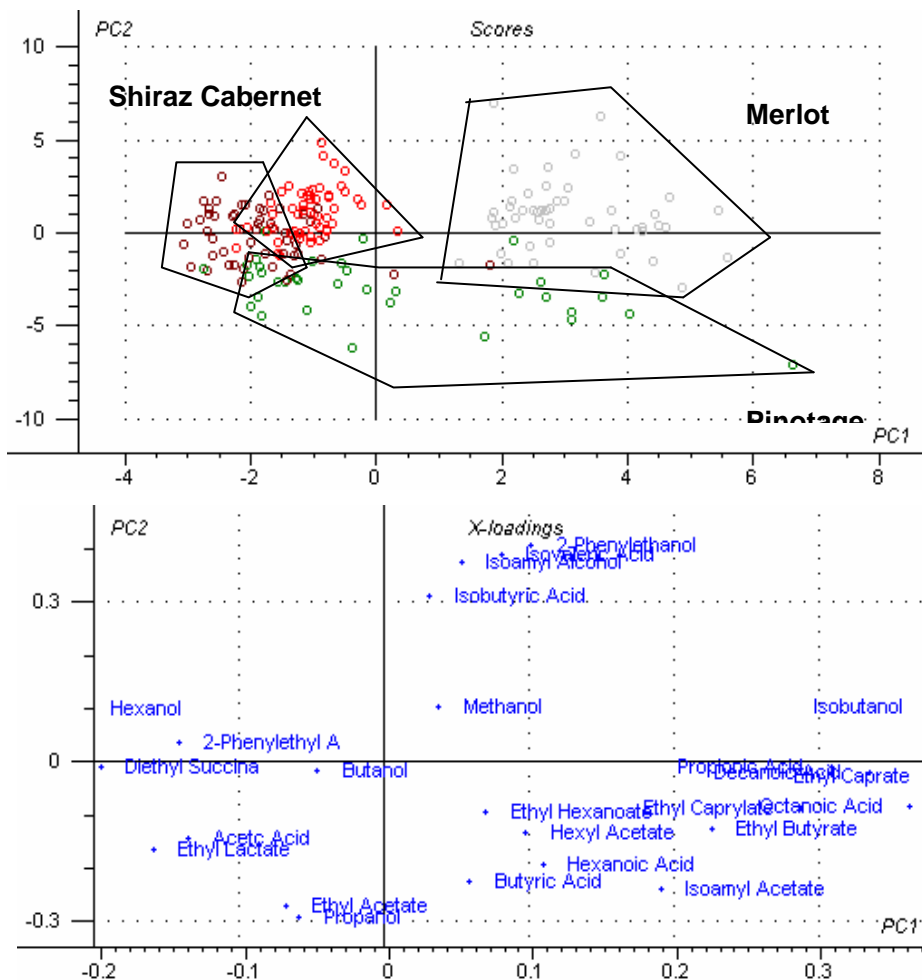


Figure 4. Separation between red cultivars from the 2005 vintage along PC 1 and PC 2. PCA were performed with volatile compounds as variables and PC 1 and PC 2 each explains 21% and 18% of the total variance respectively. The loadings plot of the X variables in a PCA investigating the influence of the volatile compounds on the differences between red wine cultivars from the 2005 vintage.

When the classical wine parameters were used in combination with the volatile compounds, the high loadings of volatile acidity contributed slightly to the separation of Pinotage wines. No cultivar groupings could be observed when the spectra was used as variables.

No clear geographical origin groupings could be observed with PCA, regardless of the variables. Principle component analysis were performed within each cultivar group as well as in vintage subsets within each cultivar group. No visible trends in terms of the rating the wines received at the Young Wine Shows could be observed.

5.3.2 PARTIAL LEAST SQUARE REGRESSION

Calibrations with PLS regression was also performed, but there were almost no correlation between wine quality and quantitative or spectral information. This could be an indication of the subjective nature of sensory evaluation of wines. However, most of the wines were scored 15 out of 20 marks, and the extreme ends of the score range were poorly represented. Such a calibration could benefit from a larger sample set that includes more highly scored and low scored wines.

5.3.3 LINEAR DISCRIMINANT ANALYSIS

Linear discriminant analysis (LDA) was applied in order to classify the wines into their respective cultivar and origin classes. Generally good classification results were achieved between the cultivar wines. The classification success rate was between 91% and 100% when the entire spectral range was used (Table 3).

Table 3. The percentage correct classification between cultivar wines with different variable sets

Cultivar	Full Spectra	Selected Wavenumbers	Volatile Compounds	Volatile Compounds + Selected Spectra	Volatile Compounds + Full Spectra
Chardonnay	100	93	59	97	100
Cabernet	91	69	63	89	94
Pinotage	96	92	92	100	100
Sauvignon blanc	100	97	94	100	100
Shiraz	97	97	70	93	100
Merlot	100	88	88	96	100
Total	97	89	77	95	99

Interestingly, classification success rate dropped when wavenumbers 5011-2970 cm^{-1} and 1543-1716 cm^{-1} , which are associated with noise caused by water absorption, were excluded from the analysis. When these selected wavenumbers were used, the wines were classified correctly between 88-97% with the exception of Cabernet Sauvignon, which were only 69% correctly classified. When the volatile components were used, the classifications was between 59 and 94% correct. The combination of the volatile compounds and the selected wavenumbers were better than the two separate variable sets, with a classification success rate of 87-100%. The best results, a 100% correct classification except for Cabernet (94%), was achieved using a

combination of the entire spectral range and the volatile components. The results for this variable combination is shown in Figure 5.

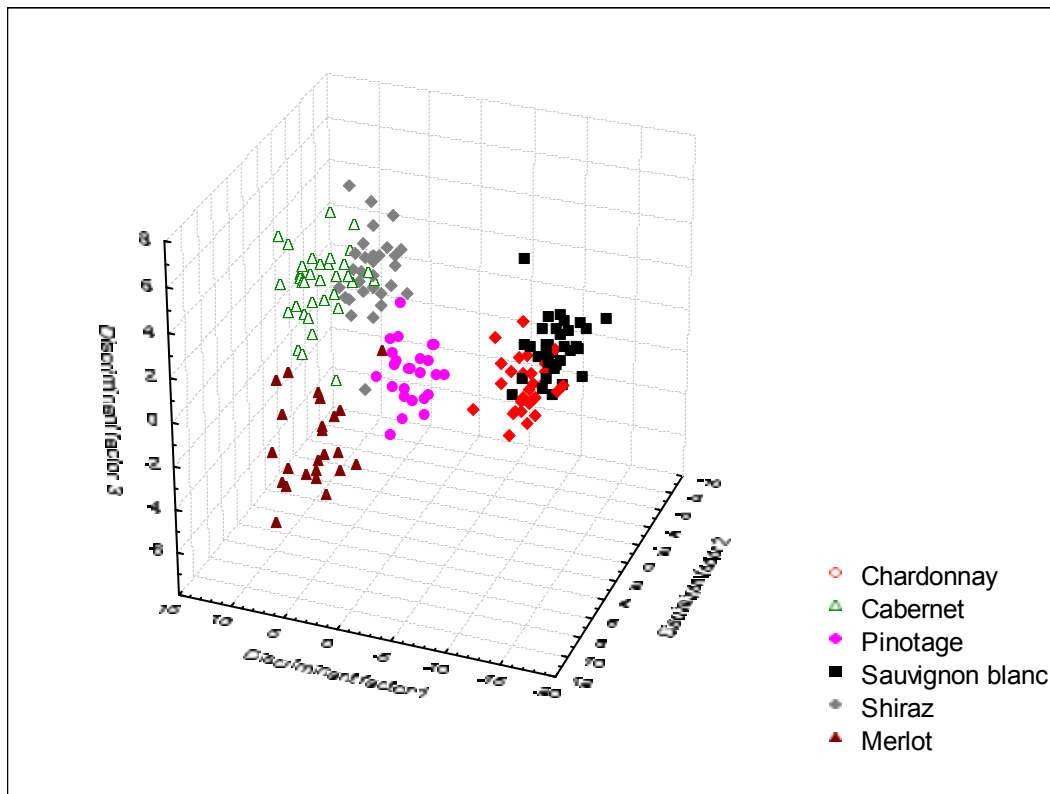


Figure 5. The results from the discriminant analysis of the cultivars wines based on volatile composition and the full spectral range. The graph shows the scores for the first three discriminant functions.

The geographical origin of the wines could not be successfully classified with the volatile compounds, spectra or classical parameters. The percentage correct classification varied between 36% and 55% (data not shown). The wines were divided into subsets containing red wines, white wines, or cultivar wines, but the classification rates did not improve significantly. It needs to be mentioned that the information of the origin of the wines used in this study is based on the geographic location of the cellar. Since South African wine cellars are allowed to purchase grapes from other wine producing areas and it can not be guaranteed that the grapes used to produce the wines are actually from the same region. However, the value of volatile compounds and infrared spectra could be better evaluated with samples of guaranteed origin. Previous studies have shown that volatile compounds could be successfully used to classify wines according to geographical origin (Marias *et al.*, 1981). The use of spectroscopy in the classification of the geographical origin of wine have not been widely explored, but some promising results have been reported (Urbano *et al.*, 2006).

In this study, the role of yeast derived volatile components, infrared spectra and major wine parameters predicted with FT-MIR technology on the variability of South African young wines were investigated. Based on PCA, it seemed that most of the variability was due to the volatile constituents of the wines, although the spectra did contribute to cultivar groupings between the white wines. However, the role of the infrared spectra was much more pronounced in terms of the

classification of the cultivar wines, where the most successful classification rate was achieved with a combination of the entire infrared spectra and volatile compounds. Unfortunately, neither the volatile components or the infrared data could indicate differences between wine regions, although this is most likely to be due to the limitations of the dataset. Lastly, it was not possible to predict the score of the wines from PLS regression models of the chemical or spectral data.

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Chapter 6

RESEARCH RESULTS

Evaluation of enzyme-linked spectrophotometric assays and high performance liquid chromatography as reference methods in the optimisation of Fourier transform mid-infrared calibrations for major wine compounds

RESEARCH RESULTS

ABSTRACT

Enzymatic assays and high performance liquid chromatography (HPLC) are often used for the determination of organic acids, sugars and glycerol in wine. Enzymatic assays are highly specific but time consuming. On the other hand, HPLC analyses allows the simultaneous determination of a variety of compounds, but previous reports indicated that the determination of organic acids are subject to substantial matrix effects, in especially red wines. The objective of this study was to evaluate and compare spectrophotometric enzymatic assays and HPLC methods for the determination of malic acid, lactic acid, glucose, fructose and glycerol. The effect of fining agents for the decolourisation of red wines as a sample preparation procedure were evaluated. Solid phase extraction and PVPP treatments were evaluated as sample preparation procedures to minimise interferences during the HPLC analysis of organic acids. Each method were evaluated in terms of matrix effects, sample preparation procedures, accuracy, repeatability and practicality. The current enzymatic assay procedures were considered suitable for the analysis of glycerol, glucose and fructose, but unsuitable for the determination of L-malic and L-lactic acid with regards to the monitoring of malolactic fermentation to the endpoint. HPLC analysis were considered suitable for the analysis of all the analytes, although higher measurement errors were observed for glucose and fructose determination compared to the enzyme assays. Low recoveries were observed for organic acids in wines treated with SPE. Higher recoveries were observed for the organic acids in wines treated with PVPP, but the method must be optimised to increase reproducibility. It was concluded that different approaches should be considered for the quantification of organic acids and alcoholic fermentation related components.

6.1 INTRODUCTION

Alcoholic fermentation and malolactic fermentation are two of the most important biological processes of winemaking and they need to be closely monitored for the purpose of exerting effective quality control. It is common practice in the wine industry to measure amongst other, pH glucose, fructose, ethanol, selected organic acids and glycerol in order to gain insight into the progress of these processes. During alcoholic fermentation, yeasts convert glucose and fructose, the two major sugars in grape must, to ethanol, CO₂ and other by-products. The changes in the glucose and fructose concentrations in fermenting must be monitored to identify problematic sluggish or stuck fermentations that can lead to off-flavours and wine spoilage (Ribereau-Gayon *et al.*, 2000). The residual sugar left in the wine after fermentation most often consists of fructose and small amounts of glucose that was unutilised by the yeast. Apart from the obvious flavour implications, high levels of residual sugar can stimulate the growth of unwanted spoilage micro-organisms, especially after bottling. During alcoholic fermentation, yeast cells also produce glycerol

during the metabolism of a small amount of sugar through the glyceropyruvic pathway (Ribereau-Gayon *et al.*, 2000). Although the sensory impact of glycerol in wine is unascertained, the role of the by-products of glyceropyruvic fermentation, including acetic acid, diacetyl and acetaldehyde, is associated with decreased wine quality (Ribereau-Gayon *et al.*, 2000).

Malolactic fermentation (MLF) is used particularly during red winemaking for the purpose of converting malic acid to lactic acid, a process mainly mediated by lactic acid bacteria (Zoeklein *et al.*, 1995). This process influences wine flavour by de-acidification and altering wine aroma and also increases the microbial stability of wine (Ribereau-Gayon *et al.*, 2000). MLF monitoring can be particularly cumbersome since its onset is frequently unpredictable and the progress slow which result in significant variation in the duration from batch to batch. This complicates the simultaneous monitoring of several fermentation vessels and also implies that large numbers of chemical analyses must be done.

The large amounts of samples that need to be analysed in industrial wine cellars, especially to monitor malolactic fermentation, requires methods that are fast and cost effective. In recent years Fourier transform mid-infrared (FTMIR) spectroscopy has been shown to be a suitable method for the determination of several of the major compounds in wine, including ethanol, sugars, organic acids, glycerol and pH (Kupina and Shrikhande, 2003; Nieuwoudt *et al.*, 2004; Patz *et al.*, 2004). The main advantages of the method resides in its speed, low analysis cost and simple sample preparation procedures, thereby making it a particularly attractive option for large-scale high sample throughput analysis. FTMIR spectroscopy is an indirect analytical method and relies on calibration models for quantification. Calibration models are established using data collected from the analysis of real samples, that are representative of samples that will be analysed in the future, with a suitable reference method. This typically involves the measurement of large numbers of samples in order to capture as much natural variance as possible. The evaluation of the suitability of analytical methods as reference methods for FTMIR calibration not only relies on the accuracy and precision of the methods, but also on practical considerations, robustness, time efficiency and sample throughput capacity.

Two commonly used analytical techniques for the quantification of organic compounds in wine are high performance liquid chromatography (HPLC) and enzyme-linked spectrophotometric assays. Both techniques are often cited in the literature as reference method of choice for the development of infrared spectroscopy-based calibration models (Guggenbichler *et al.*, 2006; Patz *et al.*, 2004; Urbano Cuadrado *et al.*, 2005). Enzymatic assays have high specificity and are relatively easy to use. However, each compound are analysed separately with this method, contrary to HPLC that allows the simultaneous analysis of several compounds (Mato *et al.*, 2005). Numerous HPLC methods have been reported for the analysis of organic acids and major fermentation products such as sugars and glycerol, although the most common methods are based on ion exclusion chromatography (Castellari *et al.*, 2000; Dopico-García *et al.*, 2007). However, HPLC analysis requires specialist training and the accuracy of the analyses relies on the purity of the peak that elutes at the retention time associated with the analyte of interest.

The UV-visible absorption detectors that are usually used for the HPLC analysis of organic acids also provide response for other common organic molecules, notably the phenolic compounds

present in wine. This detection method relies on the chromaphoric properties of the measured analytes (Rounds and Gregory, 1998). The chromaphoric properties of especially aromatic organic molecules are stronger than that of the carboxylic acid groups of the organic acids (Zotou *et al.*, 2004). Therefore, it is often observed that the large amounts of organic constituents present in red wines interfere with the analysis of organic acids (Zotou *et al.*, 2004). Solid phase extraction (SPE) is commonly used to separate organic acids and carbohydrates from phenolic compounds prior to HPLC analysis (de Villiers *et al.*, 2004). The extraction procedure is normally based on the principles of reversed phase chromatography, where a polar mobile phase and a non polar stationary phase, nowadays available in convenient pre-packed cartridge format, is used. Octadecylsilyl (C18) is the most common packing material for these SPE cartridges. The use of polymeric packing materials such as polystyrene-divinylbenzene eliminates the presence of residual silanols and also provides better pH stability and selectivity (Rounds and Gregory, 1998). Recent technology in reversed phase SPE also includes packing materials made from macroporous copolymers. Combinations of lipophilic divinylbenzene and hydrophilic N-vinylpyrrolidone monomers provide added wetting properties, thereby protecting the packing material from drying out due to air contact (Waters information center, n.d.). However, the use of extraction procedures for the determination of organic acids in wine are generally regarded as tedious, expensive and time-consuming and poor recoveries for organic acids are often reported (Mato *et al.*, 2005).

Preliminary results of PVPP treatments as an alternative sample clean-up procedure have been reported (Zotou *et al.*, 2004). In winemaking, several types of fining agents are used to remove excessive amounts of phenolic compounds from wine. Of these, polyvinyl polypyrrolidone (PVPP) and activated charcoal form the least amount of lees, making them the most practical to use in laboratory conditions. Activated charcoal adsorbs weak polar molecules, especially small phenolic compounds, and is considered the most effective colour removing agent (Zoeklein *et al.*, 1995). However, PVPP can bind larger phenolic compounds than activated charcoal making it more suitable for the removal of phenolic compounds in general (Zoeklein *et al.*, 1995).

In this paper HPLC and enzyme-linked spectrophotometric assays were evaluated as reference methods for FTMIR calibrations that can be used for the quantification of malic acid, lactic acid, glucose, fructose and glycerol. The evaluation was based on performance criteria that includes precision, accuracy, complexity of sample preparation, sample throughput and time efficiency. Sample preparation techniques that include methods to eliminate the interferences caused mainly by phenolic compounds in red wines were investigated. The optimisation of a SPE method in order to improve the recovery of the organic acids for this study was conducted in two parts: the comparison of four types of SPE cartridges, and the optimisation of the rinsing volume needed to maximise the recovery of organic acids on the optimal phase. The use of PVPP for the removal of phenolic compounds was also investigated.

6.2 METHODS AND MATERIALS

6.2.1 STANDARDS AND REAGENTS

R-Biopharm enzymatic analysis kits (AEC-Amersham, Sandton, South Africa) were used for analysis of D-glucose and D-fructose, glycerol, L-malic acid and L-lactic acid respectively. PVPP and GAT1 activated charcoal were used for decolourisation and removal of phenolic compounds in wine and were obtained from Merck (Darmstadt, Germany) and CJ Petrow Chemicals (Cape Town, South Africa) respectively. Methanol (99.8%) was obtained from Merck (Darmstadt, Germany). Water was de-ionised with a Milli-Q A10 water purifying system (from Millipore, Billerica, MA, USA) and adjusted to pH 2.5 with 1M HCl Merck (Midrand, South Africa). Analytical grade standards for L-malic acid (99.5%) and succinic acid (99.5%) were purchased from Fluka (Buchs, Switzerland). Standard solutions for L-lactic acid were prepared from sodium L-lactate (99.0%) from the same manufacturer. Tartaric acid (99.5%) and citric acid (99.5%) were from Aldrich (Steinheim, Germany). Acetic acid standard solutions were prepared from glacial acetic acid obtained from Saarchem (Waderville, South Africa). D-Glucose, D-fructose and glycerol standards were also obtained from Saarchem. Ethanol was obtained from Merck (Midrand, South Africa). H₂SO₄ (50%) was purchased from Fluka (Buchs, Switzerland).

6.2.2 DECOLOURISATION AND REMOVAL OF PHENOLIC COMPOUNDS IN WINE

Fining with activated charcoal was performed by mixing GAT1 with a red wine at a concentration of 20 g/L and allowing a two hour reaction time at room temperature. An aliquot of the red wine to which no GAT1 was added, served as control. After this treatment the wine was centrifuged for 2 minutes at 14 558 g and the supernatant was removed to be analysed further. A noticeable pellet of activated charcoal particles was observed in each wine sample after centrifugation. PVPP fining was done on the same red wine according to the method described by Zotou *et al.* (2004). PVPP powder was added to red wine at a 50 g/L dosage and stirred for 15 minutes. Following this, the murky samples were centrifuged for 15 minutes at 1055.2 g. A noticeable pellet was observed after centrifugation and the supernatant was removed and filtered with a 0.2 µm disposable syringe filter (Lased, South Africa). As in the case of activated charcoal treatment, an aliquot of the wine that was not treated by PVPP, served as control. The colour and phenol removing properties of PVPP and activated charcoal were evaluated through absorbance measurements of the supernatants at 280 nm, 420 nm, 520 nm and 720 nm in quartz cuvettes, using an Ultraspec 2000 UV/Visible spectrophotometer (Pharmacia Bio-Tek Instruments, Cambridge, England). The optimal PVPP dosage was determined by comparing the percentage colour loss caused by PVPP dosages of 10 g/L, 25 g/L, 50 g/L, 100 g/L and 150 g/L respectively. Absorbance readings were corrected for sample dilutions and for path length differences between the various cuvettes used for the spectrophotometric readings. All treatments were performed in duplicate.

6.2.3 ENZYME-LINKED SPECTROPHOTOMETRIC ASSAYS

The enzyme assays used rely on the generation or loss of spectrophotometrically active metabolites such as NADH and NADPH and in the enzyme reaction mixtures the final amounts of these compounds are stoichiometric to the initial amounts of the respective substrates. The analysis of D-glucose and D-fructose is based on the conversion of NADP⁺ to NADPH, while glycerol quantification is based on the oxidation of NADH to NAD⁺. The analysis of L-lactic acid as well as L-malic acid is based on the reduction of NAD⁺ to NADH. Full details of the enzymatic reactions are described in Addendum B of this thesis. Typically, two absorbance readings are taken for the enzymatic analysis, one before the onset of an enzymatic reaction (A_1), and one after completion of the reaction (A_2). In order to attain sufficient precision, wines were diluted with deionised H₂O where necessary, so that the absolute difference in absorbance $|A_1 - A_2|$ between the two readings was at least 0.1 AU and not exceeding 1.0 AU. Absorbance readings were taken at 340 nm using an Ultraspec 2000 UV/Visible spectrophotometer (Pharmacia Bio-Tek Instruments, Cambridge, England). Each wine sample was analysed by at least two independent assay repeats in disposable plastic cuvettes (with path length 1 cm) after verification that the cuvettes do not absorb light at 340 nm. The precision of replicate determinations was expressed as the coefficient of variation that was calculated as:

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

where s is the standard deviation and \bar{x} is the sample mean.

The assay volume recommended by the manufacturer, namely 3 mL, was reduced to 1 mL in order to increase the number of determinations performed per kit. The accuracy of the down-scaling procedure was tested on the assay controls provided with the kits.

6.2.4 HPLC

6.2.4.1 SPE equipment and optimisation of extraction conditions

Oasis HLB, C-18 Bond Elut, Chromabond HR-P and Strata SDB-L cartridges were obtained from Waters (Milford, MA, USA), Varian (Harbor City, CA, USA), Machery-Nagel (Düren, Germany) and Phenomenex (Torrance, CA, USA) respectively. The Oasis HLB cartridge is based on hydrophilic-lipophilic technology while the C-18 Bond Elut cartridge is packed with octadecylsilyl packing material. The Chromabond HR-P and Strata SDB-L cartridges both contain polystyrene-divinylbenzene packing material.

SPE was carried out at an elution rate of one drop per three seconds using a vacuum manifold (Supelco Visiprep 24 from Sigma Aldrich, Aston Manor, South Africa). The cartridges were pre-conditioned with 3 x 1 ml methanol followed by 3 x 1 ml deionised water (pH 2.5 with HCl). Subsequently, 1 ml of sample, acidified to pH 2.5 with HCl, was passed through the cartridge followed by either 2 x 1 ml, 4 x 1 ml, 6 x 1 ml or 8 x 1 ml of deionised water (pH 2.5) in order to determine the optimal solvent volume. The sample fraction and water fraction were pooled. For comparison of the different SPE cartridges the organic acids were eluted with 4 x 1 ml acidified

water. The recovery for a given treatment were determined as the ratio of the measured concentration of the treated sample to the measured concentration the control sample, i.e. a sample of the same wine that were directly injected. The composition of the mixture of standards used during the evaluation of the SPE methods are shown in Table 1.

Table 1. Concentrations of the components in the standard mixture that was used for the optimisation of the SPE method.

Standard	Concentration (g/L)
Citric acid	2.25
Tartaric acid	4.50
Malic acid	4.50
Succinic acid	2.25
Lactic acid	2.25
Acetic acid	1.80
Glucose	4.50
Fructose	4.50
Glycerol	7.17
Ethanol	11.14 ^a

^aMeasured in %v/v

6.2.4.2 Chromatography

6.2.4.2.1 Standards solutions

Individual stocks solutions of D-glucose, D-fructose, tartaric acid and citric acid (100 g/L each); L-malic acid (90 g/L); succinic acid and acetic acid (40 g/L each) and lactic acid (25 g/L) were prepared and stored at 4 °C. Mixtures of standards for the organic acids and for the sugars were prepared to establish calibrations. Ethanol and glycerol calibrations were established with separate individual standards, prepared from stock solutions with concentrations of 48 %v/v and 48 g/L respectively. The concentration ranges used to establish the calibrations were as follows: 0.05 – 5 g/L for citric acid; 0.10 – 10 g/L for tartaric acid and malic acid; 0.05 – 5 g/L for succinic acid and lactic acid; 0.04 – 4 g/L for acetic acid; 0.2 – 10 g/L for glucose and fructose; 3.2 – 16.1 g/L for glycerol and 9.65 – 24.13 %v/v for ethanol. These intervals span the concentration ranges typically found for the respective components in wine. Individual standard solutions (10 g/L) were injected of each compound for peak identification by retention time.

6.2.4.2.2 Liquid chromatography (LC)

All samples were filtered through a 0.22 µm disposable syringe filter (type of filter) (Lasec, South Africa) before HPLC analysis. Isocratic LC analysis of sugars, alcohols and acids was performed in the same run using an Aminex HPX-87H ion exclusion column with dimensions 300 mm × 7.8 mm (Biorad, Hercules, CA, USA) and an Agilent 1100 Series (Waldron, Germany) HPLC instrument equipped with diode array- (DAD) and refractive index (RID) detectors with the same

manufacturing specifications. The mobile phase was 5 mM H₂SO₄ in de-ionised water. The flow rate was 0.5 ml/min, the injection volume 10 µl unless indicated otherwise and a constant oven temperature of 55°C was maintained. The total run time for each analysis was 28 minutes. DAD quantification of organic acids was performed at 210 nm and peak integration was performed using HP Chemstation software.

6.2.5 STATISTICS

Statistical analyses were done in Microsoft Excel 2002 (Microsoft Corporation, www.microsoft.com) and Statistica 7.0 (Statsoft Inc., www.statsoft.com) software packages. Coefficient of variance (CV) and standard error of laboratory (SEL) values were calculated as described.

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

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

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

Where y_1 and y_2 are duplicate measurements of a sample and n is the number of samples (Fern, 1996).

6.3 RESULTS AND DISCUSSION

6.3.1 DECOLOURISATION AND REMOVAL OF PHENOLIC COMPOUNDS IN WINE BY PVPP AND ACTIVATED CHARCOAL

The colour removing properties of the two fining agents were tested on a red wine. Dosages of 10g/L, 25 g/L, 50 g/L, 100 g/L and 150 g/L of PVPP were used to determine the optimal dosage for maximum removal of colour and phenolic compounds. After treatment with activated charcoal, the samples were nearly colourless, with some very fine but visible particles, and a thick, black precipitate was observed after centrifugation. After treatment with PVPP, samples were clear and light pink in colour with a substantial amount of precipitate. Samples treated with 150 g/L PVPP were visibly oversaturated.

Colour was determined by absorbance at 420 nm, 520 nm and 720 nm and phenolic compounds at 280 nm. Measured absorbance values were corrected to compensate for sample dilutions and differences in the path lengths of the various cuvettes used. In order to facilitate comparison between the different readings, the corrected absorbance values were normalised by considering the absorbance of the control samples (not subjected to PVPP treatment) as 100%. The absorbance of the treated samples at each recorded wavelength was normalised and expressed as a percentage loss in colour and phenolic compounds after treatments with different PVPP dosages (Figure 1). Results showed that a PVPP dosage of 50 g/L resulted in a decrease of almost 90% in the colour (measured at 420 nm and 520 nm) and a 70 % decrease in the phenolic

content of the red wine. At PVPP dosages higher than 50 g/L, the absorbance values increased, possibly due to spectral interferences caused by over-saturation of the sample with PVPP. The use of PVPP at a 10 g/L dosage was suggested by the manufacturers of the enzymatic kits for decolouring of highly pigmented samples such as wine, but from the results shown in Figure 1, this was clearly too low for optimal removal of phenolic compounds. The results of this study are more comparable to the PVPP dosage used in the publication of Zotou *et al.* (2004) prior to HPLC analysis, namely 50 g/L.

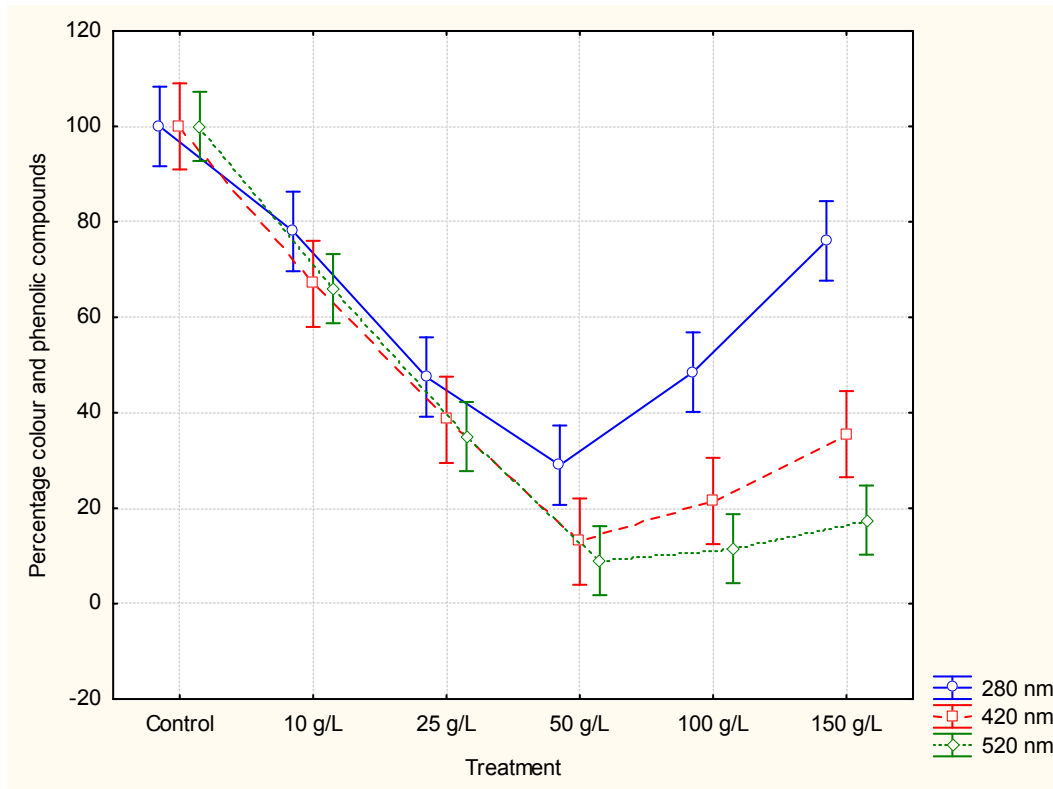


Figure 1. Percentage colour (420 nm and 520 nm) and phenolic compounds (280 nm) left in a red wine treated with different PVPP dosages in comparison to an untreated control wine. Red wine colour was measured at 420 nm and 520 nm and phenolic content at 280 nm. Error bars denote 95% confidence intervals.

A dosage of 20 g/L activated charcoal was recommended by the suppliers for 100% removal of colour in red wines. This dosage was compared to the 50 g/L dosage PVPP for the removal of coloured compounds. At these dosages, activated charcoal removed up to 22% more colour than PVPP (Figure 2) under the laboratory conditions used in this experiment.

Activated charcoal has been cited as a better colour removing agent than PVPP by some (Zoecklein *et al.*, 1995), yet it has been shown that PVPP can bind larger phenolic compounds than activated charcoal, thereby making it more suitable for the removal of phenolic compounds in general (Morris and Main, 1995).

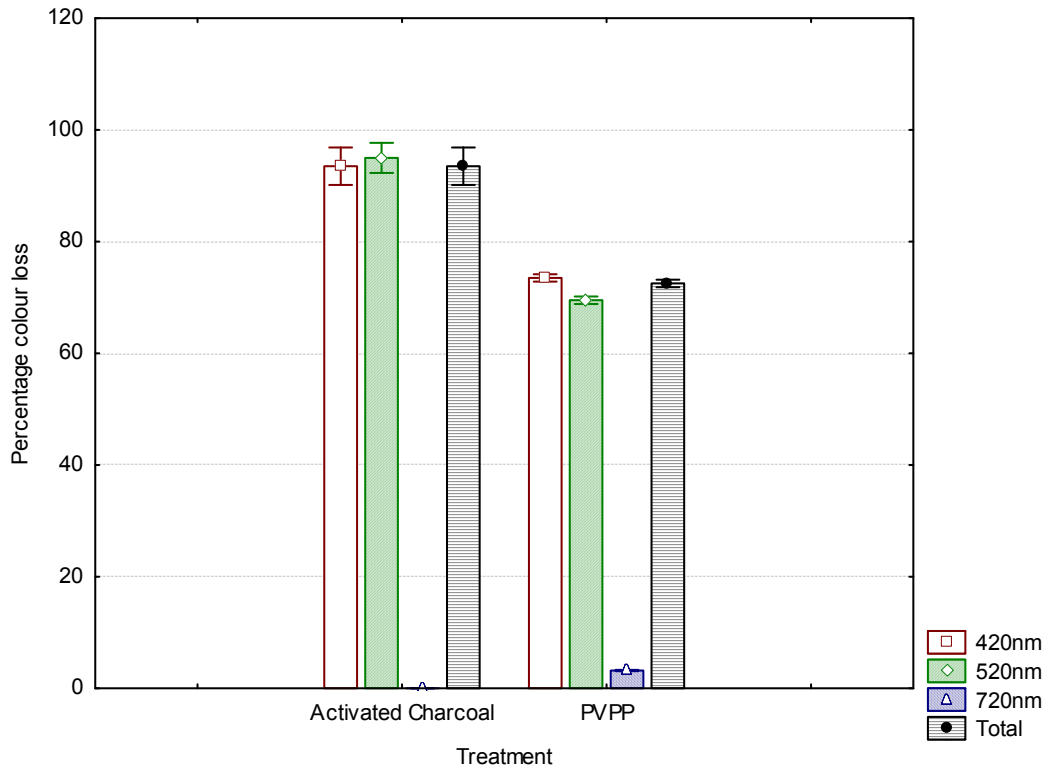


Figure 2. Percentage colour loss in red wine due to treatment with 20 g/L activated charcoal and 50 g/L PVPP. Colour measurements were made at 420 nm, 520 nm and 720 nm. Total colour density was calculated as determined as absorbance at 420nm +520nm +720nm. Error bars denote standard deviation.

6.3.2 ENZYME ASSAYS

All enzymatic analyses were performed in duplicate and a overall measurement error of less than 5% were maintained.

6.3.2.1 Downscaling of enzyme assay volumes

The effect of the downscaling of the enzyme assay volume was tested for D-glucose, L-lactic acid, L-malic acid and glycerol using the assay controls of known concentration provided with the enzyme kits. An assay control for D-fructose could not be included as it is unstable in an aqueous solution and therefore not provided as part of the D-glucose/D-fructose kit. When evaluating the effect of downscaling on the accuracy of the assays, the measurement error in the 3 ml volume assay volumes (recommended by the manufacturer) were comparable to the measurement error in the 1 ml assay volumes (Table 3). The percentage deviation of the measured concentration from the reference concentration was unbiased and lower than 4% for D-glucose, L-lactic acid and L-malic acid. For glycerol the percentage deviation was lower than 6%. It could be concluded that downscaling of the assay volume did not affect the performance of any of the abovementioned enzymatic assays.

Table 3. Evaluation of the accuracy of the enzymatic assays using 3 ml and 1 ml assay volumes respectively.

Compound	Concentration of reference solutions ^a	Assay volume 3 ml ^b		Assay volume 1 ml	
		Measured concentration	% Deviation from reference concentration ^c	Measured concentration	% Deviation from reference concentration ^c
D-glucose g/L	0.499	0.509	-2.0	0.493	1.2
L-lactic acid g/L	0.199	0.189	5.0	0.196	1.5
L-malic acid g/L	0.198	0.203	-2.5	0.206	-4.0
Glycerol g/L	0.393	0.372	5.3	0.367	6.5

^aReference solutions refer to the assay control solutions of known concentrations provided with the respective enzyme kits; ^bAssay volume recommended by manufacturer; ^cCalculated as the difference between the concentration of the reference solutions (provided by the manufacturer) and the average of the measured concentrations (duplicate determinations).

6.3.2.2 Matrix effects

The performance of the D-glucose/D-fructose kit in both red and white wine matrices was satisfactory. In each case absorbance values below 1.0 absorbance units (AU) could be maintained by using the appropriate dilution, while maintaining absorbance differences between 0.1 and 1.0 AU for the reactions. The standard error of laboratory (Table 4) was 0.04 g/L and 0.09 g/L for D-glucose and D-fructose respectively. These values are low in comparison to the average concentration of these analytes present in the samples.

Table 4. The standard error of laboratory (SEL) of the enzymatic assays in relation to the average concentration of the measured samples.

Compound	Sample no. (white wines; red wines)	Concentration range (mean \pm SD)	SEL (g/L)
Malic acid g/L	13	0.06 – 4.18 (2.20 \pm 1.90)	0.05
Lactic acid g/L	27	0.00 – 5.12 (1.00 \pm 1.12)	0.23
Glucose g/L	29	0.11 – 2.24 (0.51 \pm 0.47)	0.04
Fructose g/L	24	0.07 – 5.32 (1.29 \pm 1.77)	0.09
Glycerol g/L	27	4.02 – 16.15 (7.71 \pm 3.03)	0.32

^aStandard deviation

Absorbance differences between 0.1 and 1.0 AU could easily be achieved with the glycerol kits for red and white wines. The absorbance values were in most cases in the order of 1.5 AU, which would normally be too high. However, relatively high absorbance values were also observed in the blank samples and the assay control samples, for which fairly accurate results were observed, as indicated in Table 1. Therefore, it is unlikely that the high absorbance values observed in the wine samples were related to a matrix effect. As it did not seem to influence the accuracy of the assays, the absorbance values were considered satisfactory. The SEL for the glycerol assays were very good.

In the case of L-lactic acid determinations the observed absorbance values and absorbance differences were acceptable. However, the repeatability between duplicates was very poor as the SEL indicated a error margin up to 23% based on the average concentration of the samples (data not shown). This was observed for both red and white wines. Considering the high accuracy observed for the analysis of the assay control sample, it is possible that the poor repeatability is due to a matrix effect of the wines. However, the cause of such a matrix effect is unclear.

The suitability of the L-malic acid enzymatic kit for analysis of wine was tested on a red and white wine. The repeatability between duplicate wines of both the red and white wine was good, with a SEL of 0.05 g/L. However, the absorbance values of the red wines were very high, in the order of 1.5 to 3.0 AU. Unlike the case of the glycerol assays, these unacceptably high absorbance values were only observed during the red wine analyses and not during the analysis of the white wines, blank samples or assay control samples. Therefore, the high absorbance values were considered a matrix effect, possibly caused by the pigmented phenolic compounds present in the red wines.

It was further attempted to decrease the abovementioned matrix effect observed by diluting the red wine samples with de-ionised water. A red wine was spiked with 0.2 g/L L-malic acid and analysed undiluted, diluted two-, five-, ten- and fifteen times. In the case of the undiluted and twice diluted samples, the matrix effect was too strong, resulting in absorbance values of higher than 1.000 AU. The dilution effect was too strong at 10 and 15 times dilutions respectively and absorbance differences of below 0.100 AU were observed between successive readings. Adequate absorbance values and absorbance differences were achieved with the five times diluted sample and the malic acid in this sample was determined as 0.32 g/L. It can therefore be said that red wines with malic acid concentration exceeding 0.32 g/L can successfully be analysed with enzymatic assays using the experimental conditions as described. However, it is generally accepted that red wines that have completed malolactic fermentation normally contains less than 0.3 g/L L-malic acid. Therefore, an alternative sample preparation method will be required in order to use L-malic acid enzymatic assays as a reference method for the development of a FTMIR calibration model to monitor malolactic fermentation up to the endpoint of the fermentation process.

The effects of the colour removing sample treatments with 20 g/L activated charcoal and 50 g/L PVPP on the performance of enzymatic analysis of L-malic acid in red wines were investigated. When these fining agents were tested in an L-malic acid assay, both fining agents caused heavy spectral interferences, despite the fact that the fined samples were now nearly colourless (data not shown). These dosages of PVPP and activated charcoal are therefore not compatible with enzymatic assays of L-malic acid without further sample clean-up to remove PVPP and activated charcoal traces. However, given the objective of evaluating FTMIR calibrations, additional sample clean-up procedures would be too cumbersome. Alternatively, enzymatic assay kits from various manufacturers can be compared.

6.3.3 HPLC

HPLC analyses were done in duplicate. The overall measurement error between duplicates, calculated as CV%, were less than 5%.

6.3.3.1 Standards

Standard solutions were analysed to determine retention times and the degree of separation of the individual compounds. The peaks are identified in Figure 3 and Table 5. Fructose (peak no. 8) co-eluted with malic acid (peak no. 3). Good linearity and recovery were observed for each of the analytes.

Table 5. Relevant chromatographic information for the HPLC analysis of standard compounds. Peaks numbers correspond to those in Figure 3.

Peak	Compound	Retention time (min)	Concentration Range (g/L)	R ²	% Recovery ^a
1	Citric acid	9.57	0.05 - 5.00	0.998	96
2	Tartaric acid	10.17	0.10 - 10.00	0.997	97
3	Malic acid	11.41	0.10 - 10.00	0.999	97
4	Succinic acid	14.28	0.05 - 5.00	0.998	97
5	Lactic acid	14.95	0.05 - 5.00	0.998	97
6	Acetic acid	18.01	0.04 - 4.00	0.997	95
7	Glucose	10.83	0.2 - 10.00	0.997	95
8	Fructose	11.80	0.2 - 10.00	0.998	95
9	Glycerol	15.89	3.20 - 16.01	0.998	100
10	Ethanol	25.48	9.65 - 24.13 ^b	0.996	91

^aDetermined as the ratio of the measured concentration to the actual concentration of a given compound in the mixture of standards;

^bMeasured in %v/v

6.3.3.2 Matrix effects

During HPLC analysis of especially red wine, co-elution of phenolic compounds and organic acids takes place, which interferes with the quantification of the organic acids. This can clearly be seen in Figure 4 a-b, where the interference in the directly injected red wine (b) is more substantial than for the directly injected white wine (a). Therefore it was considered necessary to remove phenolic compounds from red wine samples to ensure accurate analysis of the organic acids. Solid phase extraction (SPE) on reversed phase packing materials, where the compounds are separated according to hydrophobicity, has been used for this purposes in several studies (Dopico-García *et al.*, 2007; de Villiers *et al.*, 2004).

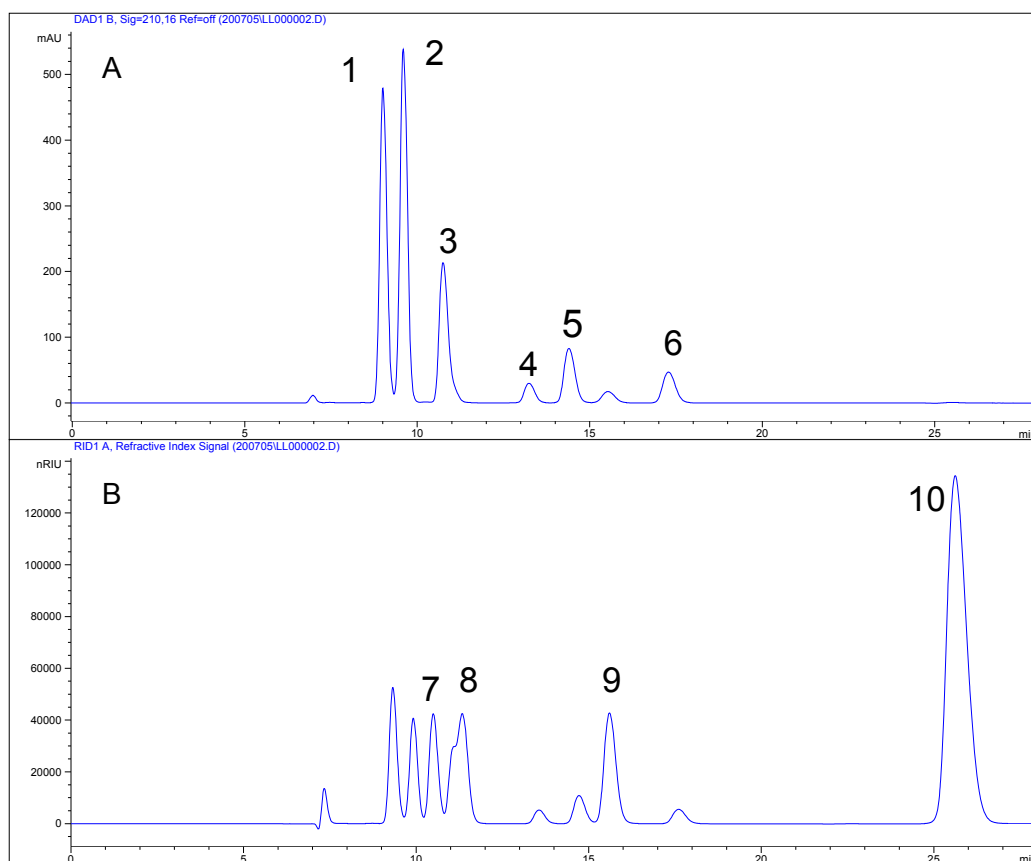


Figure 3A and B. Analysis of a mixture of standards containing organic acids, sugars and alcohols on a Aminex HPX-87H ion exclusion column with 5 mM H₂SO₄ mobile phase at a flow rate of 0.5 ml/min. Organic acid detection was performed using UV detection at 210 nm (a); sugar and alcohol detection was done with RID (b). Peak identification, using external standards, as presented in Table 1. Peak 3 (malic acid) clearly co-elutes with Peak 8 (fructose).

6.3.3.3 Comparison between SPE cartridges

During SPE some organic acids may be retained on the column and this reduces the recovery of these compounds. A selection of reversed phase cartridges, Oasis HLB, C-18 Bond Elut, Chromabond HR-P and Strata SDB-L, was compared with the objective to achieve optimal retention of phenolic compounds and maximum recovery of organic acids. Based on visual inspection of the cartridges, the Oasis HLB cartridge clearly retained a red pigmented band on the cartridge before the phenolic compounds were eluted. The SDB-L cartridge also retained the pigmented compounds fairly well, although not in a clear band. The C-18 Bond Elute cartridge did not retain the pigmented compounds as effectively, although better than the Chromabond HR-P cartridge for which elution of pigmented compounds was observed during the first rinsing step.

Compared to the control red wine (direct injection) (Figure 4b); all the cartridges removed the phenolic interferences to some extent (Figure 4c-f). Significantly more interfering compounds are detected by UV detection for the sample treated on the Chromabond HR-P cartridge (Figure 4c) compared to the Bond Elute cartridge (Figure 4d). A large, unidentified peak (b), which was also visible in the control sample, eluted at 25.09 minutes in the C-18 Bond Elute sample but did not elute in the Chromabond HR-P sample. It seems as if some of the organic acid peaks are larger in the C-18 Bond Elute sample than the Chromabond HR-P sample. The peak areas of all organic

acids were higher in the Oasis HLB (Figure 4e) and Strata SDB-L (Figure 4f) samples, compared to the C-18 Bond Elute and Chromabond HR-P samples, indicating higher recovery on the former two cartridges. Moreover, the unknown peak (a) detected at 6.9 minutes, presumably representing unretained organic molecules, was smaller in the Oasis HLB and Strata SDB-L chromatograms compared to the other two. This indicates more effective removal of interfering compounds on these cartridges. Based on these results, the Oasis HLB and Strata SDB-L cartridges were considered preferable to the other two cartridges. Differences could also be observed between the Oasis HLB and Strata SDB-L cartridges in terms of the two unknown peaks (a) and (b). Both of these peaks were less prominent on the Oasis HLB chromatogram than the Strata SDB-L chromatogram. However, the peak area of tartaric acid was higher in the Strata SDB-L chromatogram. The differences between the Oasis HLB cartridge and the Strata SDB-L cartridge seemed arbitrary and the Strata SDB-L was chosen for further analysis.

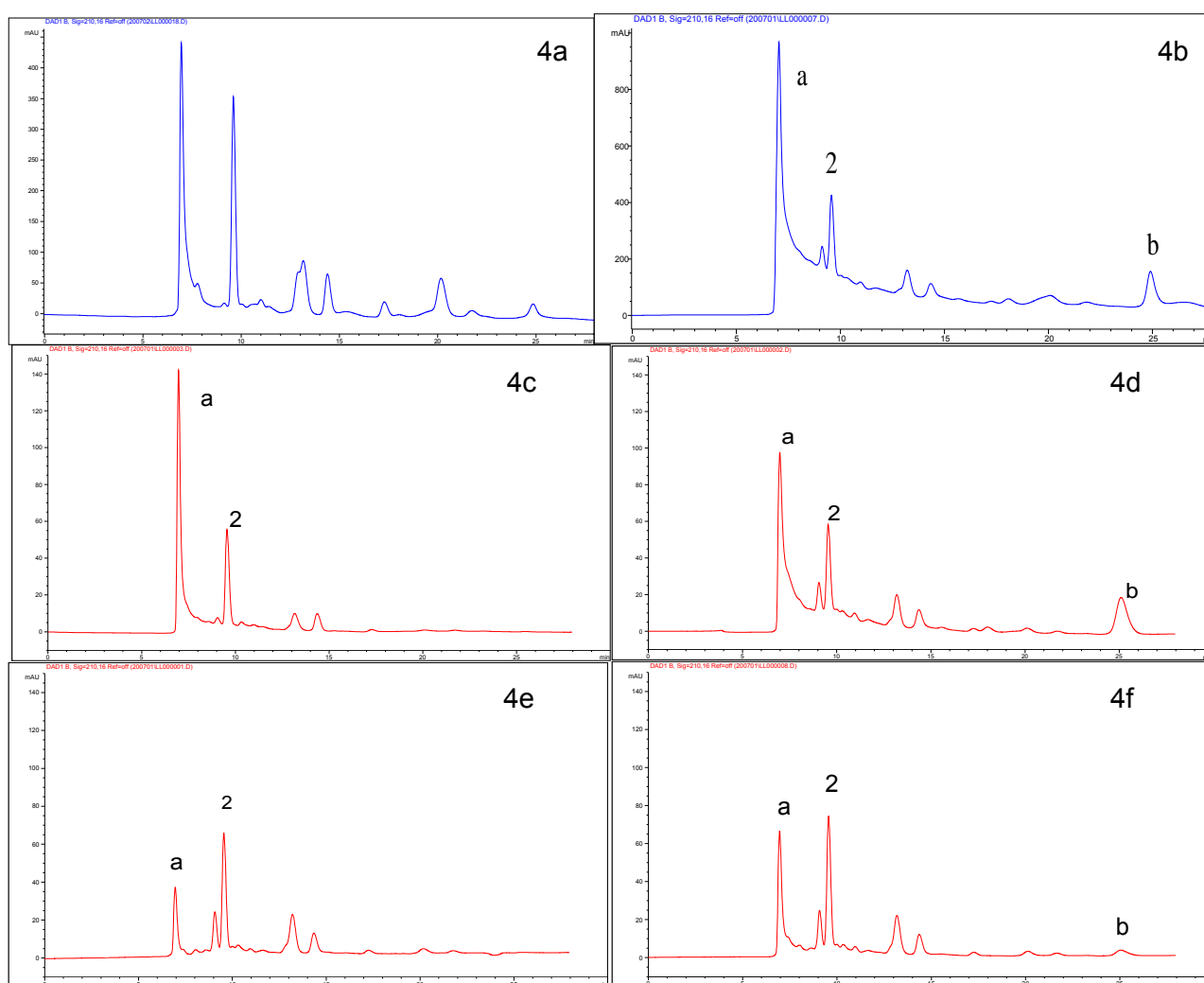


Figure 4a - f Comparison of sample clean-up treatments using SPE for improved quantification of organic acids in wine with HPLC analysis using an Aminex HPX-87H ion exclusion column (Biorad) with 5 mM H₂SO₄ mobile phase at a flow rate of 0.5 ml/min. Peak a and b denotes unidentified interfering peaks, while peak 2 is identified as tartaric acid in Table 1. Figures 4a and b represents the analysis of a direct injected white and red wine respectively. Figure 4c-f represents a red wine

that underwent SPE sample clean-up with Chromabond HR-P, C-18 Bond Elut, Oasis HLB and Strata SDB-L SPE cartridges respectively.

6.3.3.4 Optimisation of solvent volume for elution of organic acids

After conditioning of the Strata SDB-L cartridge, 1 ml of sample was passed through followed by 2 ml, 4 ml, 6 ml and 8 ml acidified water (each were passed through 1 ml at a time) to determine the smallest solvent volume that provides the highest recoveries for the compounds of interest. The experiment was executed using a red wine (spiked with 2 g/L of each organic acid, glucose and fructose using individual standards) and on a mixture of standards to exclude any matrix effects (Table 1). For the standards mixture, the highest recoveries were obtained in the 8 ml rinse fraction, although high recoveries (larger than 90% except for citric acid which was 84%) were achieved in the 6 ml rinse fraction. In the red wine, the recoveries were extremely poor for the organic acids (Table 6). The recoveries for glycerol and ethanol were better, around 100%, and although the recoveries for the sugars were high, the dilution effect on the 6 ml and 8 ml fractions were so strong that these peaks were below the limit of detection. The wines were then diluted 2 times and 10 times prior to SPE in order to improve the recoveries by reducing the matrix effect. The organic acids were eluted with 6 ml of acidified water as a compromise between the dilution effect on the sugars and the recovery of the organic acids. The recoveries of the 2 times diluted wine was still very poor, mostly around 60% (Table 6). The dilution effect was too severe in the case of the 10 times diluted wine where glucose, fructose, malic acid and lactic acid were below the limit of detection, even though the injection volume was doubled (data not shown).

Table 6. Average percentage recovery for the various analytes during sample clean up of a standard solution, a red wine and a twice diluted red wine with SPE as well as a red wine fined with 50 g/L PVPP^a. SPE analyses were performed in duplicate while PVPP analyses were carried out in six repeat measurements. Quantification was done by HPLC analysis as described in the text.

	SPE with Standards ^b	SPE with Wine	SPE with Diluted wine	PVPP
Citric Acid	84%	32%	44%	94%
Tartaric acid	97%	56%	49%	95%
Glucose	97%	nd	nd	101%
Malic Acid	96%	31%	91%	100%
Fructose	99%	nd	110%	103%
Succinic Acid	91%	44%	69%	89%
Lactic Acid	97%	43%	66%	94%
Glycerol	95%	91%	85%	104%
Acetic Acid	102%	34%	62%	97%
Ethanol	104%	122%	102%	100%

^aSPE was performed using a Strata SDB-L cartridge with an elution volume of 6 ml acidified water; ^bRefer to Table 1.

6.3.3.5 PVPP fining for the removal of phenolic compounds

A red wine spiked with 2 g/L of each organic acid, fructose and glucose were treated eight independent times with 50 g/L PVPP (Figure 5). Six of the samples were diluted two times while

the other two were analysed undiluted. No difference were observed between the diluted and undiluted wines. In both cases the recoveries were between 89% and 104% for all the compounds and the repeatability was excellent. The reproducibility, determined by treating five different non-spiked wines, was somewhat inconsistent. Despite this, the recoveries for each compound in at least three of the wines corresponded by more than 98 % (Table 7). Poor recoveries were observed for malic acid in these five wines.

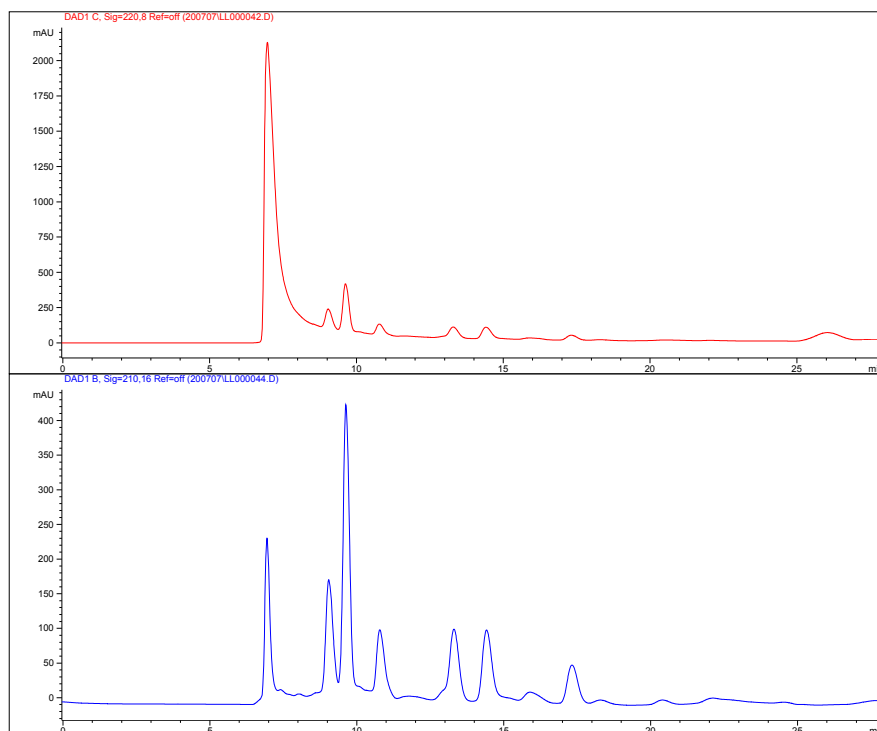


Figure 5. HPLC analysis of an undiluted red wine before (top) and after (bottom) treatment with 50 g/L PVPP.

Table 7. Recoveries of organic compounds in five independent wines after treatment with 50 g/L PVPP. Quantification was done with HPLC analysis as described in the text.

	Wine 1	Wine 2	Wine 3	Wine 4	Wine 5
Citric Acid	186%	113%	114%	31%	112%
Tartaric acid	85%	101%	114%	103%	102%
Glucose	102%	98%	nd	95%	99%
Malic acid	nd	49%	26%	25%	40%
Fructose	111%	94%	75%	98%	96%
Lactic acid	84%	74%	92%	89%	92%
Glycerol	109%	102%	58%	101%	102%
Acetic acid	96%	99%	106%	106%	97%
Ethanol	92%	100%	98%	99%	100%

6.3.4. WINE ANALYSIS

Enzymatic assays and HPLC methods were compared based on the precision of wine analysis. The standard error of laboratory (SEL) indicates the measurement error between duplicate samples. A summary of the SEL values for the enzymatic and HPLC analyses of malic acid, lactic

acid, glucose, fructose and glycerol is given in Table 8. In the case of glucose and fructose, lower measurement errors were observed during enzymatic analysis. In the case of lactic acid, malic acid and glycerol, lower laboratory errors were observed during HPLC analysis.

Table 8. Summary of the standard error of laboratory (SEL) for the determination of selected quality control analytes with enzymatic assays and HPLC.

Analyte	SEL ^a for enzymatic assays (g/L)	SEL for HPLC analysis (g/L)
Malic acid	0.05	0.034
Lactic acid	0.23	0.089
Glucose	0.04	0.112
Fructose	0.09	0.229
Glycerol	0.32	0.058

^aStandard error of laboratory.

6.4 CONCLUSIONS

Enzymatic assays were found suitable for the determination of glucose, fructose and glycerol in red and white wines as well as malic acid in white wines and red wines containing more than 0.32 g/L malic acid. It was determined that six samples could be measured in duplicate simultaneously. The accumulated reaction time for the determination of all these compounds in six duplicate samples is estimated as 41 to 63 minutes. The time spent preparing the enzymatic reactions, performing the spectrophotometric analyses and changing between assay kits still needs to be taken into account. The total runtime of the HPLC analyses is 28 minutes during which the abovementioned compounds as well as lactic acid, ethanol and additional organic acids are determined in a single sample. Therefore, the accumulated runtime for six samples analysed in duplicate is estimated at 336 minutes or 5 hours and 36 minutes. This excludes the amount of time spent preparing mobile phases, establishing calibration curves, sample preparation and integration of the resulting chromatograms. Therefore, ion exclusion HPLC analysis should not be regarded a quick and easy way to determine a variety of reference values in a single run. For the determination of glucose, fructose, glycerol and malic acid it seems that enzymatic assays are a faster method, although HPLC holds the advantage of a lower limit of quantification for malic acid in red wines as well as the ability to generate data for additional compounds.

The two sample clean-up methods for HPLC analysis evaluated in this study, SPE and PVPP fining, both effectively removed interfering phenolic compounds. On a practical level, both methods were time consuming and labour intensive. The PVPP fining method was considerably less expensive than the SPE method and provided better recoveries for the organic acids.

None of these methods are ideal and the choice between these methods involves a compromise between limit of quantification, recovery, and reproducibility as well as time and cost efficiency. Based on standard error of laboratory, glucose and fructose can be measured more reliably with enzymatic assays than with HPLC while the opposite is true for glycerol, malic acid and lactic acid. Ideally, the suitability of the two methods should have been evaluated by the performance of preliminary calibration models established with data from each method respectively. According to results reported by Blieke in 2005, better calibration models were

obtained for enzymatically determined analytes than for HPLC determined analytes. However, it was suggested that the automation of the enzymatic method used increased the stability of the calibrations (Blieke, 2005). Although not the ideal practical solution, it is clearly more reliable to use different approaches for the determination of organic acids and alcoholic fermentation related analytes.

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Chapter 7

RESEARCH RESULTS

Optimisation of the quantification of major wine parameters in South African young wines using Fourier transform mid-infrared spectroscopy

RESEARCH RESULTS

ABSTRACT

Fourier transform mid-infrared (FTMIR) spectroscopy plays an important role in wine quality control by providing a rapid and cost effective method to determine a range of major wine constituents. The objective of this study was to optimise the quantification of major wine constituents with FTMIR. A sample selection procedure based on principal component analyses was used to optimise the degree of variance in the calibration sample set. A selection of degassing procedures were evaluated and compared in terms of efficient CO₂ removal. The performance of commercial FTMIR calibrations in a young wine matrix were evaluated. New preliminary calibration models were established for young wines and evaluated in terms of coefficient of determination, bias and prediction error. The effect of wavenumber selection were also evaluated. Vacuum filtration was regarded as the most efficient degassing procedure. The commercial calibration models for VA, malic acid and lactic acid performed well in a young wine matrix. The new preliminary models for the prediction of titratable acidity, malic acid, VA, lactic acid, glucose, fructose and glycerol performed better using a small selection of highly correlated wavenumbers. Better prediction models for pH and ethanol were obtained using a larger wavenumber region. In conclusion, the establishment of FTMIR calibrations should not be performed using a recipe-like approach but with careful consideration of the limitations of the datasets, matrix effects and wavenumber selection.

7.1 INTRODUCTION

There are several chemical parameters that need to be monitored during wine production. Titratable acidity and pH play an important role in the organoleptic properties and microbial stability of the wine. Volatile acidity (VA) influences the quality of a wine and large amounts of this parameter can indicate microbial spoilage. The malic acid and lactic acid content in wine are influenced by malolactic fermentation, a process during which lactic acid bacteria converts malic acid to lactic acid. This process plays an important role in the sensory properties and stability of wine and therefore, it is important to monitor the levels of malic and lactic acid in wine during malolactic fermentation. Winemakers need to measure the glucose and fructose levels in wine during alcoholic fermentation to monitor the process and to identify problematic fermentations. High levels of residual sugar in wine can stimulate the growth of unwanted spoilage microorganisms, which can have detrimental results, especially after bottling. The fermentation process can also be followed by the increase in ethanol concentration in the wine. The final ethanol concentration in wine is also of high importance as there are certain legal limits that need to be adhered to. In addition, ethanol plays a subtle but important role in the flavour of wine. The organoleptic impact of glycerol in wine is debatable, but the by-products formed during glycerol

production play an important role. These by-products include acetic acid, acetaldehyde and diacetyl which are all associated with decreased wine quality (Ribereau-Gayon *et al.*, 2000).

Due to the high sample throughput in wine laboratories during the harvest season, it is important to be able to determine these compounds in a time and cost effective way. The advantages of Fourier transform infrared (FTMIR) spectroscopy for rapid wine screening and quality control during winemaking have already been reported by several authors (Patz *et al.*, 2004; Kupina and Shrikhande, 2003; Gishen and Holdstock, 2000). This technique measures the absorption of infrared radiation by covalent bonds contained in molecules such as C-H, O-H, C-O, C=O and N-H groups in the mid-infrared region of the electromagnetic spectrum that is usually defined as ranging from 4000 to 400 cm^{-1} , or in terms of nanometers from 25000 to 2500 nm (Skoog *et al.*, 1997). The absorption data at all the infrared wavenumbers are captured simultaneously at the detector in the form of an interferogram which is then converted with the Fourier transform algorithm to a transmittance or absorbance spectrum. The resulting spectrum can be converted to quantitative data by means of a calibration process that involves chemometric techniques such as partial least square regression (PLS) (Wehling, 1998). This process establishes a correlation between the amount of absorption of infrared radiation at specific wavenumbers and the concentrations of a specific compound as measured with an appropriate reference method. The correlation is typically described by a linear algorithm. The concentrations of a compound of interest in future samples are then predicted on the basis of the algorithm and the FTMIR spectrum of the sample. The selection of suitable wavenumbers is an important part of the calibration process. Not all wavenumbers in the mid infrared spectral region contains useful information that can be correlated to wine compounds of interest. The wavenumber regions 3626-2970 cm^{-1} and 1716-1543 cm^{-1} has been reported to contain spectral noise largely caused by water absorbance, while very little useful wine-related information is captured in the 5011-3630 cm^{-1} regions (Nieuwoudt, *et al.*, 2004; Patz, *et al.*, 2004).

The first purpose-built FTMIR spectrometer dedicated to wine analysis was marketed in 1998 (Foss Analytical, Denmark) and the instrument is fitted with useful ready-to-use commercial calibrations, with the software accompanying the instrumentation. These calibrations were developed using wine samples mostly from European origin and are to be used as a starting point for the quantitative analysis in a wine laboratory. The software of the instrumentation does facilitate adjustment of the slope and/or intercept of the calibration algorithms if it is necessary to improve the prediction error of samples analysed in the laboratory (WineScan FT120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001). The software can also be used to create new calibration algorithms for compounds of interest.

The success of quantification using FTMIR spectroscopy relies on the quality of the spectra and the quality of the reference sample set. The quality of mid-infrared spectra can be negatively influenced by high levels of CO_2 in the sample. Poor spectral repeatability has been reported for samples containing high levels of CO_2 (Bevin *et al.*, 2006).

The reference sample set that will be used to establish the calibration prediction models must meet two very important criteria. Firstly the reference values must be accurate and will therefore rely on the performance of the reference method. Secondly, it is also important that the reference

samples are representative of the samples that will be analysed in the future and all variation to be expected in future unknown samples analysed by FTMIR must be accounted for in the calibration model. Several aspects of the sample matrix must be taken into account, including the concentration range of the compounds of interest, the colour and style of the wine, the cultivar and the production stage (Nieuwoudt *et al.*, 2004).

Young wines used in this study are defined as single cultivar, unwooded wines that have not been bottled for commercial release yet. Although young wines contain similar amounts of the abovementioned analytes as bottled wines, they have not necessarily been subjected to blending, ageing, fining treatments or stabilisation processes. The inherent instability of young wines could be the source of a matrix effect that might influence the prediction abilities of commercial calibrations.

This study had two main aims. The first was to evaluate the performance of global FTMIR calibrations of the Winescan FT 120™ spectrometer (Foss Analytical, Denmark) for quantification of pH, titratable acidity, volatile acidity, malic acid, lactic acid, glucose, fructose, ethanol and glycerol in South African young wines. Secondly, preliminary new calibrations set up specifically for a young wine matrix were established and the performance of the respective calibration models was evaluated and optimised using different strategies of wavenumber selection and sample selection procedures. In addition, some elementary sample preparation methods, particularly efficient CO₂ removal in wine samples which is required for FTMIR spectroscopy were evaluated.

7.2 MATERIALS AND METHODS

7.2.1 WINE SAMPLES

Wines were collected from the 2005 and 2006 South African Young Wine Shows and supplemented with wines from the Stellenbosch University experimental cellar and stored at 4-8°C till analysed. Wines were specifically chosen to include important South African cultivars in order to be representative of South African wines. The wine samples included Sauvignon blanc, Chardonnay, Chenin blanc, Pinotage, Merlot, Cabernet Sauvignon and Shiraz wines. Wines were selected from 4 main winemaking regions in South Africa, namely Paarl, Stellenbosch, Robertson and Worcester in order to include spectral variation related to the geographic origin of the samples in the FTMIR spectra.

7.2.2 REFERENCE ANALYSES

Reference analyses for pH, titratable acidity, volatile acidity and ethanol were done using methods recommended by the Office International de la Vigne et du Vin (<http://www.oiv.com>). pH was determined using a Unitrode pH meter (Metrohm, Switzerland). Certified buffers (pH 7.00 and pH 4.00, LASEC, SA) were used to calibrate the electrode. Titratable acidity (expressed as g/L tartaric acid) measured by potentiometric titration using a 702 SM Titrino (Metrohm, Switzerland) and standardised 0.33 N sodium hydroxide (LASEC, Cape Town, SA) to the end point of pH 7.00 as

described by Zoecklein *et al.* (1999). Volatile acidity and ethanol analyses were outsourced and were determined by cash still distillation and pycnometry respectively. Glycerol was determined by enzymatic analysis using a R-Biopharm Glycerol kit (AEC Amersham, Sandton, South Africa). Malic acid, lactic acid, glucose and fructose determinations were done by HPLC analyses (refer to sections 2.4.2.1 and 2.4.2.2 in Chapter 6).

7.2.3 FTMIR SPECTROSCOPY

7.2.3.1 Sample preparation

Centrifugation, sonication and multiple filtration were evaluated for efficient CO₂ removal on a commercial wine. Samples were centrifuged for five minutes at 2993.3 g (RC 5C centrifuge with a S1-50T rotor from Sorvall, Newtown, South Africa). Sonication was performed in an ultrasound water bath for ten minutes. Samples were filtered with a filtration unit (type 79500, FOSS Analytical, Denmark) connected to a vacuum pump. Filter paper disks graded with pore size 20 to 25 µm and diameter 185 mm (Schleicher & Schuell, Germany, catalogue No. 10312714) were used for filtration. The amounts of CO₂ present in the wine after these treatments were measured with FTMIR spectroscopy. Wines used for the calibrations were filtered two and three times for red and white wines respectively. Statistical analysis (ANOVA) of the sample preparation treatments was done in Statistica 7.0 (StatSoft. Inc, Tulsa, USA).

7.2.3.2 Generation of FTMIR spectra

Instrument: The FTMIR spectra of the wines were generated in the wavenumber region 5011-929 cm⁻¹ with a WineScan FT 120 spectrometer (Foss Analytical, Denmark, 2001).

Spectral acquisition and processing: Samples (7 mL) were pumped through the CaF₂-lined cuvette (path length 37 µm) at a constant temperature of 40°C. Samples were scanned from 5011-929 cm⁻¹ at 4 cm⁻¹ intervals. The amount of infrared radiation transmitted by the sample were recorded at the detector and used to generate an interferogram that is calculated from a total of 20 scans. Subsequently the interferogram is converted to a single beam transmittance spectrum by the Fourier transformation. (WineScan FT120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001).

7.2.4 EVALUATION OF GLOBAL FTMIR SPECTROSCOPY CALIBRATION MODELS

The descriptive statistics of the wine samples used to establish the global WineScan FTMIR calibrations are given in Table 1.

Table 1. Descriptive statistics of wine samples used to establish global WineScan FTMIR calibrations^a.

Parameter	Value range of calibration samples mean (min.- max.) ^b	Reference method	Reference ^a
pH	3.55 (2.82 -4.04)	potentiometer	Application note 137 ^b P/N 1025274
Titrateable Acidity g/L ^c	4.01 (2.45 – 10.31)	titration using NaOH	Application note 139, P/N 1025275
Volatile Acidity g/L	0.37 (0.04 – 1.07)	distillation and titration; titration using NaOH	Application note 140, P/N 1025277
Malic Acid g/L	0.82 (0.0 – 5.20)	HPLC, enzymatic	Application note 136, P/N 1025273
Lactic Acid g/L	1.16 (0.0 – 3.87)	HPLC, enzymatic	Application note 135, P/N 1025272
Glucose g/L	2.23 (0.0 – 15.70)	enzymatic	Application note 134, P/N 1025271
Fructose g/L	3.38 (0.0 – 23.30)	enzymatic	Application note 132, P/N 1025269
Ethanol %v/v	12.12 (8.5 – 14.75)	electronic density meter, distillation	Application note 131, P/N 1025268
Glycerol g/L	5.11 (3.40 – 10.78)	enzymatic	Application note 191, P/N 1025415

^aApplication notes for WineScan FT 120 Type 77110 and 77310, Issue 2GB, October 2001, Foss Analytical, Denmark. <http://www.foss.dk>; ^bminimum to maximum range; ^cmeasured as g/L tartaric acid.

The suitability of the global calibrations for the South African young wine matrix was evaluated by fitting the reference sample set (consisting of FTMIR wine spectra and corresponding reference values for the compounds of interest) as an independent validation set onto the global calibrations. Goodness-of-fit was evaluated by partial least squares regression 1 (PLS1) using the Advanced Performance software module version 2.2.2 of the FTMIR spectrometer (WineScan FT120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001). The performance of the global calibrations was evaluated by the statistical indicators describes in section 2.6.

7.2.5 ESTABLISHMENT OF NEW FTMIR SPECTROSCOPY CALIBRATION MODELS

7.2.5.1 Selection of calibration sample sets

For the evaluation of the preliminary new young wine calibration models, the reference sample set for each compound was divided into a calibration and validation set containing 60% and 40% of the samples respectively. The samples for the calibration set was selected by performing principal component analysis (PCA) on the FTMIR spectra and selecting equal numbers of samples from each quadrant of the resulting score plot (Naes *et al.*, 2002). Histograms were plotted of the full reference sample set and the calibration set to ensure that the latter covers the entire concentration range of the former. The calibration set selection procedure was performed with The Unscrambler 9.2 software (Camo ASA, Trondheim, Norway).

7.2.5.2 Establishment of new FTMIR calibration models

New calibrations were established in the Advanced Performance software package of the WineScan instrument using PLS1 regression. The calibration errors were calculated using ten-segmented cross-validation as pre-programmed by the software. By default the software automatically selects 15 filters that consist of single wavenumbers or a small number of adjacent wavenumbers that collectively capture the maximum variation in the concentrations of the analyte, or y-variable, under investigation. Typically with this selection strategy typically not more than 25 individual wavenumbers are selected for calibration out of a total spectrum based on 1056 wavenumbers. Each new calibration model was validated using an independent validation sample set and PLS1.

Calibration models were also established by using larger wavenumber regions than those recommended by the WineScan software in order to evaluate the impact of wavenumber selection on the accuracy of the predicted values generated by the respective calibration models. These calibrations were performed with The Unscrambler 9.2 software using PLS1 regression and the same calibration and validation sample sets, established before. Ten-segmented cross validation was used in order to mimic the Advanced Performance software calibration procedure as closely as possible. These calibrations were also validated further with independent validation sample sets.

7.2.6 STATISTICAL INDICATORS

Standard error of laboratory (SEL) and standard deviation of difference between repeated measurements of the reference values (SDD) values were calculated for each reference method as discussed in Chapter 3. The accuracy of the predictive ability of the calibration model, relative to the reference data, was expressed as standard error of cross validation (SECV) when based on the calibration samples and as standard error of prediction (SEP) when based on independent validation sets. The performance of the calibrations was evaluated in terms of bias (which gives an indication of a systematic error in the predicted data), coefficient of determination (R^2), and the ratio between SECV:SEL and SEP:SDD. The residual predictive deviation (RPD) was used as broad indicator of the performance of the calibration models when using independent validation (Williams, 1995). RPD is defined as the ratio of the standard deviation of the reference values to the standard error of the predicted values. These criteria are discussed in detail in Chapter 3 and a summary of the criteria and the proposed interpretation thereof are given in Table 2. The criteria limits are categorised according to the suitability of a calibration for a specific purpose. Quantification refers to the determination of a quantitative value while screening refers to ability to distinguish between high, medium and low values.

Table 2. Summary of criteria used to interpret the for performance the precision of infrared calibrations

Performance criterium	Fit for quantification	Fit for quantification	Fit for screening	Unsuitable for quantification
R ² ^a	>0.9	0.7 - 0.9	0.5 – 0.7	> 0.5
SECV:SEL ^b	<1.5	2 - 3	n/a	n/a
SEP:SDD ^c	<2	<2	n/a	n/a
RPD ^d	>5	>5	3-5	<3

^aR² Coefficient of determination (Shenk and Westerhaus, 1996); ^bSECV: SEL: ratio of standard error of cross validation to standard error of laboratory (Shenk and Westerhaus, 1996); ^cSEP: SDD: ratio of standard error of prediction to standard deviation of difference for reference samples (Esbensen, 2002); ^dRPD: Residual prediction deviation (Williams, 1995).

7.3 RESULTS AND DISCUSSION

7.3.1 DESCRIPTIVE STATISTICS OF REFERENCE SAMPLES

The descriptive statistics of the wine samples used in this study are given in Table 3. The concentration range of the reference sample sets for all the wine parameters, except pH, were representative of healthy South African dry table wines. The pH values of the sample set used in this study covers a range up to 3.90 units (Table 3) and therefore the pH calibration models discussed in this section are only valid up to pH 3.9. This is not entirely representative of the pH ranges found in South African wines, which have been known to have pH values well over 4 units.

Table 3. Descriptive statistics of South African young wines used as a reference set to evaluate the performance of the global WineScan FTMIR calibrations^a and to establish new calibration models for quantification of pH, titratable acidity, volatile acidity, malic acid, lactic acid, glucose, fructose, ethanol and glycerol for the young wines.

Wine parameter	Sample No. (white:red) ^b	Value range (min. – max.) ^c	Mean ± SD ^d	SDD ^e	SEL ^f
pH	38 (20;18)	3.19 -3.90	3.58 ± 0.17	0.093	0.045
Titratable acidity g/L ^g	38 (20;18)	4.93 - 8.67	5.90 ± 0.73	0.063	0.068
Volatile acidity g/L	20 (10;10)	0.19 -0.81	0.49 ± 0.16	n/a	
Malic acid g/L	61 (41;20)	0.11 – 5.72	2.42 ± 1.89	0.056	0.034
Lactic acid g/L	61 (40;21)	0.09 – 3.55	0.65 ± 0.75	0.121	0.089
Glucose g/L	62 (54;8)	0.22-4.05	2.14 ± 0.85	0.104	0.112
Fructose g/L	67 (54;13)	0.21-6.39	2.52 ± 1.35	0.265	0.229
Ethanol %v/v	27 (14;13)	11.43-15.24	13.34 ± 0.82	n/a	n/a
Glycerol g/L	27 (14;13)	4.02-16.15	7.71 ± 3.03	0.460	0.321

^aFoss Analytical, Denmark. <http://www.foss.dk>; ^bSample number (white wine; red wine); ^cMinimum to maximum value; ^dStandard deviation; ^eStandard deviation of difference for reference samples; ^fStandard error of laboratory; ^gMeasured as g/L tartaric acid

A comparison between the concentration ranges for the global calibrations (Table 1) and the ranges for South African samples (Table 3) showed that for malic acid, alcohol and glycerol the wines used in this study fell outside the calibration ranges of the global models. This result indicated that new calibration models would have to be established for the South African samples.

7.3.2 SELECTION OF CALIBRATION SAMPLES

A typical spectrum of a wine sample is shown in Figure 1. The two areas where water absorbs, respectively $3626 - 2970 \text{ cm}^{-1}$ and $1716 - 1543 \text{ cm}^{-1}$, could be clearly distinguished by visual inspection. These regions were typically broad and covered several hundreds of wavenumbers.

In selecting calibration samples, it is important to capture as much of the spectral variation in the FTMIR spectra as possible. The variation can reside in both the x -variables (wavenumbers in this study) and the y -variables (reference values for the respective compounds in this study) a useful way of modeling the total variability is through principal component analysis of the spectra (Esbensen, 2002). The outcomes of this selection strategy are illustrated for the selection of calibration samples for malic acid (Figure 2). Samples with highest loadings on principal component 1 (PC1) and PC2 were chosen first. The calibration sets for all components consisted of the 60% of the number of samples in the reference set. Calibration samples that had relatively high loadings on PC1 and PC2 were selected first in such a way that the selected samples were evenly distributed over all four quadrants of the PCA score plot.

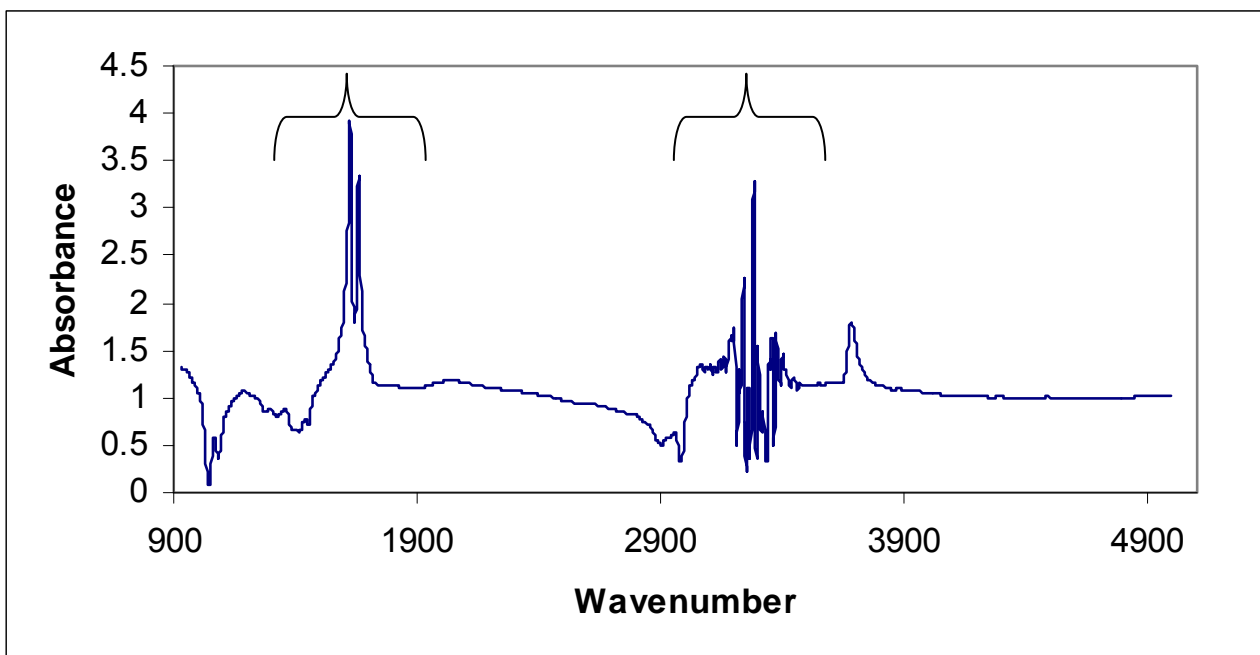


Figure 1. A typical FTMIR spectrum of an young wine generated in the wavenumber region 929 to 5011 cm^{-1} . The water 1 and water 2 regions refer to the wavenumber regions $1716-1543 \text{ cm}^{-1}$ and $2970-3626 \text{ cm}^{-1}$ respectively and is considered to contain largely spectral noise caused by intense water absorbance.

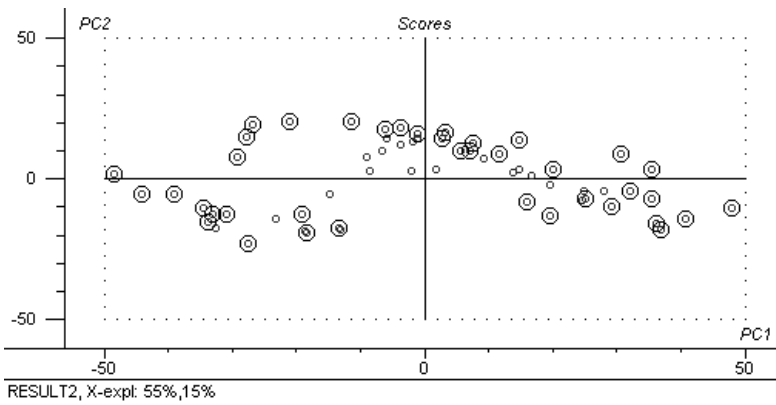


Figure 2. Graphic illustration of the selection strategy used to select calibration samples for establishing a FT-MIR spectroscopy calibration. Markers in the PCA score plot represent FT-MIR spectra of wines and the encircled markers represent samples selected for the calibration set. Uncircled markers represent samples that were used in an independent validation set.

The reference sample sets were each split up into a calibration set and a validation set by using a PCA-based sample selection method where samples were selected from. The concentration range of the calibration set samples were compared to the range of the entire reference set. A comparison between the malic acid reference set and calibration set are shown in Figure 3. The malic acid calibration samples covered the same concentration range as the reference sample set. Similar results were observed for calibration and reference sets for the other wine parameters.

In the case of fructose, the red samples clustered in one quadrant of the score plot leading to the selection of red and white calibration samples in separate PCA score plots.

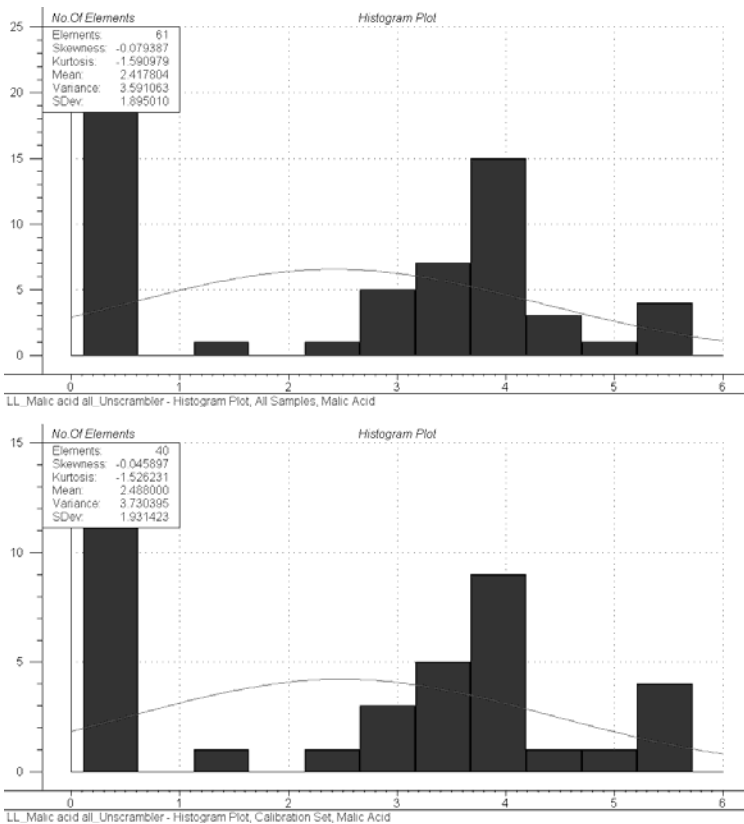


Figure 3. The malic acid distribution in the reference sample set (top) and the calibration sample set (bottom).

7.3.3 SAMPLE PREPARATION FOR FTMIR SPECTROSCOPY

Sample CO₂ levels of less than 300 mg/L were considered acceptable for FTMIR analyses. Three different procedures to remove CO₂ gas from wine samples were compared as shown in Figure 4. The control wine already contained a relatively low amount of CO₂ compared to the values that can be expected from young wines. All treatments contained significantly lower amounts of CO₂ compared to the control. The best results were obtained after multiple filtrations under vacuum, after which the sample contained just more than a third of the CO₂ present in the control sample. This method was chosen to degas the wine samples in all further analysis, with spot checks at regular intervals to ensure that the CO₂ levels in the sample wines are in fact below the required 300 mg/L.

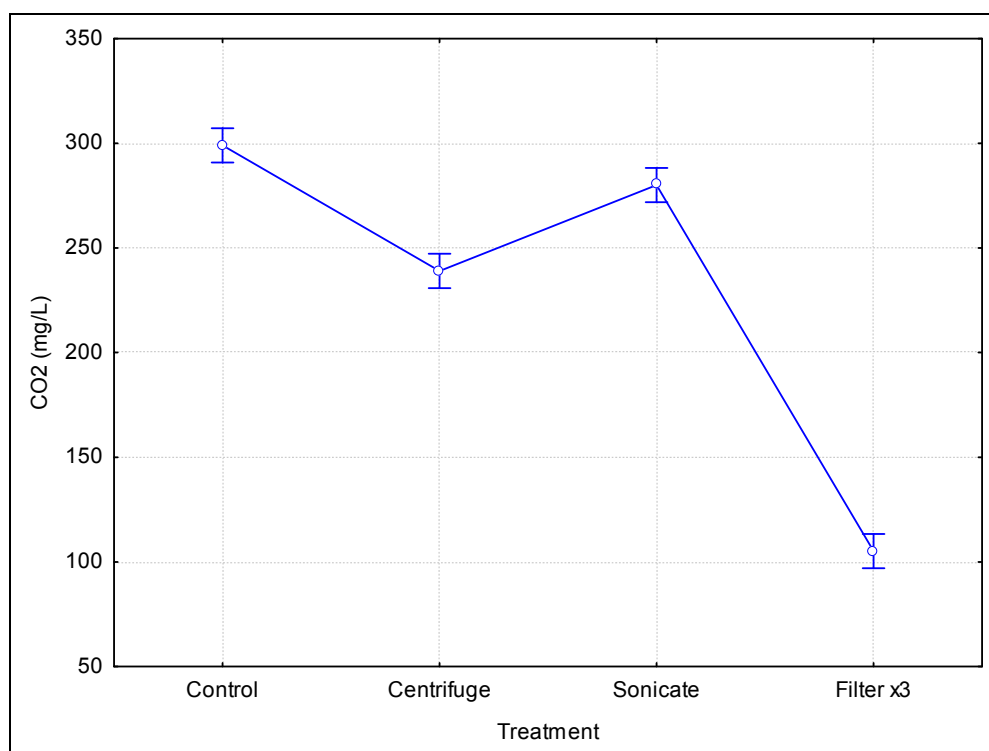


Figure 4. The CO₂ levels in a red wine after three different sample treatment procedures. Treatments were performed in triplicate using FTMIR spectroscopy. Error bars denote 95% confidence intervals .

7.3.4 EVALUATION OF GLOBAL CALIBRATIONS

The performance of the WineScan FT 120 global calibrations were evaluated in terms of the performance parameters indicated in Table 3. A summary of the validation statistics using South African young wines are given in Table 4.

7.3.4.1 pH

The pH reference values fitted reasonably well onto the commercial calibration model (Table 4). The R² for the pH model was close to 0.9, the bias was fairly low and the SEP was smaller than 2 × SDD. However, the prediction error was large relative to the standard deviation in the sample set, resulting in a RPD value much lower than 3. This could be expected, considering that the

sample set covers a relatively small range of pH values. Nevertheless, the model can be considered fit for the quantification of pH in young wines.

7.3.4.2 Titratable acidity

There was almost no correlation between the titratable acidity values determined with the reference method and the values predicted by the commercial calibration model (data not shown). The model was characterised by a high prediction error and could not be used for either screening of quantification or titratable acidity in young wines.

Table 4. The validation statistics of the performance of the WineScan FT 120 global calibrations using young South Africa wines as independent test sets.^a

Parameter	pH	Titratable Acidity g/L ^b	Volatile Acidity g/L	Malic Acid g/L	Lactic Acid g/L	Glucose g/L	Fructose g/L	Ethanol v/v%	Glycerol g/L
Number of samples (white:red) ^c	20;18	20;18	10;10	41;20	40;21	54;8	54;13	14;13	14;13
Min-Max. ^d	3.19-3.90	4.93-8.67	0.19-0.81	0.11-0.72	0.09-3.55	0.22-4.05	0.21-6.39	11.43-15.24	4.02-16.15
Mean \pm SD ^e	3.58 \pm 0.17	5.90 \pm 0.73	0.49 \pm 0.16	2.42 \pm 1.89	0.65 \pm 0.75	2.14 \pm 0.85	2.52 \pm 1.36	13.34 \pm 0.82	7.71 \pm 3.03
R ^{2f}	0.90	0.32	0.92	0.99	0.97	0.00	0.38	0.79	0.83
Bias	0.13	-0.42	-0.01	-0.41	-0.30	-1.72	-1.03	0.25	1.61
SEP ^g	0.14	0.64	0.05	0.60	0.13	1.99	1.48	0.50	2.05
SEP:SDD ^h	1.52	10.16	n/a	10.75	1.03	19.13	5.78	n/a	4.45
RPD ⁱ	1.21	1.14	3.25	3.13	6.00	0.43	1.07	1.63	1.48

^aFoss Analytical, Denmark. <http://www.foss.dk>; ^bMeasured as g/L tartaric acid; ^cNumber of white wines; number of red wines; ^dMinimum to maximum range; ^eStandard deviation; ^fCoefficient of determination; ^gStandard error of prediction; ^hRatio of standard error of prediction to standard deviation of difference for reference samples; ⁱResidual predictive deviation.

7.3.4.3 Volatile acidity

The high R² value indicates that the commercial calibration was suitable to quantify volatile acidity in young wines. In addition, the bias was very low. The prediction error was reasonable, being smaller than 10% of the average VA concentration of the reference samples. However, the RPD value was below 5, suggesting that the calibration was only suitable for the screening of young wines. The model for volatile acidity prediction could not be evaluated in terms of the SEP:SDD ratio as only one reference analysis was done per sample. At best it can be stated that the

calibration is suitable for screening and possibly for quantification. However, additional evidence of the accuracy of the reference method would be required to come to a final conclusion.

7.3.4.4 Malic acid

The reference values for malic acid fitted very well onto the commercial calibration model, with a R^2 of 0.99. Due to a high prediction error, the RPD was between 3 and 5, indicating that the model is suitable for screening only. Moreover, the SEP value was more than $2 \times$ SDD, showing that, despite the high coefficient of determination, the prediction error had to be decreased to ensure accurate quantitative determination of malic acid in young wines.

7.3.4.5 Lactic acid

The commercial calibration was suitable for the quantification of lactic acid in young wines, with a RPD value of 6 and a SEP:SDD ratio very close to 1. In addition, the R^2 was 0.97, indicating an excellent correlation between the lactic acid concentration measured with the reference method and lactic acid concentration predicted with the commercial calibration model.

7.3.4.6 Glucose

The commercial calibration was not suitable for the quantification or screening of glucose in young wines. There was no correlation between the glucose concentration measured with the reference and the concentrations predicted by the commercial calibration. Moreover, the RPD value was very low and indicated that the model was not suitable for a young wine matrix. The bias of the commercial calibration was also very high. Nevertheless, the prediction error was lower than $2 \times$ SDD.

7.3.4.7 Fructose

The fit of the calibration sample set onto the commercial fructose calibration was very poor. The R^2 value was very low and the bias was very high. Furthermore, the calibration was characterised by a high prediction error. These factors indicated that the commercial calibration was unsuitable for screening or quantification of fructose in young wines.

7.3.4.8 Ethanol

The performance of the commercial calibration in a young wine matrix was dubious. The R^2 was reasonable at 0.79, indicating that the model was fit for quantification. The bias was low and the prediction error of the commercial calibration was comparable to the legal limit for laboratory error of 0.5 v/v%. However, the RPD was well below 3, showing that the model is not suitable for screening. Unfortunately, it was not possible to determine the SDD of the reference method, and therefore the SEP:SDD ratio, as samples were not analysed in replicates.

7.3.4.9 Glycerol

The correlation between the glycerol concentrations determined with the reference method and the values predicted from the commercial calibration model was suitable for quantification. However, the prediction error was too high to be suitable for quantification or screening, as can be observed from the low RPD value and high SEP:SDD ratio. Moreover, the bias was very high, indicating a large systematic error. Hence, the prediction abilities of the commercial calibration was negatively influenced by the young wine matrix.

7.3.5 EVALUATION OF NEW PRELIMINARY YOUNG WINE CALIBRATIONS

New, preliminary calibration models were established for the determination of the abovementioned parameters in young wines. Each calibration model was established using PLS-regression and validated with an independent validation sample set. In order to improve the quality of the calibrations that did not sufficiently comply to the precision criteria, the number of wavenumbers used for the calibrations were enlarged. It is possible that all the variance in the data were not modeled with the selected wavenumbers calculated by the instrument software. Calibrations were performed according to the procedure of the instrument software. Two options were investigated, using the full spectral range as variables and using the intervals 2966-1720 cm^{-1} and 1539-925 cm^{-1} as variables. The latter intervals are the spectral regions that remains after the water absorbance regions were discarded and will henceforth be referred to as the extended spectral region. Results are shown in Table 5.

Table 5. A comparison of important validation statistics for calibrations using a variety of spectral regions.

Model	Filter Selection			Full Spectrum			Extended Spectrum		
	R ^{2a}	SEP ^b	RPD ^c	R ²	SEP	RPD	R ²	SEP	RPD
pH	0.85	0.09	3.32	-0.17	0.16	1.98	0.95	0.04	8.20
Titrateable acidity	0.92	0.24	2.35	0.82	0.34	1.66	0.87	0.30	1.91
VA ^d	0.89	0.07	2.47	0.39	0.14	1.16	0.65	0.11	1.49
Glucose	0.84	0.29	2.16	0.79	0.40	1.57	0.86	0.44	1.43
Ethanol	0.51	0.50	1.14	0.73	0.36	1.60	0.89	0.31	1.83
Glycerol	0.97	1.01	2.94	0.77	1.74	1.71	0.88	1.33	2.24

^a Coefficient of determination; ^b Standard error of prediction; ^c Residual prediction deviation ^d Calculated with The Unscrambler Software using the entire reference sample set

7.3.5.1 pH

The new preliminary calibration model for pH performed well. The Coefficient of determination indicated good precision and the bias was close to zero (Table 6). Furthermore, the SEP:SDD and

SECV:SEL ratios denoted excellent precision. The RPD value was between 3 and 5, judging the model suitable for screening purposes. Overall the model was fit for quantification.

The model for pH based on the extended spectral region was a significant improvement on the previous model (Figure 5). The validation statistics show a coefficient of determination larger than 0.9 and a RPD value much larger than 5, indicating that this model can be used for quantification. The SEP of this model was in fact much lower than the SDD, confirming the excellent performance of the model. However, the sample set should be expanded to include pH values higher than 4 units.

Table 6. The calibration and validation statistics for the evaluation of the new preliminary young wine calibrations of pH, titratable acidity and malic acid.

Calibration				Validation			
Parameter	pH	Titratable acidity (g/L)	Malic acid (g/L)	Parameter	pH	Titratable acidity (g/L)	Malic acid (g/L)
Number of Samples	23	24	36	Number of Samples	10	11	22
Range	3.20 -3.90	5.01 - 8.68	0.11 -5.72	Range	3.32 -3.74	5.12 -6.69	0.12 -4.49
Mean \pm SD ^a	3.59 \pm 0.20	6.03 \pm 0.80	2.53 \pm 1.93	Mean \pm SD ^a	3.56 \pm 0.31	5.97 \pm 0.57	2.28 \pm 1.86
Number of Components	7	15	10	R ^{2e}	0.85	0.92	0.98
AR ^b	0.00	0.05	0.03	Bias	-0.04	0.19	-0.03
SECV ^c	0.05	0.13	0.20	SEP ^f	0.09	0.24	0.26
SECV:SEL ^d	1.21	1.85	5.92	SEP:SDD ^g	1.01	3.84	4.66
				RPD ^h	3.32	2.35	7.13

^a Standard deviation; ^b Absolute repeatability; ^c Standard error of cross validation; ^d Standard error of cross validation to standard error of laboratory; ^e Coefficient of determination; ^f Standard error of prediction; ^g Standard error of prediction to standard deviation of difference; ^h Residual prediction deviation

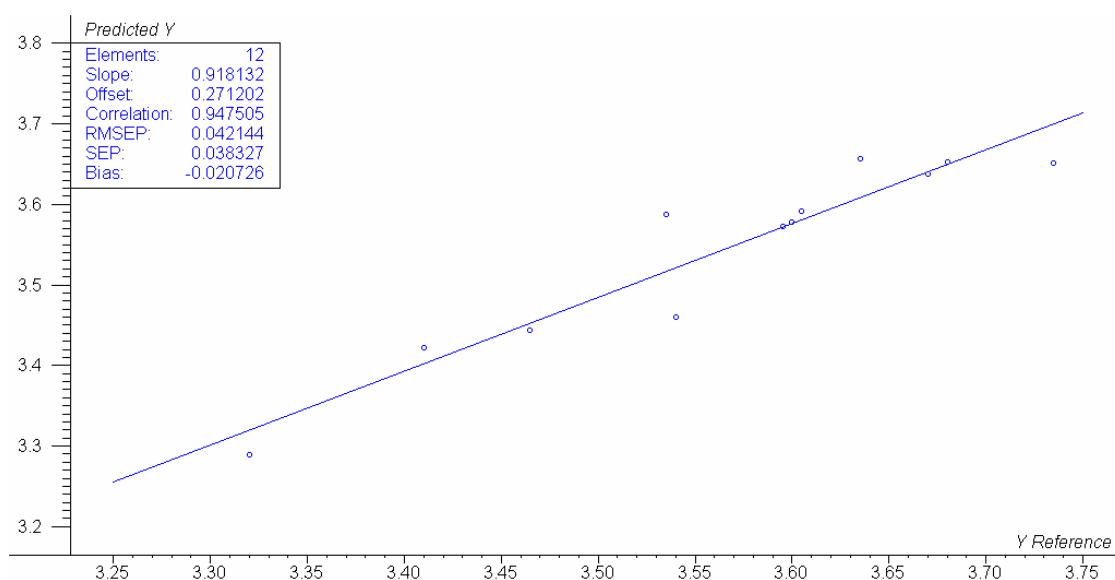


Figure 5. The preliminary new FTMIR calibration model for pH in young wines using PLS-regression and the extended area of the mid-infrared spectra.

7.3.5.2 Titratable acidity

The titratable acidity model based on young wines performed well in the calibration phase (Figure 6). The combination of a R^2 exceeding 0.9 and a SECV:SEL ratio of 1.8 indicates good precision (Table 6). However, the SEP of the young wine model did not compare well to the standard deviation or the SDD value of the independent validation set. This implies that the model was not strong enough to uphold its performance when applied to independent samples. The performance parameters worsened when enlarged spectral regions were used for the calibration model.

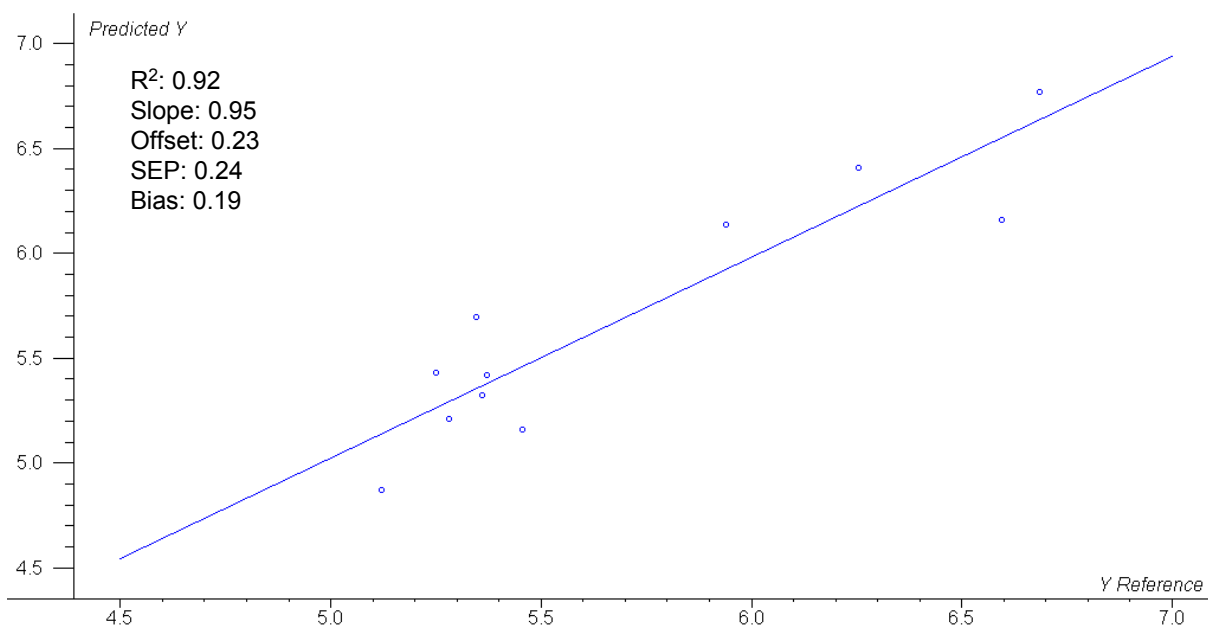


Figure 6. The preliminary new FTMIR calibration model for titratable acidity in young wines using PLS-regression and selected highly correlated wavenumber filters.

7.3.5.3 Malic acid

The preliminary calibration set up with the young wine sample set performed very well (Figure 7). The bias was close to zero and the R^2 was excellent. The SEP was low and the RPD value was above 7. The model did not meet the SEP:SDD ratio or SECV:SEL criteria, suggesting that the RPD value, based on a high standard deviation, might be an overoptimistic indication of the model's prediction abilities. However, in the light of the high coefficient of determination the model was considered suitable for quantification of malic acid in young wines.

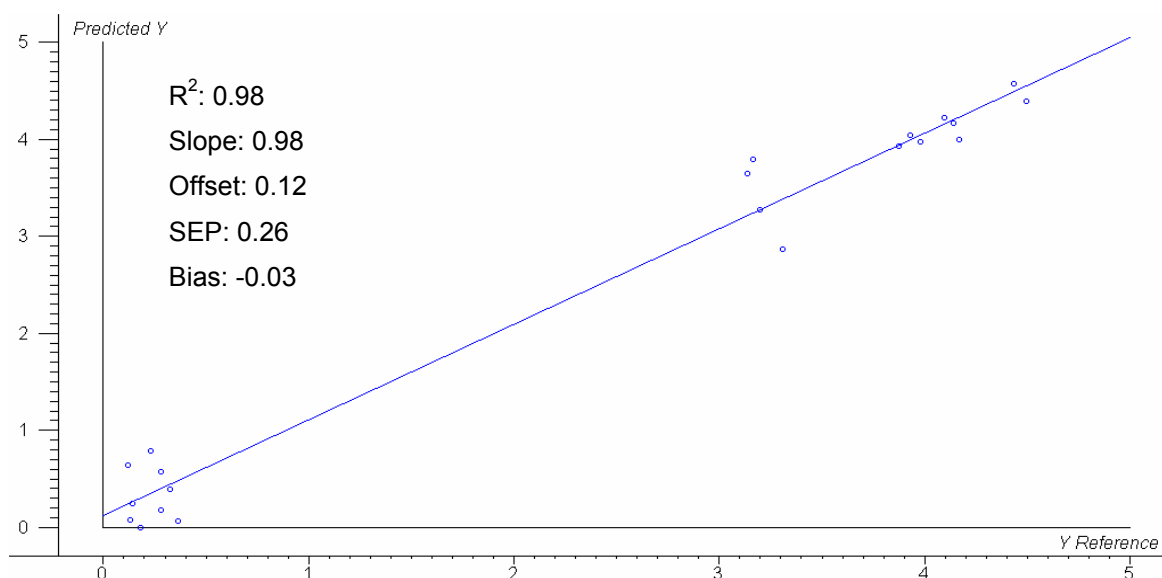


Figure 7. Performance of a preliminary new FT-MIR calibration model for malic acid in young wines using PLS regression and a selection of highly correlated wavenumber filters

7.3.5.4 Volatile acidity

The number of samples in the reference set was too little to validate a calibration model established with young wines with an independent validation set. Instead, the calibration was set up with the entire reference set and validated with cross validation (Figure 8). The R^2 was sufficient for quantification. However, the prediction error was high compared to the average of the reference set and the RPD was too low to be considered suitable for screening (Table 4). It could be possible that a larger sample set might improve the results. The preliminary calibrations using the full spectral region or the extended region were considerably worse than the calibration using selected wavenumber filters. In the case of volatile acidity, the use of a refined selection of wavenumbers seems to be the most efficient.

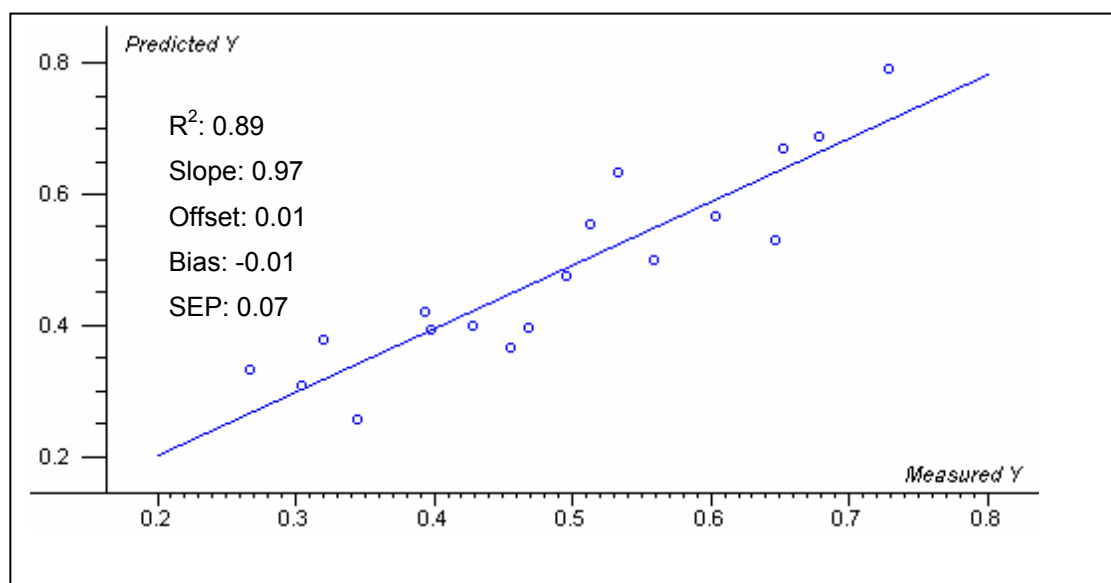


Figure 8. Performance of a preliminary new FT-MIR calibration model for volatile acidity in young wines using PLS regression and a selection of highly correlated wavenumber filters

Table 7. The calibration and validation statistics for the evaluation of the new preliminary young wine calibrations of lactic acid, glucose and fructose.

Calibration				Validation			
Parameter	Lactic acid (g/L)	Glucose (g/L)	Fructose (g/L)	Parameter	Lactic acid (g/L)	Glucose (g/L)	Fructose (g/L)
Number of Samples	42	27	36	Number of Samples	25	26	23
Range	0.09-3.55	0.22-4.05	0.21-6.38	Range	0.15-1.94	0.34-3.11	0.22-4.83
Mean \pm SD ^a	0.70 \pm 0.84	2.03 \pm 1.00	2.56 \pm 1.44	Mean \pm SD ^b	0.44 \pm 0.51	2.18 \pm 0.62	2.41 \pm 1.27
Number of Components	10	6	8	R ^{2e}	0.96	0.84	0.98
AR ^b	0.04	0.06	0.06	Bias	0.04	0.07	-0.10
SECV ^c	0.19	0.32	0.26	SEP ^f	0.16	0.29	0.20
SECV:SEL ^d	2.18	2.81	1.12	SEP:SDD ^g	1.29	2.78	0.40
				RPD ^h	3.28	2.16	6.22

^aStandard deviation; ^bAbsolute repeatability; ^cStandard error of cross validation; ^dStandard error of cross validation to standard error of laboratory; ^eCoefficient of determination; ^fStandard error of prediction; ^gStandard error of prediction to standard deviation of difference; ^hResidual prediction deviation.

7.3.5.5 Lactic acid

The preliminary lactic acid model for young wines performed well in terms of R² and bias (Figure 9). In addition, the SECV:SEL and SEP:SDD ratios indicated good precision (Table 7). Although the RPD value was below 5, the model was considered suitable for quantification.

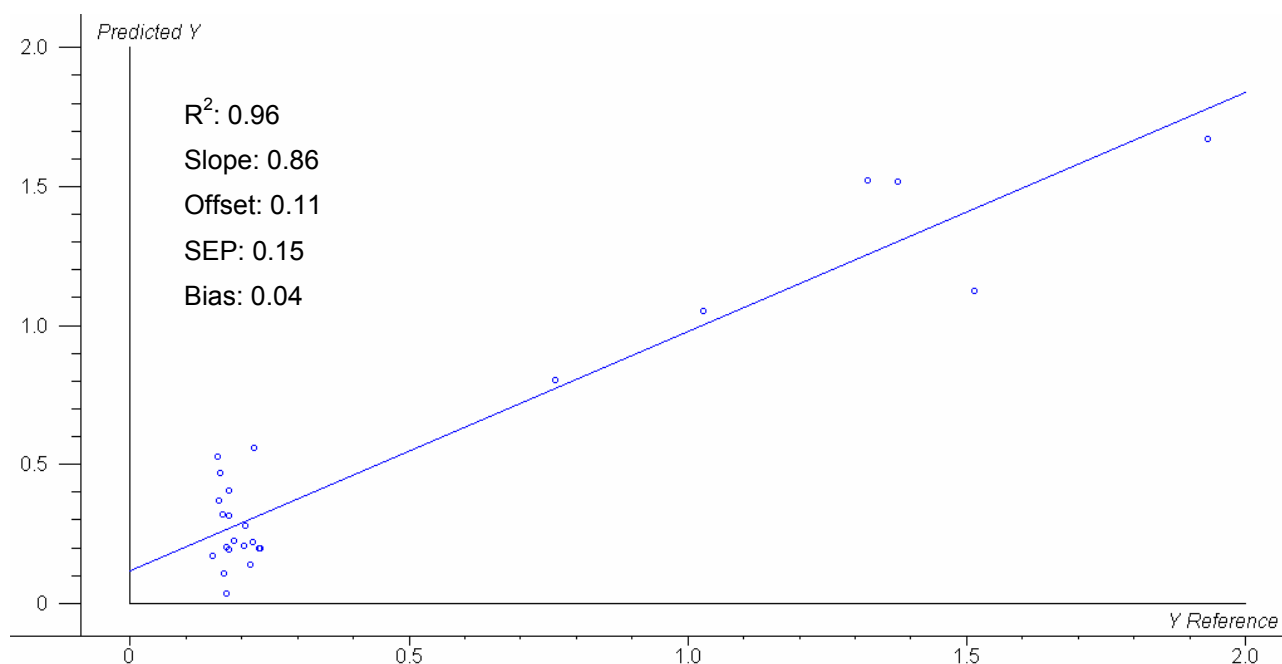


Figure 9. Performance of a preliminary new FTMIR calibration model for lactic acid in young wines using PLS regression and a selection of highly correlated wavenumber filters

7.3.5.6 Glucose

The calibration statistics for the preliminary glucose calibration for young wine samples were satisfactory, with a R^2 of 0.84 and the ratio of SECV to SEL was between 2 and 3 (Table 7). After validation with an independent sample set, the prediction error was too high, causing a RPD value below 3 and a SEP:SDD ration higher than 2. This indicated that the calibration (Figure 10) could not be used for quantification. It was attempted to improve the calibration statistics by using less restricted spectral areas to build the regression model. The validation statistics worsened where the whole mid-infrared wavenumber range was used. Although the coefficient of determination improved slightly when using the extended spectral region, the prediction error was much higher than that of the model using the wavenumber filters. None of the calibration options attempted in this study was suitable for the quantification of glucose in young wines. It has previously been reported that spectral interferences could occur during the quantification of sugars in dry wines with FTMIR (Moreira, 2004). It was stated that the most important absorption bands for sugar determination (C-O and H-O) are similar to that of organic acids, which occurs in much larger quantities in dry wines. It would be more sensible to include off-dry wines (> 5g/L residual sugar) into the sample set with the purpose to establish a screening model to distinguish between dry and non-dry wines.

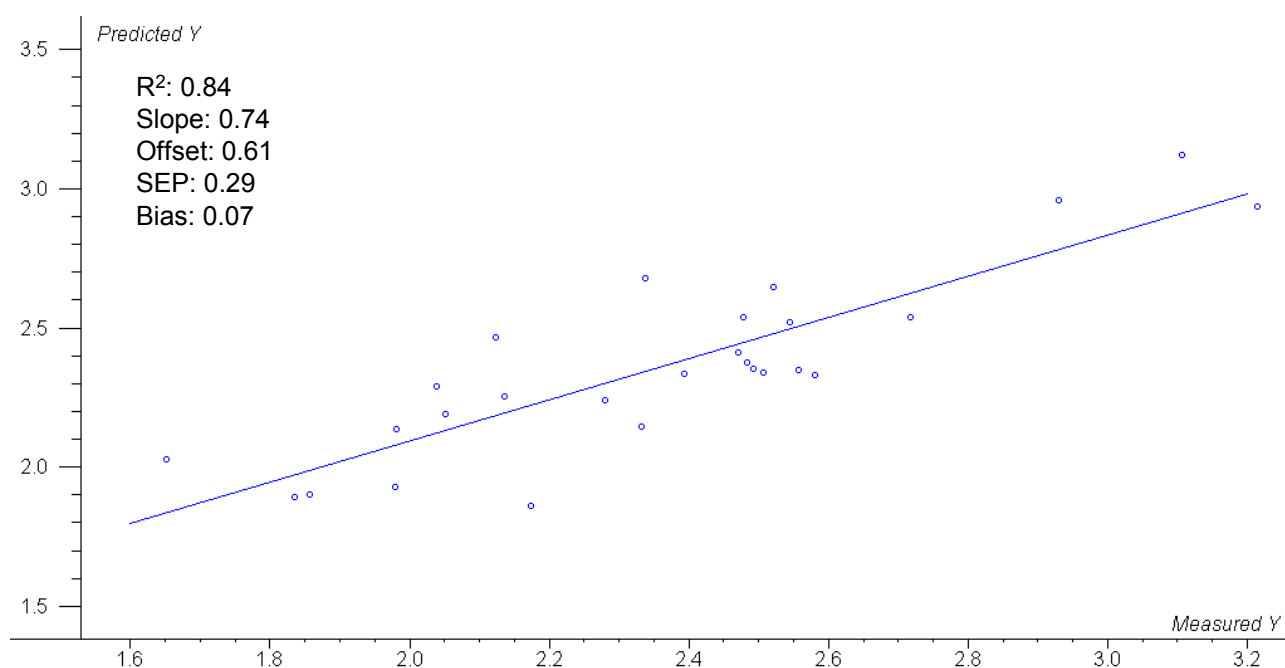


Figure 10. Performance of a preliminary new FTMIR calibration model for glucose in young wines using PLS regression and a selection of highly correlated wavenumber filters

7.3.5.7 Fructose

The preliminary calibration with the young wine sample set performed very well (Figure 11). The coefficient of determination was 0.98 and the RPD value 6.2 meaning that the calibration can be used for the quantification of fructose in young wines (Table 7). These results were supported by

the SEP:SDD and SECV:SEL ratios which also complied to the requirements for good precision in a calibration model.

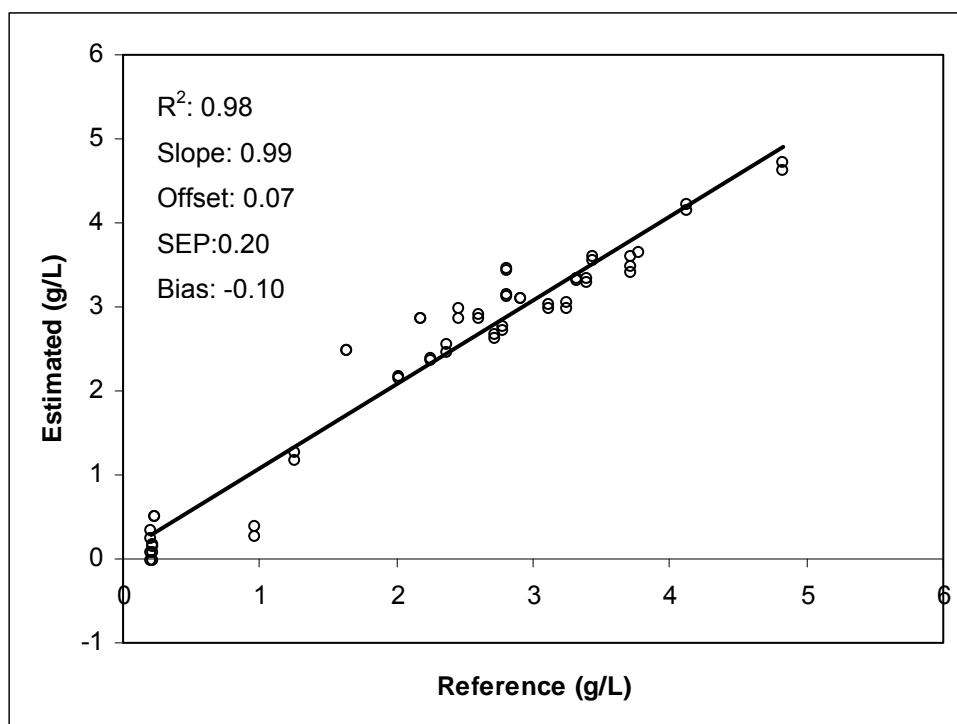


Figure 11. Performance of a preliminary new FTMIR calibration model for fructose in young wines using PLS regression and a selection of highly correlated wavenumber filters

Table 8. The calibration and validation statistics for the evaluation of the new preliminary young wine calibrations of ethanol and glycerol.

Parameter	Calibration		Parameter	Validation	
	Ethanol (v/v%)	Glycerol (g/L)		Ethanol (v/v%)	Glycerol (g/L)
Number of Samples	18	16	Number of Samples	10	9
Range	11.43-15.24	4.02-16.18	Range	12.28-14.11	4.52-12.99
Mean \pm SD ^a	13.40 \pm 0.95	7.48 \pm 3.37	Mean \pm SD ^b	13.25 \pm 0.57	8.42 \pm 2.98
Number of Components	15	15	R ^{2e}	0.52	0.97
AR ^b	0.05	0.09	Bias	-0.24	-0.42
SECV ^c	0.22	1.11	SEP ^f	0.50	1.02
SECV:SEL ^d	n/a	3.47	SEP:SDD ^g	n/a	2.20
			RPD ^h	1.14	2.94

^aStandard deviation; ^bAbsolute repeatability; ^cStandard error of cross validation; ^dStandard error of cross validation to standard error of laboratory; ^eCoefficient of determination; ^fStandard error of prediction; ^gStandard error of prediction to standard deviation of difference; ^hResidual predictive deviation.

7.3.5.8 Ethanol

Unsatisfactory results were obtained when the preliminary calibration was set up using young wine samples with selected wavenumbers and the calibration was unsuitable for screening. The results improved slightly when the spectral range used for the calibration was enlarged. The full spectral

range resulted in a coefficient of determination suitable for quantification, and the SEP was much lower than the legal limit for the laboratory error of ethanol determinations (Table 4). The best calibration model was achieved using the extended spectral region, where a fairly good coefficient of determination of 0.89 was observed (Figure 12). The RPD value was still below the limit for screening purposes. A better indication of the precision of the calibrations would have been possible if the reference samples were measured in duplicate.

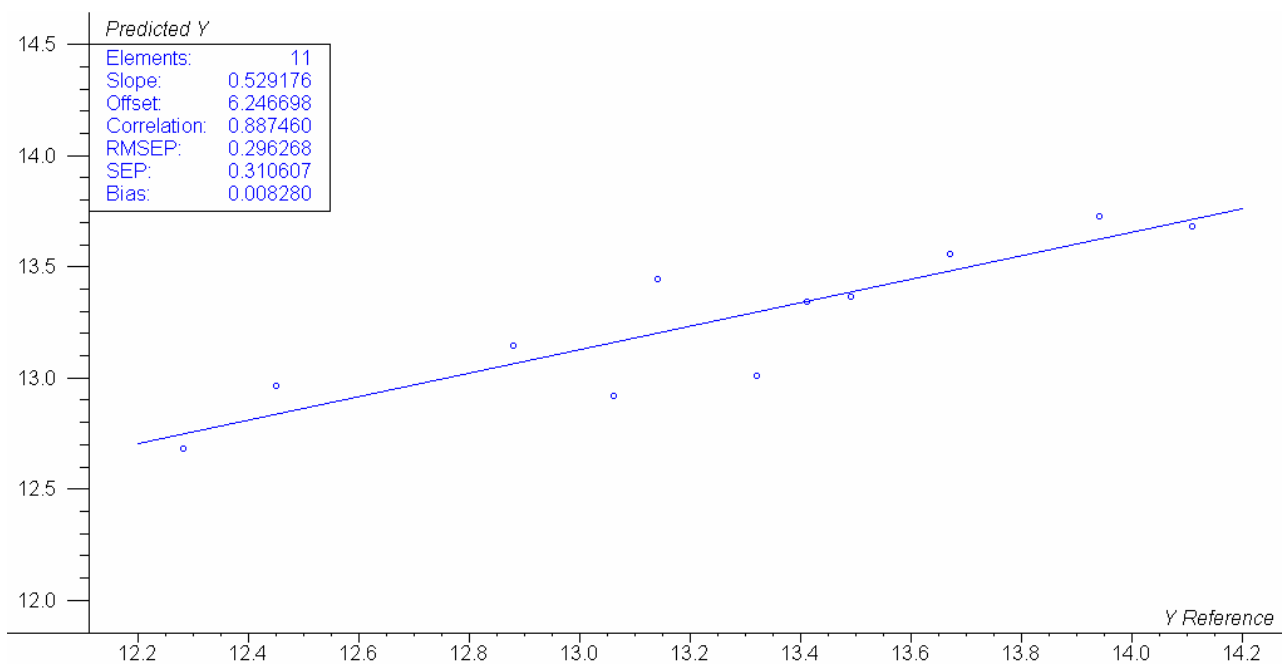


Figure 12. The preliminary new FTMIR calibration model for ethanol in young wines using PLS-regression and the extended spectral area of the mid-infrared spectra.

7.3.5.9 Glycerol

The coefficient of determination of the new preliminary calibration for glycerol in young wines was satisfactory at 0.96 (Figure 13). However, the prediction error was too high compared to the error of the reference method. The results could not be improved by using larger areas of the spectral region.

A possible solution was to set up separate calibrations for red and white wines, as red wines usually contain much more glycerol than white wines. This would result in a more specified range of values in the calibration model and possibly a smaller prediction error. However, the sample set used in this study is too small to be split up as this without compromising the use of an independent validation set. A very rough calibration model was established for white and red wines individually using selected wavenumber filters. After three badly predicted samples were removed, the cross validation error of the rough red wine model (Table 9) was lower than that of the model for both types of wines. The ratio of SECV to SEL for the preliminary red wine model was 2.6, indicating good precision. The absolute repeatability of the red wine model was also lower than the model for all the wines and the red wine model also needed fewer components to reach the minimum residual variance level. It seems promising to investigate the use of a separate glycerol

calibration for young red wines. Unfortunately, the calibration results for glycerol in young white wines were worse than the calibration for all the wines. However, these results are based on very small sample sets without proper validation with an independent sample set.

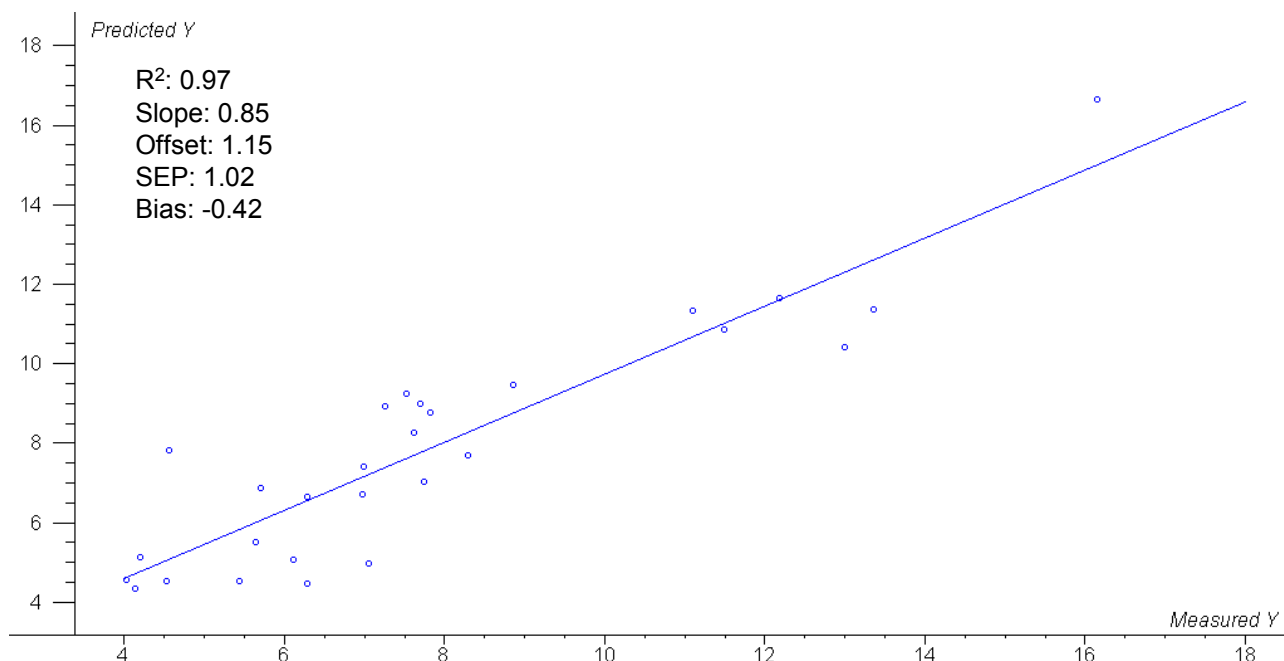


Figure 13. Performance of a preliminary new FT-MIR calibration model for glycerol in young wines using PLS regression and a selection of highly correlated wavenumber filters

Table 9. Comparison between selected calibration statistics of Glycerol prediction models for all wines, red wines and white wines.

Model	Number of Components	AR ^a	SECV ^b	SECV: SEL ^c
All Samples	15	0.094	1.114	3.470
Red Samples	4	0.036	0.835	2.601
White Samples	1	0.018	1.256	3.912

^aAbsolute repeatability; ^bStandard error of cross validation; ^cStandard error of cross validation to standard error of laboratory.

To conclude the results of this study it is most important to note that all compounds can not be quantified in a generic fashion with FT-MIR, as was seen with the young wines used in this study. Some of the components could be quantified with a global commercial calibration, even though it was not set up for a young wine matrix, while others were better suited to a more specific calibration. In some cases the calibrations performed better when only a few highly correlated wavenumbers were used, while in other cases a larger selection of wavenumbers gave more precise results. In future studies, where the samples that need to be analysed deviates from the samples used to establish the calibration, the matrix effects on the performance of the calibration needs to be established. It also seems useful to evaluate the effect of varying spectral ranges in order to avoid over- or under fitting of the model. In addition, the performance of calibration models

should not be evaluated with a recipe-like approach, but rather by comparing various performance criteria while keeping the properties of the sample set in mind.

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Chapter 8

RESEARCH RESULTS

**Quantification of volatile compounds in wine
with FTMIR spectroscopy**

RESEARCH RESULTS

ABSTRACT

Yeast starter cultures and fermentation related products are continuously being developed to improve amongst other, the efficiency of alcoholic fermentation and flavour properties of wine. Volatile compounds formed during alcoholic fermentation have an important impact on wine quality and aroma. The objective of this study was to evaluate Fourier transform infrared spectroscopy (FTMIR) as a rapid analytical tool to predict the concentrations of yeast derived volatile compounds. The volatile compounds that served as reference values for these calibrations were determined with gas chromatography flame ionisation detection (GC-FID) in young South African wines. FTMIR spectroscopy calibration models for four groups of volatile compounds, namely total alcohols, total fatty acids, total esters and esters (total esters, but excluding ethyl acetate) were developed. The performance of the models was evaluated in terms of R^2 , bias, prediction error and the relationship between the prediction error and the measurement error of the reference analytical method (GC-FID). Good linearity were observed for the “total alcohols”, “total fatty acids” and “esters” groups. An interesting polynomial trend was observed for the “total esters group”. Some possible spectroscopic interferences were observed. All four preliminary calibration models were considered suitable for screening purposes, although further investigations with regards to sample matrices should be investigated.

8.1 INTRODUCTION

During wine production, product development and quality control play an important role in maintaining a competitive edge. The most important process during winemaking is alcoholic fermentation where yeast converts sugar to ethanol. Apart from the conversion of sugar to ethanol, yeasts have an enormous influence on the aroma characteristics of wine (Lambrechts and Pretorius, 2000).

Several of the most abundant aroma compounds present in wine are formed by yeast metabolism during alcoholic fermentation. Among these compounds are higher alcohols, fatty acids and esters. The most prominent alcohols in wine, besides ethanol, are 1-propanol, isobutanol, isoamylalcohol and 2-phenylethanol. Higher alcohols have a strong, heady smell at high concentrations, but at less than 0.3 g/L they add to the complexity of wine. The alcohol, 2-phenylethanol is associated with honey, spicy and rose-like aromas in wine (Francis and Newton, 2005). The most important esters present in wine are ethyl esters and acetate esters. Esters are generally associated with a fruity, pleasant aroma. The low molecular weight ester, ethyl acetate, tends to smell like varnish and is generally associated with wine spoilage (Lambrechts and Pretorius, 2000). Aliphatic saturated fatty acids are the most common fatty acids found in wine and chain lengths of up to 14 carbon atoms have been reported. Some of the most common fatty acids

in wine are acetic, hexanoic, octanoic and decanoic acids. At high levels, these compounds are associated with rancid, cheesy and vinegar-like aromas, but in healthy wines they are usually present below their detection threshold (Schreier, 1979).

The advantages of Fourier transform mid-infrared (FTMIR) spectroscopy for rapid wine screening and quality control have been reported by several authors (Patz *et al.*, 2004; Kupina and Shrikhande, 2003; Gishen and Holdstock, 2000). FTMIR spectroscopy determines the absorption of infrared radiation by molecules that contain amongst other, C-H, O-H, C-O, C=O and N-H molecular bonds and typically the data are presented in the form of a spectrum of absorbance or transmittance versus wavenumbers. The spectra can be used to establish algorithms or calibration models to predict the concentration of organic components of interest, by the application of chemometric techniques such as partial least squares regression1 (PLS1.) (Wehling, 1998). This is done by correlating the concentration of a specific compound measured with a reference method to the absorption of infrared radiation at specific wavenumbers. The selection of suitable wavenumbers is an important part of the calibration process. The wavenumber regions 3626-2970 cm^{-1} and 1716-1543 cm^{-1} are mostly associated with interference caused by water absorbance, while it has also been reported the 5011-3630 cm^{-1} regions contains very little useful wine-related information (Nieuwoudt, *et al.*, 2004; Patz, *et al.*, 2004).

The use of GC-FID to quantify volatile compounds in wine is possibly one of the most frequently used techniques for routine analysis of these compounds in wine. Some situations require that large numbers of samples must be analysed, typically where experimental wines are produced in yeast development programs. In these cases some aspects of GC-FID analysis are time consuming and expensive. The aim of this study was to investigate the use of FTMIR spectroscopy for the rapid screening of yeast derived volatile compounds in young wines. Due to the low concentrations of some individual volatile compounds, the FTMIR screening abilities were evaluated for groups of compounds defined by common chemical characteristics. Three main groups were defined: total alcohols, consisting of higher alcohols and the aromatic alcohol 2-phenylethanol, total fatty acids and total esters. Another group, esters, was also defined. The latter group does not include ethyl acetate due to its association with wine spoilage. The role of FTMIR as a rapid screening method for volatile composition in wine has not been previously reported in the literature to our knowledge.

8.2 METHODS AND MATERIALS

8.2.1 WINE SAMPLES

Bottled wine samples (n = 200) were collected from the South African Young Wine Show 2006 and were stored at 4-8°C till analysed. Wines were specifically chosen to include important South African cultivars in order to be representative of South African wines. The wine samples included Sauvignon blanc, Chardonnay, Pinotage, Merlot, Cabernet Sauvignon and Shiraz wines.

8.2.2 GC-FID ANALYSIS

8.2.2.1 Chemicals, standards and wine simulant

Ethyl acetate and isoamyl acetate was purchased from Riedel de Haën (Seelze, Germany). Methanol, hexanol, acetic acid and 2-phenylethanol standards were from Merck (Darmstadt, Germany). Ethyl butyrate, propanol, isobutanol, butanol, hexyl acetate, ethyl lactate, propionic acid, iso-butyric acid butyric acid, iso-valeric acid, diethyl succinate, valeric acid, 2-phenylethyl acetate, 4-methyl-2-pentanol and hexane were from Fluka (Buchs, Switzerland). Hexanoic acid, octanoic acid, isoamyl alcohol, ethyl caprylate, ethyl caprate were from Aldrich (Steinheim, Germany). Decanoic acid and ethyl hexanoate were purchased from Sigma (St. Louis, USA). Diethyl ether, ethanol and NaSO₄ were also purchased from Merck (Darmstadt, Germany).

The internal standard and volatile standards were dissolved in a wine simulant consisting of 12 %v/v ethanol and 2.5 g/L tartaric acid (Merck) in de-ionised water from a MilliQ system, pH adjusted to 3.5 with 0.1 M NaOH (Merck).

Calibrations for quantification of individual volatile compounds were established as described in Addendum A.

8.2.2.2 Extraction of volatile compounds

Five millilitres of wine with internal standard, 4-Methyl-2-Pentanol, (100µl of 0.5mg/l solution in wine simulant) were extracted with 1 millilitres of diethyl ether by sonicating the ether/wine mixture for five minutes. The wine/ether mixture was then centrifuged at 3600 g for 3 minutes. The ether layer was removed and dried on NaSO₄. Each extract was injected into the GC-FID in triplicate.

8.2.2.3 Gas Chromatography conditions

A J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length × 0.32 mm i. d. × 0.5 µm f.t was used. The initial oven temperature was 33°C for 17 minutes after which the temperature was increased by 12°C/min to 240°C, at which it was held for 5 minutes. Three µl of the diethyl extract was injected at 200°C. The split ratio was 15:1 and the split flow rate 49.5 ml/min. The column flow rate was 3.3 ml/min and the total run time was 50 minutes. The detector temperature was 250°C. After each sample run, a post run of 5 minutes at oven temperature 240°C, with a column flow of 6 ml/min cleaned the column from high boiling contaminants. After every 30 samples the column was thermally cleaned by injecting hexane several times isothermally, holding it for 10 minutes per injection at an oven temperature of 220°C.

8.2.3 FTMIR SPECTROSCOPY

8.2.3.1 Sample preparation

Samples were filtered with a filtration unit (type 79500, FOSS Analytical, Denmark) connected to a vacuum pump. Filter paper disks graded with pore size 20 to 25 µm and diameter 185 mm (Schleicher & Schuell, Germany, catalogue No. 10312714) were used for filtration. Red wines were

filtered twice and white wines three times before FTMIR spectroscopy in order to keep the CO₂ levels of the wines lower than 300 mg/L. Quantification of CO₂ was done using the WineScan FT 120 spectrometer.

8.2.3.2 Generation of FTMIR spectra

Instrument: A WineScan FT 120 spectrometer (WineScan FT120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001).

Spectral acquisition and processing: Degassed wine samples (7 mL) were pumped through the CaF₂-lined cuvette (path length 37 μ m) of the spectrometer at a constant temperature of 40°C. Samples were scanned from 5011 - 929 cm⁻¹ at 4 cm⁻¹ intervals. The amounts of infrared radiation transmitted by the samples were recorded at the detector and used to generate an interferogram that is calculated from a total of 20 repeat scans. Subsequently the interferogram was converted to a single beam transmittance spectrum by Fourier transformation (WineScan FT120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001).

8.2.4 FTMIR SPECTROSCOPY CALIBRATIONS

8.2.4.1 Definition of groups of volatile compounds

The amounts of the individual compounds analysed with gas chromatography were added together to form four analyte groups: total alcohols, total fatty acids, total esters and esters, respectively. The compound groups and their composites are listed in Table 1. The esters group consisted of all esters analysed under the specific GC-FID conditions used in this study, except ethyl acetate. Each of these groups of compounds was considered as a new y -variable for the purposes of the PLS1 calibration discussed in section 2.4.3.

Table 1. Volatile compounds included in each group of volatile compounds .

Total Alcohols	Total Fatty Acids	Total Esters	Esters
Methanol	Acetic Acid	Ethyl Acetate	Ethyl Butyrate
Propanol	Propionic Acid	Ethyl Butyrate	Isoamyl Acetate
Isobutanol	Iso-Butyric Acid	Isoamyl Acetate	Ethyl Hexanoate
Butanol	Butyric Acid	Ethyl Hexanoate	Hexyl Acetate
Isoamyl Alcohol	Iso-Valeric Acid	Hexyl Acetate	Ethyl Lactate
Hexanol	Valeric Acid	Ethyl Lactate	Ethyl Caprylate
2-Phenylethanol	Hexanoic Acid	Ethyl Caprylate	Ethyl Caprate
	Octanoic Acid	Ethyl Caprate	Diethyl Succinate
	Decanoic Acid	Diethyl Succinate	2-Phenylethyl Acetate
		2-Phenylethyl Acetate	

8.2.4.2 Selection of calibration samples

The reference sample set of 200 wines was divided into a calibration and validation set containing 116 and 84 samples respectively. The samples for the calibration set was selected by sample selection method using principal component analysis as described in section 2.3 in Chapter 7. The concentration range of the full sample set and the calibration set were compared with histograms to ensure that they cover the same concentration range for each of the analyte groups. The statistical analysis for the sample selection was performed with The Unscrambler 9.2 software (Camo ASA, Trondheim, Norway).

8.2.4.3 Establishment of calibration models for groups of volatile compounds

New calibrations were set up using PLS1 regression in the Advanced Performance software module version 2.2.2 supplied by the manufacturers of the FTIR spectrometer (WineScan FT120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001). PLS1 has been described in detail in Chapter 3 of this thesis. Calibration models were validated using ten segmented cross-validation as pre-programmed by the WineScan software. Fifteen filters (individual wavenumbers or small groups of adjacent wavenumbers) were automatically selected by the software in such a way that the selected wavenumbers collectively captured the maximum information related to the variation in the concentrations of the y-variables. Each new calibration was validated using independent test set validation sample sets.

8.2.5 STATISTICAL INDICATORS

The precision of the reference methods were evaluated by calculating the standard error of laboratory (SEL) and the standard deviation of difference (SDD) as discussed in Chapter 3. The performance of the calibrations were evaluated in terms of bias, coefficient of determination (R^2), standard error of cross validation (SECV) and standard error of prediction (SEP). In order to interpret these errors the ratios of SECV:SEL, SEP:SDD and residual predictive deviation (RPD) were used. These criteria are discussed in detail in Chapter 3 and a summary of the interpretations attached to these ratios are given in Table 2.

Table 2. Summary of performance criteria for evaluating the FTMIR spectroscopy calibrations.

Performance criterium	Excellent for quantification	Good for quantification	Reasonable for screening	Unsuitable for quantification	for
R^2 ^a	>0.9	0.7 – 0.9	0.5 – 0.7	> 0.5	
SECV:SEL ^b	<1.5	2 – 3	n/a ^c	n/a ^c	
SEP:SDD ^d	<2	<2	n/a ^c	n/a ^c	
RPD ^e	>5	>5	3-5	<3	

^a R^2 : Coefficient of determination; ^bRatio standard error of cross validation (SECV) to standard error of laboratory (SEL) (Shenk and Westerhaus, 1996); ^cn/a: not applicable; ^dRatio of standard error of prediction (SEP) to standard deviation of differences between repeat reference measurements (Esbensen, 2002); ^eRPD: Residual predictive deviation (Williams, 1995).

8.3 RESULTS AND DISCUSSION

8.3.1 VOLATILE COMPOSITION OF WINES

Descriptive statistics of the wine samples classified according to the groups of components are given in Table 3. Histograms of the distribution of concentrations for each group of compounds in the calibration set were compared to the reference set. In each case the calibration sets covered a similar range of values as the corresponding reference set (data not shown).

Table 3. Descriptive statistics of South African young wines used as a reference set for the establishment of FT-MIR calibrations of volatile compounds in young wines.

Group of compounds ^a	Sample No. (white;red) ^b	Value range (min. – max.) ^c	Mean ± SD ^d
Total alcohols (g/L)	200 (73;127)	0.22 – 1.14	0.56 ± 0.22
Total fatty acids (g/L)	200 (73;127)	0.12 – 1.02	0.50 ± 0.16
Total esters (g/L)	200 (73;127)	0.33 – 1.62	0.77 ± 0.28
Esters (g/L)	200 (73;127)	0.28 – 1.57	0.70 ± 0.28

^aDefinition of groups of compounds according to Table 1; ^bsample number (white wine; red wine); ^cMinimum to maximum value; ^dStandard deviation.

8.3.2 TOTAL ALCOHOLS

The first three wave number filters that were selected accounted for 83% of the total explained variance in the dataset (Table 4). These wave numbers corresponded to the absorption areas of O-H and C-O molecular bonds (Wehling, 1998). The fact that the most important wave number corresponded to absorbance by the O-H bond was expected as -OH is the functional group of alcohols. The importance of the C-O stretch probably refers to the molecular bond between the main carbon chain of the alcohol and the O-H group.

The linearity of the calibration indicated good precision (Shenk and Westerhaus, 1996), especially considering the low concentrations of higher alcohols present in the wines (Table 5 and Figure 1). The bias of the regression curve was close to zero. The precision parameters referring to the ratio of the prediction errors to the error of the reference in each case just fell short of the criteria limits. The SECV and SEP values are less 15% of the average concentration of the calibration and validation set respectively. In this context the prediction error of the calibration curve is not excessively large. However, when compared to the very small error associated with the reference measurements, the prediction error is out of bounds. It is important to note that the quantification of the “total alcohols” in wine will rely on similar wave numbers as for the quantification of ethanol. Ethanol occurs in ten times higher concentration in wine compared to

other alcohols and absorbance by ethanol bonds are likely to overshadow that of the other alcohols. According to Moreira and Santos (2004) interferences could be expected in the infrared calibration of analytes that occur in low concentrations if their major absorbance wavelengths are similar to that of more abundant compounds. In this light the “total alcohols” calibration curve seems quite positive and could be used to screen wines in terms of high, medium or low concentrations of higher alcohols.

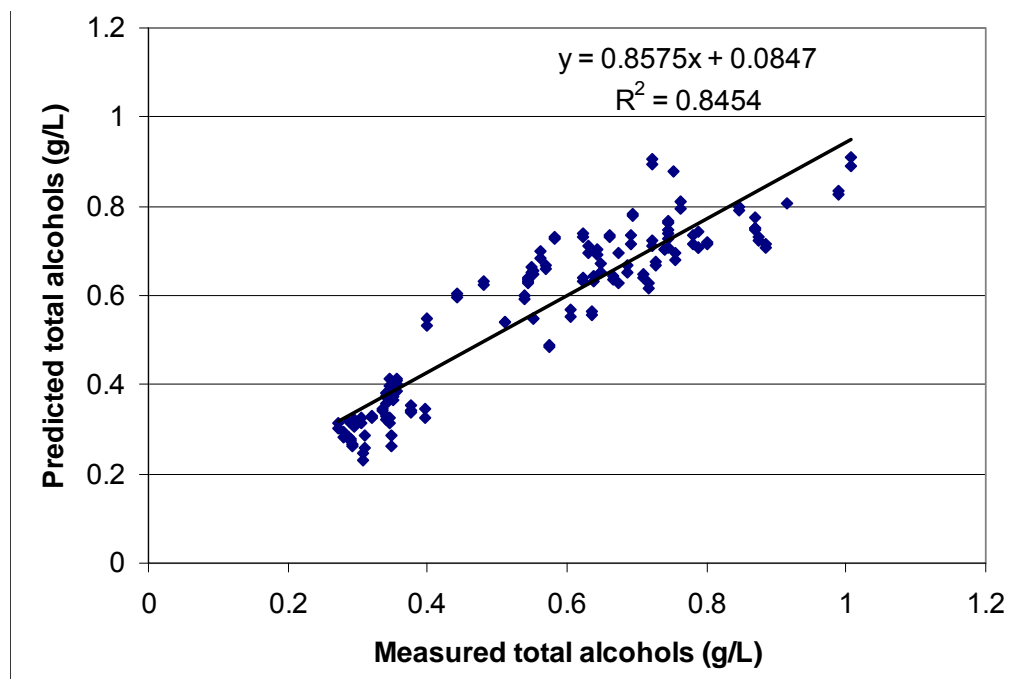


Figure 1. Regression curve of measured vs. predicted concentrations of the total alcohols in young wines.

Table 4. The wave numbers that explained the most variance within each compound group and the functional groupings they are associated with.

Total Alcohols		Total Fatty Acids		Total Esters		Esters	
Wave Number	Accumulated Explained Variance	Wave Number	Accumulated Explained Variance	Wave Number	Accumulated Explained Variance	Wave Number	Accumulated Explained Variance
1412	63%	1412	21%	1412	54%	1412	56%
1118	71%	1118-1122	24%	1118-1122	64%	1342	73%
1087	83%	1184-1188	31%	1342	72%	1736	75%
1339	86%	1265-1269	48%	1073	75%	1068-1072	77%
1728-1730	87%	1523	52%	1736	76%	1119-1120	79%
1146-1148	87%	1736	56%	1524	78%	1524	82%
1524	89%	1342	63%	1181	80%	1154	84%
1069	90%	1087	73%	2180	80%	1717	86%

Table 5. Validation and Calibration Statistics

Parameter	Total alcohols	Total esters	Esters excl. ethyl acetate	Total Fatty Acids
Number of components	15	15	15	12
Number of Filters	15	15	15	15
Number of Samples: Calibration (white; red)	111 (41; 70)	110 (40; 70)	108 (40; 68)	109 (39; 70)
SECV	0.077	0.1025	0.100	0.065
AR	0.015	0.0146	0.016	0.0141
Range (g/L)	0.220-1.092	0.331-1.615	0.284 – 1.159	0.120-0.922
Mean \pm SD	0.533 \pm 0.228	0.742 \pm 0.281	0.637 \pm 0.252	0.483 \pm 0.172
SEL	0.016	0.004	0.001	0.008
SECV:SEL	4.813	25.625	100	8.112
Number of Samples: Validation (white; red)	75 (27; 48)	79 (29;50)	75 (27;48)	78 (28;50)
Concentration range	0.271 – 1.008	0.389 -1.273	0.337-1.14	0.197-1.025
Mean \pm SD ^a	0.573 \pm 0.198	0.801 \pm 0.248	0.680 \pm 0.250	0.503 \pm 0.142
Bias	0.003	0.001	0.013	0.003
R ² , ^b	0.845	0.866 ^f	0.875	0.818
SEP ^c	0.077	0.122	0.089	0.061
SDD ^d	0.030	0.012	0.011	0.006
SEP:SDD	5.5	30.5	89	6.1
RPD ^e	2.571	2.033	2.809	2.323

^aStandard deviation; ^bCoefficient of determination; ^cStandard error of prediction; ^dStandard deviation of difference;

^eResidual predictive deviation; ^fPolynomial trend line

8.3.3 TOTAL FATTY ACIDS

Each of the selected wave number filters explained only a small amount of the variance in the dataset (Table 4). These wave numbers corresponded to the absorption areas of –OH, C-O and C=O molecular bonds which are characteristic of the –COOH functional group of fatty acids (Wehling, 1998).

The coefficient of determination of the “total fatty acid” calibration indicated good precision (Shenk and Westerhaus, 1996), especially considering the low concentrations of fatty acids present in the wines (Table 5 and Figure 2). The bias of the regression curve was very low. The SECV and SEP values were lower than 14% of the average concentration of the calibration and validation set respectively. When the low concentrations of the fatty acids are taken into account, these error parameters are reasonable. However, like with the “total alcohols”, when compared to the very small error associated with the reference measurements, the prediction error does not reflect well in terms of the precision criteria. Some spectroscopic interference could be caused by other, non-volatile, organic acids in wine. Important acids like tartaric acid, malic acid and lactic acid contains more than one –COOH functional group and/or additional –OH groups, as opposed

to the volatile fatty acids that contain only a single $-\text{COOH}$ group. This, together with the high levels of these acids present in wine would cause the absorbance by these acids to be stronger than that of the fatty acids at the specified wave numbers. In the light of these observations, especially considering that the coefficient of determination was reasonable, this calibration curve could be used to screen wines in terms of high, medium or low concentrations of fatty acids.

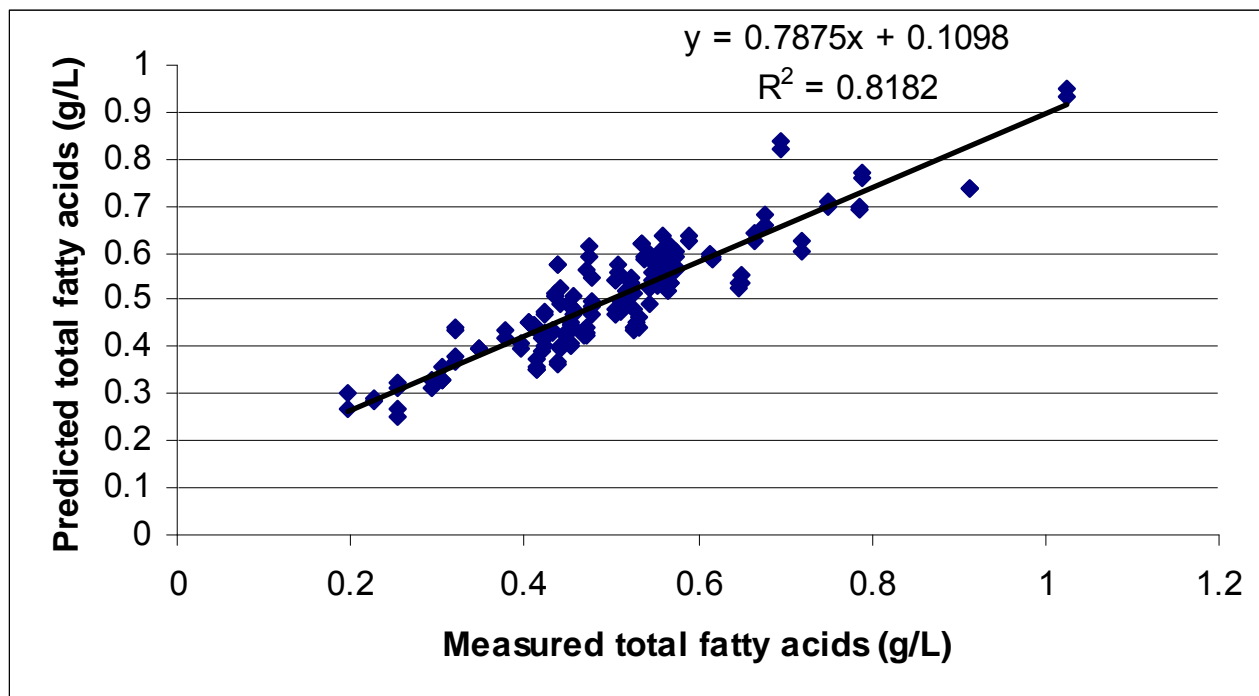


Figure 3. Fatty acid concentration measured with the reference method vs. concentration predicted with FTIR.

8.3.4 ESTERS AND TOTAL ESTERS

More than 70% of the variance in the “esters” data set was accounted for by three wave number filters. These filters corresponded to absorbance by $-\text{OH}$ and C-O molecular bonds. Of the “total esters” filters, 70% of the total variance were explained by two wave numbers in the $-\text{OH}$ absorbance region. The next two filters explained only 4% additional variance and were characteristic of absorbance by C-O and C=O bonds (Wehling, 1998). It is interesting that the $-\text{OH}$ bonds were more influential than the C-O and C=O bonds, as the latter are more characteristic of the COCOOH functional group of esters. Unlike fatty acids and alcohols, esters do not share their particular molecular structure with any major wine constituent. The fact that the most important wave numbers used in the calibration of both ester groups corresponds with $-\text{OH}$ absorbance means that more interference can be expected from alcohols and acids.

The regression plot for “esters” showed good linearity (Figure 5) according to Shenk and Westerhaus. Interestingly, the calibration curve for “total esters” followed a polynomial trend rather than a linear trend (Figure 5). It seems as if the curve follows a linear trend up to concentrations of 0.9 g/L while higher concentrations enforced a more polynomial trend. The bias of the regression curve was very low. The SECV values was 13% and 16% of the average concentration of the

calibration set of total esters and esters calibrations respectively. The SEP values was 15 and 13% of the average concentration of the validation set of the total esters and esters calibrations respectively. When samples with total ester concentrations higher than 0.9 g/L were excluded from the validation set, the SEP for total esters dropped from 0.122 to 0.104 (data not shown). In this context the prediction error of the calibration curves are not ideal yet not excessively large. However, when compared to the very small error associated with the reference measurements, the prediction error is out of bounds. Spectroscopic interferences could be expected from alcohols and acids present in wine.

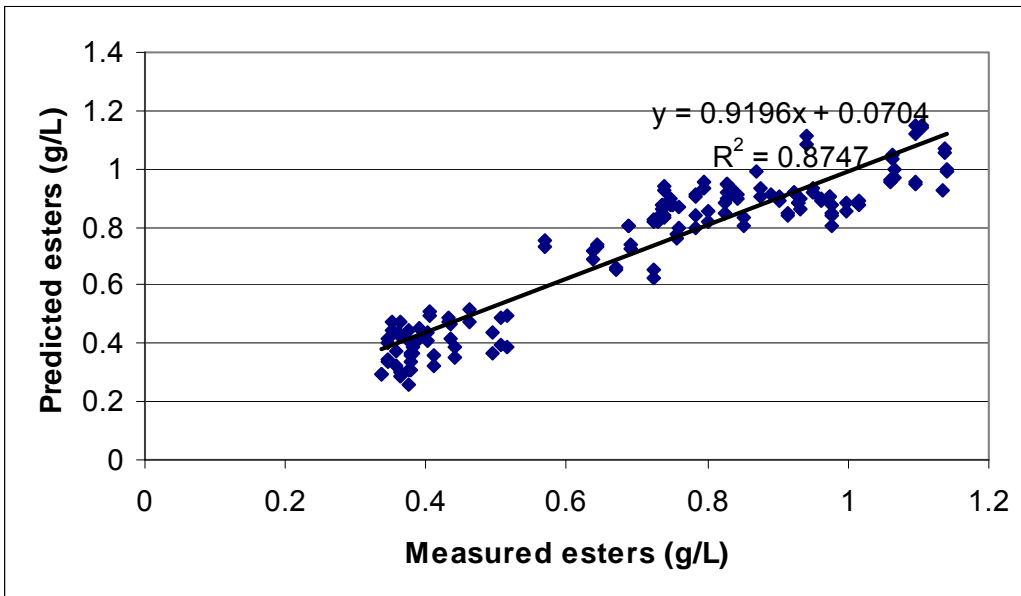


Figure 4. Calibration curve for esters predicted with FTIR.

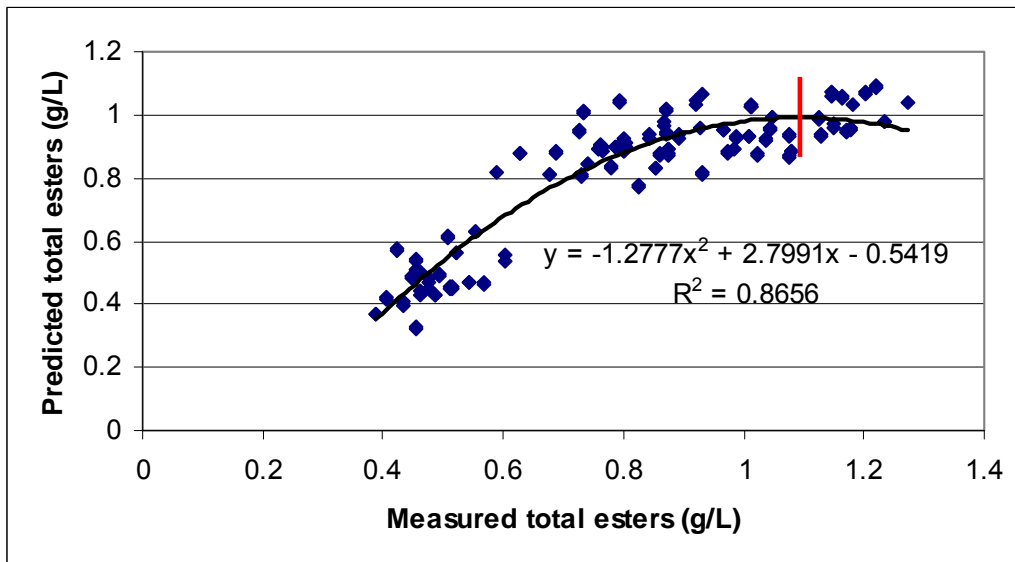


Figure 5. Calibration curve for Total esters. The curve follows a polynomial trend, although it seems as if concentrations below 0.9 g/L follow a linear trend.

8.4 CONCLUSION

The preliminary calibrations for the volatile compound groups “total alcohols”, “total fatty acids”, “total esters” and “esters” are promising for the screening purposes in wine and should definitely be investigated further. The ability of the models to distinguish between high, medium and low values for these groups could be evaluated with SIMCA models (soft independent modelling of class analogies). As discussed in Chapter 4, the levels at which these compounds occur in red and white wines are very different, and high levels of higher alcohols in white wines are close to the value of low levels of higher alcohols in red wines. This would make the distinction between high, medium and low levels of these compound groups more difficult. Possible research strategies include the analysis of more reference samples in order to establish separated screening models for red and white wines. Alternatively, the compound groups could be refined in order to minimise the variation between wine types. Screening models for volatile compounds in synthetic fermentation media could also be investigated.

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Chapter 9

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

Wine characterisation relies on two equally important fields of study: chemical analysis and data analysis. The sophisticated analytical techniques provide a wealth of chemical information while a combination of univariate and multivariate data analysis allows the interpretation of the chemical information. Thus, the first issue to address in a study on wine characterisation is the performance of the analytical method and therefore the reliability of the data. Two analytical methods were used in this study, Fourier transform infrared spectroscopy and gas chromatography.

Fourier transform infrared spectroscopy can be applied toward wine characterisation in two different ways. Firstly, the spectra generated during analysis can be examined with multivariate data analysis to gather information inherent to the sample matrix. As infrared spectroscopy is a measurement of the response of molecular bonds to infrared radiation, infrared spectra contain a wealth of compositional information that does not necessarily have to be connected to a specific compound. To fully exploit the amount of information present in the spectra it is important to limit the amount of noise present in the spectra. CO₂ is a common cause of spectral noise and must be eliminated during sample preparation. Several sample preparation techniques have been explored in this study and it was concluded that multiple filtrations using a vacuum filter was more efficient than centrifugation or sonication to decrease the CO₂ levels in young wines to below 300 mg/L.

Fourier transform infrared spectroscopy can also be used as a quantitative method. The spectral information can be correlated to the concentration of specific compounds by means of PLS regression. FTMIR calibration models was used for the of characterising South African young wines in terms of the most important wine constituents, namely tartaric acid, pH, volatile acidity, malic acid, lactic acid, glucose, fructose, ethanol and glycerol. These parameters are of paramount importance during wine quality control. In order to establish a good calibration model for the prediction of compositional information it is of crucial to obtain good reference values. Two commonly reported reference methods for infrared calibrations of organic acids, sugars and glycerol are HPLC and enzymatic analysis. These two methods were evaluated and several important observations were made.

Enzymatic assays were found suitable for the determination of glucose, fructose and glycerol in red and white wines as well as malic acid in white wines and in red wines containing more than 0.32 g/L malic acid. The enzymatic analysis of lactic acid was characterised by high laboratory errors. The reason for this could be explored further. The analysis of malic acid in red wine was hampered by a matrix effect, presumably caused by the red pigments in wine. The matrix effect could not be reduced by dilution if the samples contained low amounts of malic acid. Decolouring of the samples with activated charcoal and PVPP both resulted in heavy spectral interferences.

HPLC was more effective for the analysis of organic acids than enzymatic assays. However, a matrix effect was observed during the analysis of red wines which was caused by interfering organic molecules, especially phenolic compounds. This matrix effect and the necessity of sample

clean-up methods have been widely reported (de Villiers *et al.*, 2004; Zotou *et al.*, 2004). The two sample clean-up methods evaluated in this study, SPE and PVPP fining, both effectively removed interfering phenolic compounds. Although both methods were labour intensive and time consuming, PVPP fining was notably less expensive than SPE and provided better recoveries for the organic acids. However, the reproducibility of PVPP fining must still be optimised. None of these methods are ideal and the choice between these methods involves a compromise between limit of quantification, recovery, and reproducibility as well as time and cost efficiency. There are also other SPE methods to consider. The reverse phase SPE cartridges used in this study could be replaced with ion exchange cartridges such as SAX (strong anion exchange) cartridges. The ion exchange separation mechanism relies on the pH and ionic strength of the mobile phase rather than on polarity (Rounds and Gregory, 1998). It should be noted that varying results in terms of the precision and recovery of organic acid analysis using SAX cartridges has been reported. Moreover, the neutral carbohydrates and alcohols will be removed with the phenolic compounds during sample clean-up. However, this fraction could be analysed separately to quantify the sugars and alcohols and would not require additional sample clean-up steps as the phenolic compounds has no influence during the refractive index detection of sugars and alcohols. Alternatively, if the organic acids are analysed using SAX cartridges, the sugars and glycerol could successfully and reliably be determined with enzymatic assays (Castellari *et al.*, 2000; Mato, *et al.*, 2005; Zotou *et al.*, 2004).

An alternative method for the determination of organic acids is capillary electrophoresis (CE). The method is sensitive enough to determine low concentrations of malic acid and requires little sample preparation. However, CE analysis requires specific expertise and is characterised by poor reproducibility compared to HPLC (de Villiers *et al.*, 2003; Santalad *et al.*, 2007). Furthermore, large prediction errors have been reported for FTIR organic acid calibrations using CE analysis as a reference method (Kupina and Shrikhande, 2003).

Although not the ideal practical solution, it is evident from the results of this study that the determination of reference values for FTMIR calibrations should be approached differently.

The same statement can be made regarding the establishment of FTMIR calibrations. Young wines were analysed with reference methods in order to determine whether commercial FTMIR calibrations are suitable for a South African young wine matrix. In the cases of total acidity, pH, glucose, fructose, malic acid, ethanol and glycerol, better performance were observed for brand new calibrations based on young wine reference samples. This clearly indicated that the South African conditions had a significant effect on the quantification abilities of the commercial calibrations that was set up under European conditions. The efficiency of wavenumber selection for the establishment of preliminary young wine calibrations was evaluated. Very refined wavenumber selections were the most suitable for total acidity, volatile acidity and glycerol, while the use of broader spectral regions was more efficient for the calibration of pH and ethanol. From the glycerol calibration statistics it was observed that a separate calibration for red wine samples performed

better than a calibration including both red and white wines. Thus, it seems that FTMIR calibration models should not be approached in a generic fashion for all analytes.

Promising results were obtained for the preliminary calibration models for the determination of volatile compounds in young wines. Four compound groups, based on chemical structure and sensory impact on wine, were chosen: total alcohols, fatty acids, total esters and esters. A distinction was made between “esters” and “total esters”, where the former does not include ethyl acetate. The reason for this is that the sensory contribution of ethyl acetate in wine is often associated with volatile acidity rather than esters. In the case of total alcohols and fatty acids, the most significant wavenumbers corresponded to the absorbance areas of molecular bonds that are characteristic of these compounds. Interestingly, the wavenumbers corresponding to O-H bonds were more influential than those corresponding to C-O and C=O bonds, which are more characteristic of the COCOOH functional group of esters. The preliminary calibrations for total alcohols, fatty acids and esters performed well in terms of linearity and bias. The total esters calibration followed an interesting polynomial trend. The prediction errors of the calibration were high, but not excessively so in terms of the average concentration of the sample sets. However, compared to the analytical error of the reference method, the prediction error of all four calibrations were unacceptable. The high prediction errors can partially be explained by spectroscopic interferences caused by more abundant wine constituents that absorbs strongly in similar spectral regions.

The proposed gas chromatography method for the analysis of volatile compounds was considered suitable after validation. Slight matrix effects were observed between red and white wines. The following factors in the protocol had a significant influence on the results and should be closely adhered to: amount of diethyl ether, sample volume, length of sonication and the temperature of the water bath. The amount of NaSO₄ salt, ethanol concentration and pH of the sample did not influence the extraction efficiency significantly. No trends were observed between analyses of the same extract or wine on the same day or over time. Large variations in concentrations were observed during methanol analyses over time. These results are a major contribution towards the establishment of a routine analytical method for the quantification of volatile compounds in our environment.

The data analysis aspect of wine characterisation can be approached in terms of descriptive analysis and pattern recognition. Young South African wines from six cultivar groups and four production areas were analysed with FTMIR spectroscopy and gas chromatography over two vintages. An overview of the volatile composition of South African young wines was described by univariate statistics. In addition, FTMIR spectra, major chemical composition determined with FTMIR spectroscopy and the volatile composition determined with GC-FID were subjected to pattern recognition techniques to identify trends and patterns in the data relating to vintage, wine style, grape cultivar, production region and wine quality.

The main source of variation between the wines was the distinction between red and white wines. Significant differences were observed between the volatile composition of red and white wines that were in agreement with results from previous studies (Gil *et al.*, 2006). PCA scores plots showed a clear distinction between the volatile and major chemical composition and FTIR spectra of red and white wines. The most influential volatile compounds in the separation between red and white wines were in agreement with the results obtained from univariate statistics. The most important major chemical components responsible for the distinction between red and white wines were related to malolactic fermentation.

Significant differences were observed between the composition of the 2005 and 2006 vintage wines. These differences were cultivar dependent, which means that the changes in the chemical composition of wines between different vintages are not consistent for each cultivar. A probable reason for this observation is that the different climatic events that occurred during each ripening season, such as the heavy precipitation in October 2004 (Boom, 2005), did not affect cultivars that were in different phenological stages to the same extent. It would be beneficial to confirm these results by analysis of another vintage. Red and white wines had to be separated to observe differentiation between these two vintages with PCA. The FTIR spectra and major chemical components did not contribute to the distinction observed between vintages on PCA scores plots. The 2005 and 2006 white wines were mostly separated by diethyl succinate and isoamyl acetate. Butanol, decanoic acid and isobutanol caused the distinction between the 2005 and 2006 red wines. The role of isobutanol was especially interesting, since the univariate statistics indicated that the isobutanol content of the 2005 and 2006 red wines were not significantly different. However, significant cultivar-vintage interaction was observed between the variance in isobutanol levels. The role of multivariate data analysis to uncover the influence of isobutanol concentration on the differences between vintages underlines the fact that multivariate statistics are more appropriate where there are correlations between variables than univariate statistics.

The compounds that were most influential in the differentiation between cultivar wines could be linked by their role in yeast metabolism. Their roles in the differentiation between red wines were confirmed by previous studies (Ferreira *et al.*, 2000; Marias *et al.*, 1981). Interestingly, the volatile composition of Pinotage wines was more comparable to white wines than the other red wines. Results from univariate statistics and PCA shows that isoamyl acetate, isobutanol, isobutyric acid and 2-phenylethyl acetate were influential in the distinction between Pinotage and the other red wines. The fact that Pinotage specifically stands apart from the other red wine cultivars could have important implications for the production and marketing strategies of Pinotage wines. Pinotage wines have long been the proverbial black sheep of South African red wine cultivars, and perhaps the preliminary results from this study is another indication that Pinotage wines should be considered in a class of their own. It seems valuable to investigate the use of winemaking strategies that deviates from the standard red winemaking practices for the production of Pinotage and to establish Pinotage as a unique and distinguishable wine style.

The role of infrared spectra in the discrimination between cultivar wines with PCA was limited to the white cultivars. It was observed from PCA that the volatile composition contributed more to the differences between red wine cultivars than the spectra. However, the role of the spectra was more pronounced in LDA, where the most powerful classification model for cultivar groupings was achieved with a combination of the entire infrared spectra and volatile compounds. Surprisingly, better classification results were obtained using the entire FTMIR spectra than using selected spectral regions containing minimum amounts of noise. This highlights the value of FTMIR spectra as an information rich and non-selective instrumental signal.

Higher alcohols and fatty acids contributed the most to differences between production regions. Based on univariate analysis of their volatile composition, Paarl and Stellenbosch wines were very similar, whereas Robertson and Worcester wines differed from each other and from the other regions. However, no differences were observed between the regions through multivariate data analysis of the FTMIR or volatile data. This could be due to the fact that the regions were allocated to each wine based on the location of the wine cellar. South African wine cellars may lawfully buy grapes from other production regions, and therefore the actual origin of the wines used in this study is not guaranteed. In future studies it would be advisable to use estate wines, or wines of which the origin is guaranteed.

Lastly, it was not possible to predict the score of the wines from PLS regression models of the chemical or spectral data. Wines with very high and very low score ratings were under represented in the calibrations, a fact that possibly hampered the prediction abilities of the models. Further attempts to calibrate wine quality from chemical data should ideally include a larger number of high and low quality wines.

The important role of sound and validated analytical methods to generated high quality analytical data, and the subsequent application of chemometric techniques to model the data for the purpose of wine characterisation have been thoroughly explored in this study. After critical evaluation of the analytical methods used in this study, a variety of statistical methods were applied to uncover the chemical core of South African cultivar wines. The use of multivariate data analysis has revealed some limitations in the dataset. From the observations made in terms of the unsuccessful classification of production region and wine quality, it must be said that wine characterisation is not just reliant on sophisticated analytical chemistry and advanced data analytical techniques, but also on high quality sample sets.

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Addendum A

Method Validation Report
GC-FID method for the determination of
alcohols, esters and fatty acids in wine

ADDENDUM A

OBJECTIVE AND SCOPE OF THE METHOD

A method suitable for the extraction, analysis and quantification of aroma compounds (volatile alcohols, esters and acids) with gas chromatography and Flame Ionization Detection.

TYPE OF COMPOUNDS AND MATRIX

Volatile alcohols, esters and acids in red and white wine, using 4-Methyl-2-Pentanol as internal standard (IS).

CHEMICALS / MATERIALS

All chemicals were of the highest purity as obtained from the manufacturer and were used without further purification.

Standards:

Ethyl Acetate, Riedel de Haën, 99.7%; Methanol, Merck, 99.8%; Ethyl Butyrate, Fluka, 99%; Propanol, Fluka, 99%; Isobutanol, Fluka, 99.5%; Isoamyl Acetate, Riedel de Haën, 98%; Butanol, Fluka, GC grade; Isoamyl Alcohol, Aldrich, 99%; Ethyl Hexanoate, Schuchardt Munchen, 98%; Hexyl Acetate, Fluka, 99%; Ethyl Lactate, Fluka, 99%; Hexanol, Merck, Synthesis grade; Ethyl Caprylate, Aldrich, 99%; Acetic Acid, Merck, 98%; Propionic Acid, Fluka, 99.5%; Iso-Butyric acid, Fluka, 99.5%; Ethyl Caprate, Aldrich, 99%; Butyric Acid, Fluka, 99.5%, Fluka; Iso-Valeric Acid, Fluka, 98%; Diethyl Succinate, Fluka, 99%; Valeric Acid, Fluka, 99%; 2-Phenylethyl Acetate, Fluka, 98%; Hexanoic Acid, Aldrich, 99.5%; 2-Phenylethanol, Merck, 99%; Octanoic Acid, Aldrich, 99.5%; Decanoic Acid, Sigma, 98%.

Internal Standard:

4-Methyl-2-Pentanol, Fluka

Wine simulant

12% Ethanol (Merck, 99.7-100%), 2.5g/L tartaric acid (Merck, 99.5%), pH adjusted to 3.5 with a solution of NaOH 0.1M (Merck).

Deionized water was obtained with a Millipore system.

Volatile components were extracted from the wine with diethyl ether (Merck, 99.5%).

The ether extract was dried on NaSO₄ (Merck, 99%).

Gas:

UHP hydrogen was used as carrier gas, UHP Nitrogen as make up flow for the detector and UHP Air for the Flame Ionization detector. All these were purchased from AFROX.

Column:

A J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m Length \times 0.32 mm i. d. \times 0.5 μ m f.t, was used.

METHOD PARAMETERS

• **Instrumental Parameters**

Initial temperature: 33°C

Initial time: 17 min

Ramp: 12°C/min to 240°C, hold for 5 minutes

Front inlet:

Injection volume: 3 μ l

Mode: Split

Split Ratio: 15:1

Split Flow: 49.5 ml/min

Injector temperature: 200°C

Initial pressure: 84.5 kPa

Flow mode: constant flow

Column flow: 3.3 ml/min

Column:

DB-FFAP, 60 m \times 0.32 mm \times 0.5 μ m f.t

Detector:

Temperature: 250°C

H₂ flow: 30 ml/min

Air flow: 350 ml/min

Make up flow: N₂ 30 ml/min

After each sample run, a post run of 5 minutes at oven temperature 240°C, with a gas flow of 6ml/min cleans the column. After every 30 samples the column is thermally and chemically cleaned by injecting hexane at an oven temperature of 220°C.

- **STANDARDS AND SAMPLES**

Five millilitres of wine with internal standard, 4-Methyl-2-Pentanol, (100µl of 0.5mg/l solution in wine simulant) is extracted with 1 millilitre of diethyl ether by placing the ether/wine mixture in an ultrasonic bath for 5 minutes. The wine/ether mixture is then centrifuged at 4000 rpm for 3 minutes. The ether layer is removed and dried on NaSO₄. This extract is then injected into the GC-FID.

EQUIPMENT

Hewlett Packard 6890 Plus GC (Little Falls, USA) equipped with a split/splitless injector and an FID detector.

PROCEDURES

• STANDARDS AND SAMPLE PREP

Concentration of standards in calibration solutions were as follows:

Table 1. Concentrations and amounts of standards (in 100 ml wine simulant) used to prepare Standard solution 1

Compound	Concentration, ppm	Amounts
Ethyl Acetate	360.8	36.08
Methanol	901.74	90.174
Ethyl Butyrate	21.95	2.195
Propanol	201	20.1
Isobutanol	100.38	10.0375
Isoamyl Acetate	19.27	1.9272
Butanol	20.28	2.0275
Isoamyl Alcohol	477.31	47.731
Ethyl Hexanoate	30.56	3.0555
Hexyl Acetate	21.9	2.19
Ethyl Lactate	500.16	50.016
Hexanol	30.93	3.0932
Ethyl Caprylate	3.51	0.3512
Acetic Acid	1804.79	180.428
Propionic Acid	29.79	2.979
Iso-Butyric Acid	20.90	2.09
Butyric Acid	21.21	2.1208
Ethyl Caprate	3.45	0.3448
Iso-Valeric Acid	31.41	3.141
Diethyl Succinate	39.35	3.9354
Valeric Acid	20.66	2.0658
2-Phenylethyl Acetate	20.6	2.06
Hexanoic Acid	29.664	2.9664
2-Phenylethanol	50.95	5.095
Octanoic Acid	40.04	4.004
Decanoic Acid	50.01	5.64

Standard solution 1 is prepared by dissolving the appropriate standards amounts in 100mL wine simulant in a volumetric flask.

The other standard solutions were prepared by diluting standard solution 1 to the following solutions with wine simulant.

Table 2. Dilution levels of standard solutions

Standard Solution no.	Level, %
1	100
2	95
3	50
4	33
5	16
6	9
7	5

IS solution is prepared by adding 619 μ L of 4-Methyl -2-Pentanol to 10mL Ethanol. This solution is diluted a thousand times by adding 100 μ L of this solution to 10 mL wine simulant. A 100 μ L of this diluted standard is used during sample preparation.

STATISTICS

Equations for *regression*, *standard deviations (STD)* and *relative standard deviations (%RSD)* were calculated with Microsoft Excel.

Data analysis was performed using the ChemStation software; integration was done with the enhanced integrator, using the suitable integration parameters function.

The limit of detection (*LOD*) and limit of quantitation (*LOQ*) were calculated as follows¹:

$$y = ax + b$$

Where y = detector response (*i.e.* area), a = slope, x = concentration and b = y-intercept at origin.

$$\text{sensitivity} = \frac{\text{area}}{\text{concentration}} = \frac{y}{x}$$

Therefore:

$$LOD = \frac{\text{noise} \times 3}{\text{sensitivity}}$$

$$LOQ = \frac{\text{noise} \times 10}{\text{sensitivity}}$$

And

¹ *Quantitative Chemical Analysis*, Daniel C Harris, WH Freeman and Company, NY, 2000

In the data analysis, all compound peak areas are divided by the area of the internal standard.

$$\text{Corrected Area} = \frac{\text{Compound peak area}}{\text{IS area}}$$

For recovery experiments, the following calculations were used:

$$\% \text{ Recovery} = \frac{100 \times (\text{Concentration}^{\text{Spiked wine}} - \text{Concentration}^{\text{non-spiked wine}})}{\text{Concentration}^{\text{spiked with}}}$$

RESULTS

- **Selectivity**

The selectivity was tested by injecting in consecutive runs, a 9% dilution mixture of all the standards, the matrices (red and white wines), and the spiked matrices (red and white wines spiked with a 6.25% dilution of the mixture of standards). Peaks were identified by the retention times resulting from the injection of authentic standards. Peaks detected in the sample matrices corresponded to the retention times of the injected standards. Peak identities and retention times are given in Figure 1, Figure 2 and Table 3.

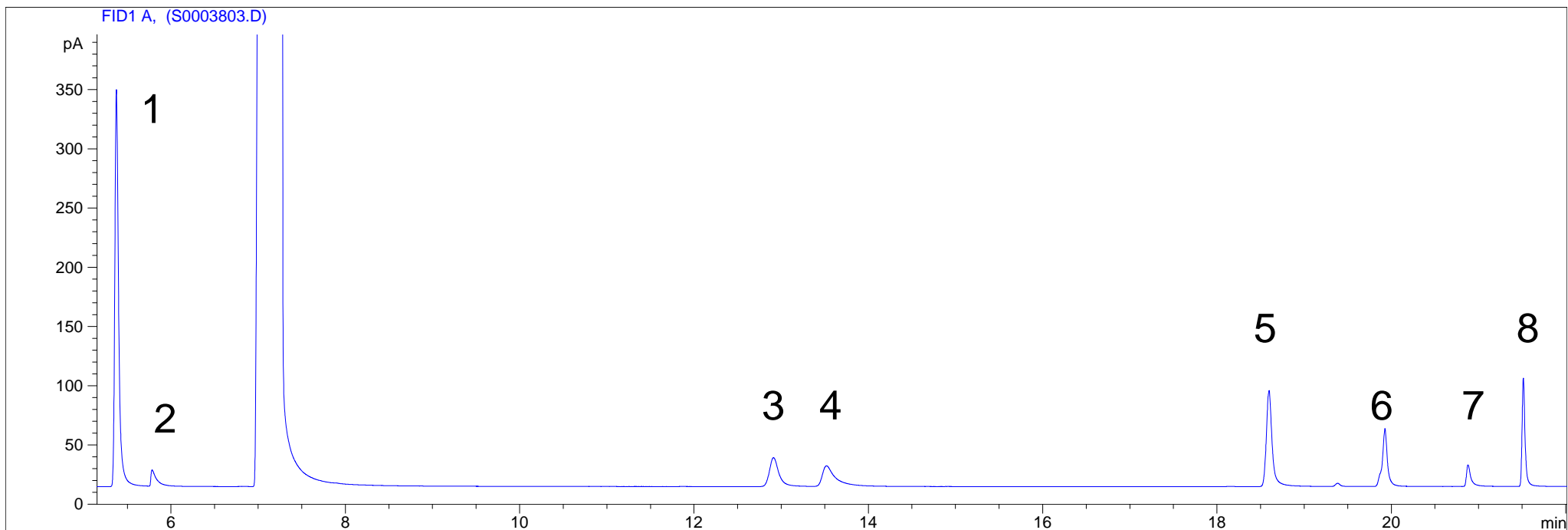


Figure 1. GC-FID analysis of a mixture of standards in a wine simulant solution from 5 - 22 minutes. Peak identities are given in Table 3.

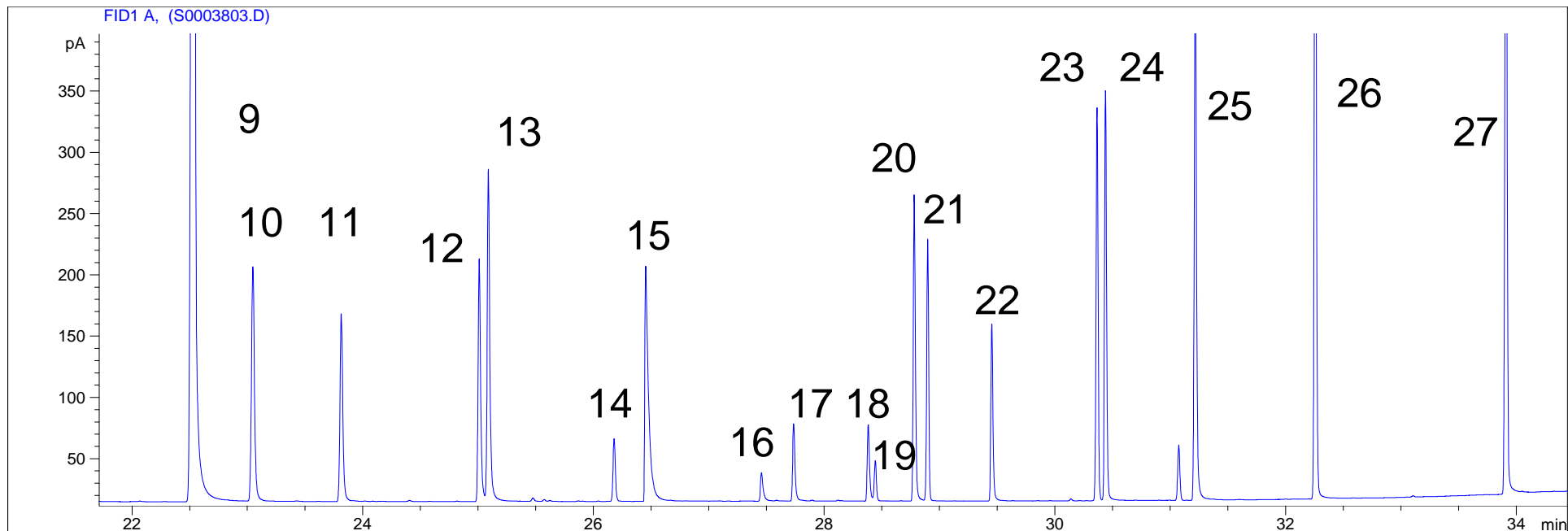


Figure 2. GC-FID analysis of a mixture of standards in a wine simulant solution from 22-35 minutes. Peak identities are given in Table 3.

- **Linearity**

Table 3. Calibration was performed for standards at the following levels:

Compound	Peak no	Retention Time (min)	Calibration levels, ppm
Ethyl Acetate	1	5.374	17.2 - 360.8
Methanol	2	5.784	42.9 - 901.7
Ethyl Butyrate	3	12.911	1.0 - 22.0
Propanol	4	13.520	9.6 - 201.0
Isobutanol	5	18.597	4.8 - 100.4
Isoamyl Acetate	6	19.926	0.9 - 19.3
Butanol	7	20.878	1.0 - 20.3
4-Methyl-2-pentanol	8	21.515	Internal standard
Isoamyl Alcohol	9	22.533	22.7 - 477.3
Ethyl Hexanoate	10	23.048	1.5 - 30.6
Hexyl Acetate	11	23.814	1.0 - 21.9
Ethyl Lactate	12	25.010	23.8 - 500.2
Hexanol	13	25.089	1.5 - 30.9
Ethyl Caprylate	14	26.180	0.2 - 3.5
Acetic Acid	15	26.454	85.9 - 1804.8
Propionic Acid	16	24.457	1.4 - 29.8
Iso-Butyric Acid	17	27.736	1.0 - 20.9
Butyric Acid	18	28.383	1.0 - 21.2
Ethyl Caprate	19	28.444	0.2 - 3.5
Iso-Valeric Acid	20	28.781	1.5 - 31.4
Diethyl Succinate	21	28.898	1.9 - 39.4
Valeric Acid	22	29.454	1.0 - 20.7
2-Phenylethyl Acetate	23	30.366	1.0 - 20.6
Hexanoic Acid	24	30.438	1.4 - 29.7
2-Phenylethanol	25	31.218	2.4 - 51.0
Octanoic Acid	26	32.256	1.9 - 40.0
Decanoic Acid	27	33.912	2.7 - 50.0

The value range for the concentrations was determined by evaluating results from Distell, South Africa for the same analysis.

The corrected peak areas gave linear responses over the concentration intervals tested. For results, see tables and figures below.

Table 4. Equations and R² of the calibration curves for the individual analytes

Compound	Equation	R² Area
Ethyl Acetate	$Y = 0.2004x + 0.0392$	0.99839
Methanol	$Y = 0.0056x - 0.0184$	0.99011
Ethyl Butyrate	$Y = 0.7806x - 0.0056$	0.99974
Propanol	$Y = 0.0943x - 0.0430$	0.99853
Isobutanol	$Y = 0.3348x - 0.0285$	0.99972
Isoamyl Acetate	$Y = 1.0021x - 0.0101$	0.99985
Butanol	$Y = 0.3174x - 0.0089$	0.99951
Isoamyl Alcohol	$Y = 0.7569x - 0.0805$	0.99988
Ethyl Hexanoate	$Y = 1.1331x - 0.0224$	0.99987
Hexyl Acetate	$Y = 1.1394x - 0.0144$	0.99989
Ethyl Lactate	$Y = 0.0632x - 0.0492$	0.99903
Hexanol	$Y = 1.3512x - 0.0032$	0.99993
Ethyl Caprylate	$Y = 1.3493x - 0.0075$	0.99949
Acetic Acid	$Y = 0.0277x - 0.1207$	0.99775
Propionic Acid	$Y = 0.1241x - 0.0061$	0.99910
Iso-Butyric Acid	$Y = 0.3987x - 0.0104$	0.99976
Butyric Acid	$Y = 1.5416x - 0.0129$	0.99766
Ethyl Caprate	$Y = 0.3859x - 0.0115$	0.99966
Iso-Valeric Acid	$Y = 0.7824x - 0.0201$	0.99993
Diethyl Succinate	$Y = 0.7519x - 0.0116$	0.99991
Valeric Acid	$Y = 0.8159x - 0.0156$	0.99991
2-Phenylethyl Acetate	$Y = 1.7003x + 0.0144$	0.99960
Hexanoic Acid	$Y = 1.3154x - 0.0219$	0.99997
2-Phenylethanol	$Y = 1.1614x - 0.0459$	0.99982
Octanoic Acid	$Y = 1.7327x - 0.0354$	0.99995
Decanoic Acid	$Y = 2.5490x - 0.2904$	0.99810

Where Y = area ratio and x = amount ratio
Each calibration level was injected three times.

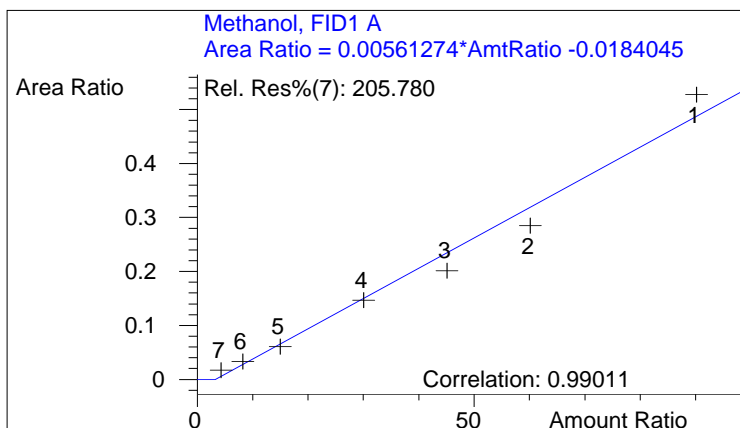


Figure 3. Calibration curve for Ethyl Acetate.

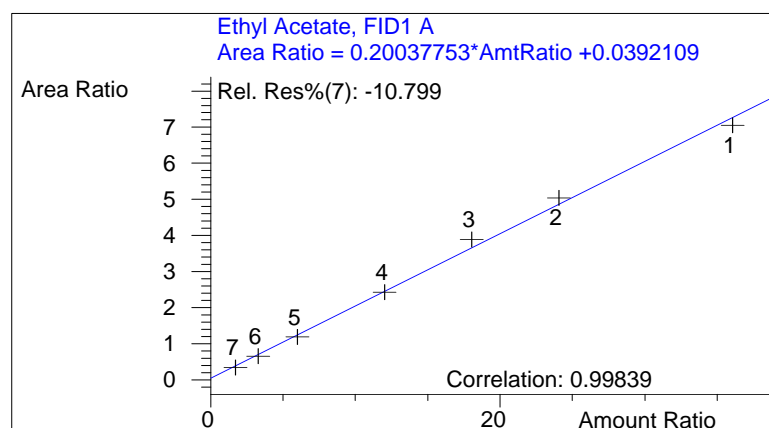


Figure 4. Calibration curve for Methanol.

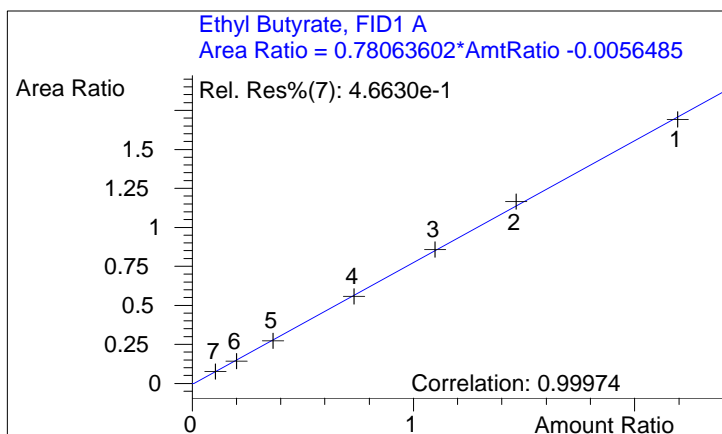


Figure 5. Calibration curve for Ethyl Butyrate.

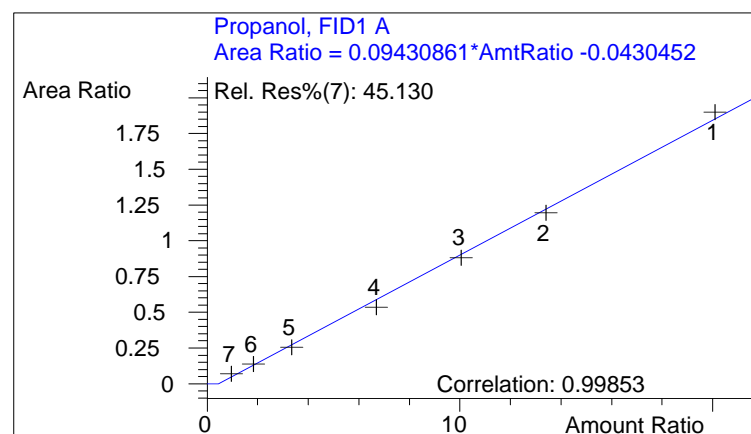


Figure 6. Calibration curve for Propanol.

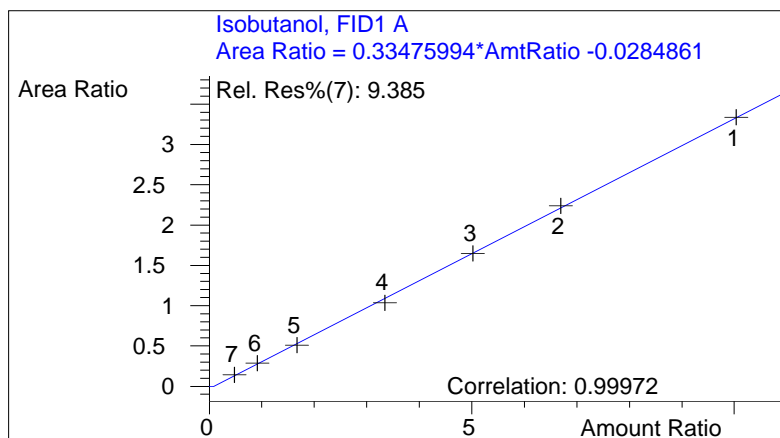


Figure 7. Calibration curve for Isobutanol.

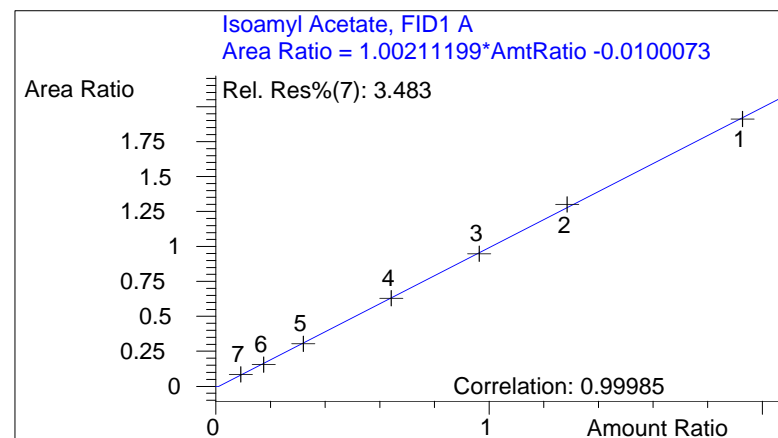


Figure 8. Calibration curve for Isoamyl Acetate.

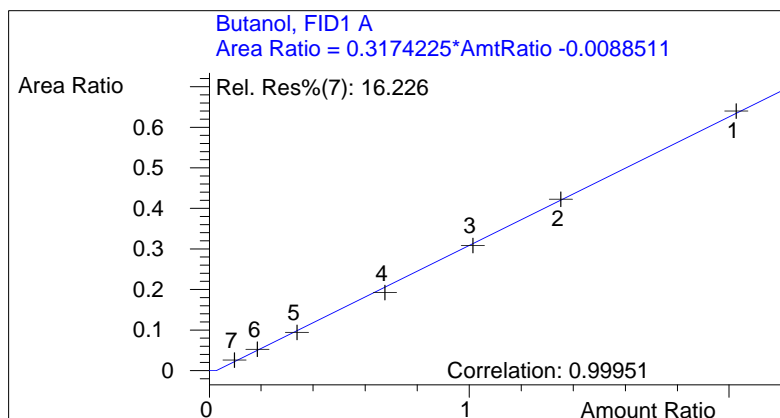


Figure 9. Calibration curve for Butanol.

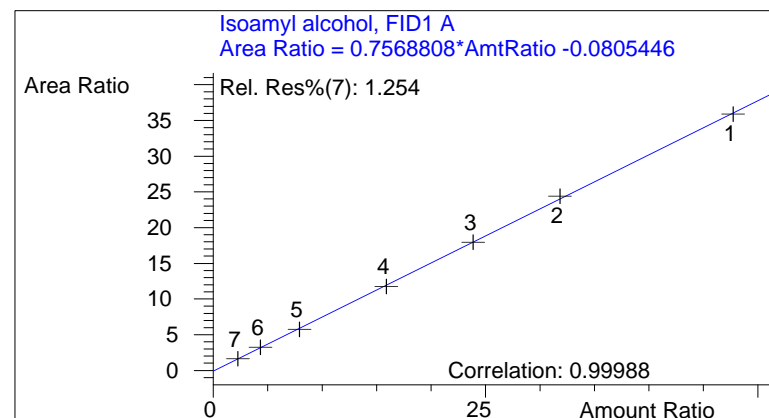


Figure 10. Calibration curve for Isoamyl Alcohol.

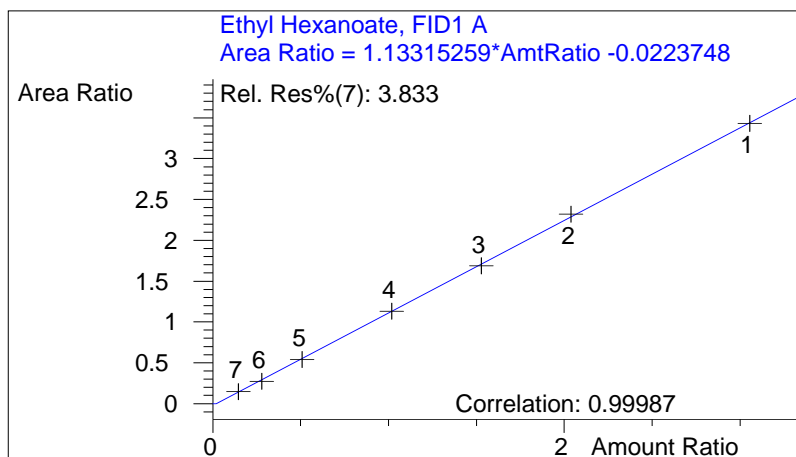


Figure 11. Calibration curve for Ethyl Hexanoate.

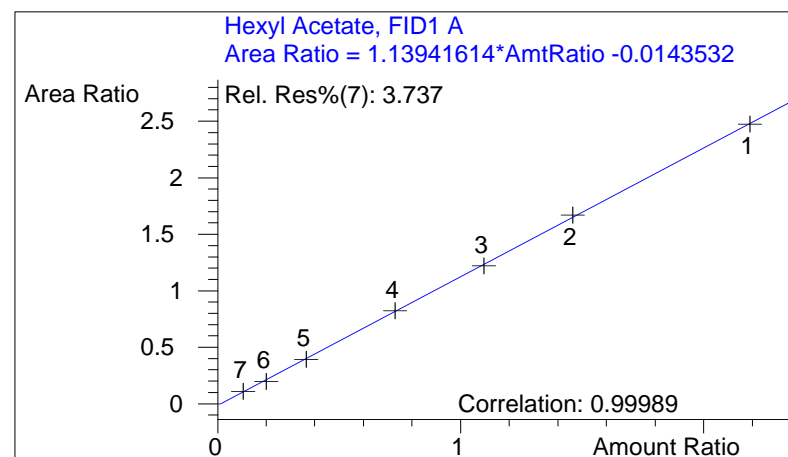


Figure 12. Calibration curve for Hexyl Acetate.

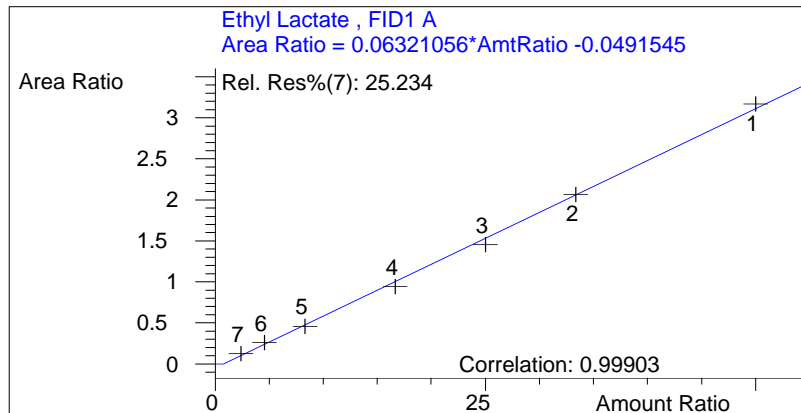


Figure 13. Calibration curve for Ethyl Lactate.

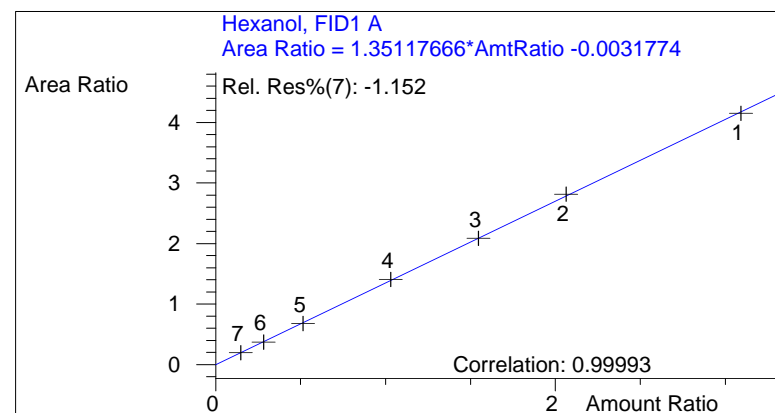


Figure 14. Calibration curve for Hexanol.

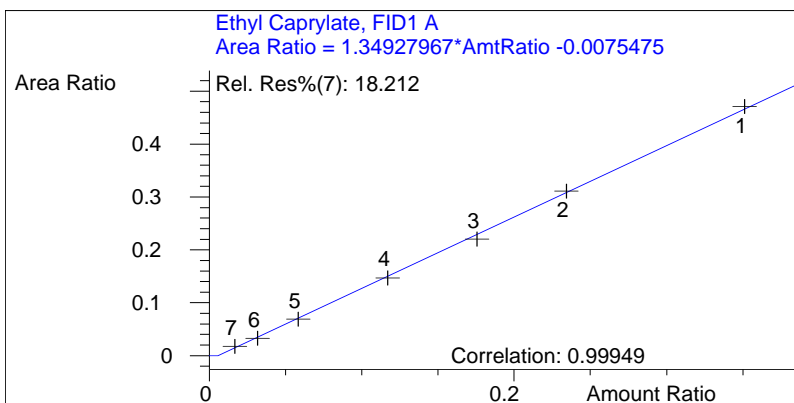


Figure 15. Calibration curve for Ethyl Caprylate.

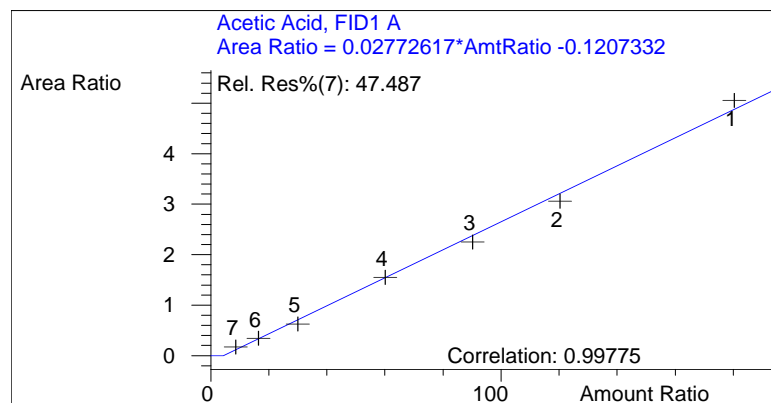


Figure 16. Calibration curve for Acetic Acid.

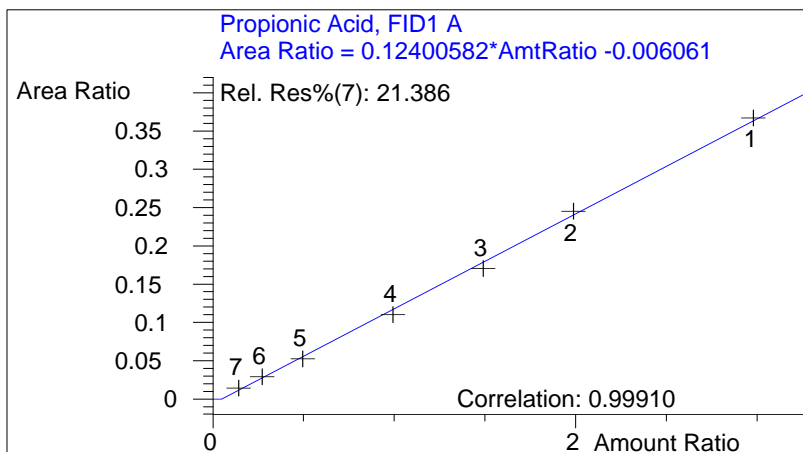


Figure 17. Calibration curve for Propionic Acid.

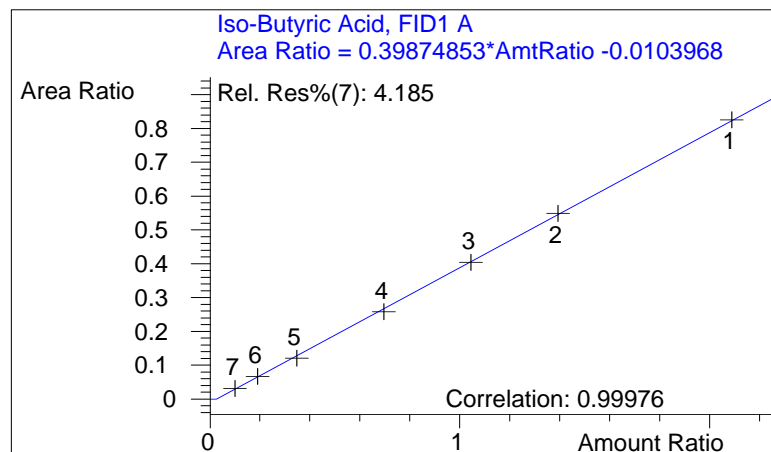


Figure 18. Calibration curve for Isobutyric Acid.

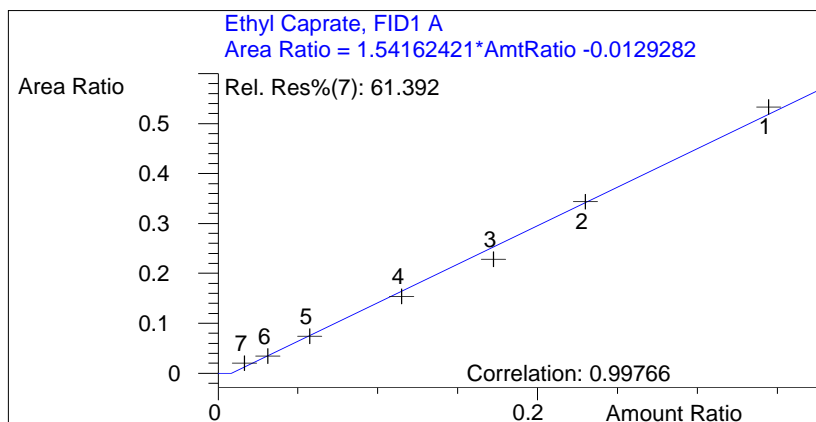


Figure 20. Calibration curve for Butyric Acid.

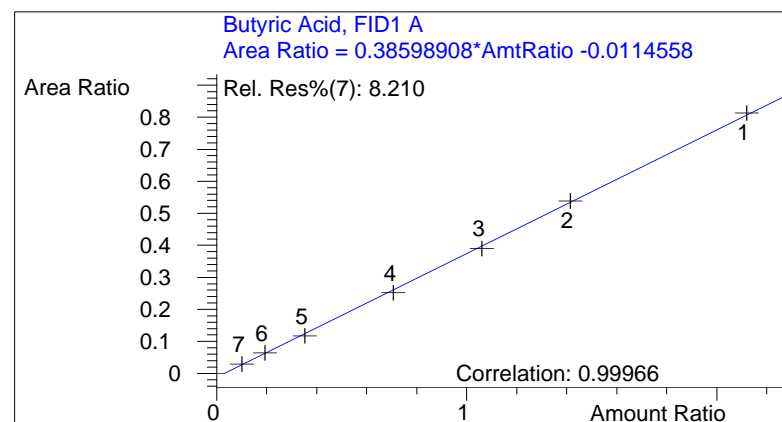


Figure 19. Calibration curve for Ethyl Caprate.

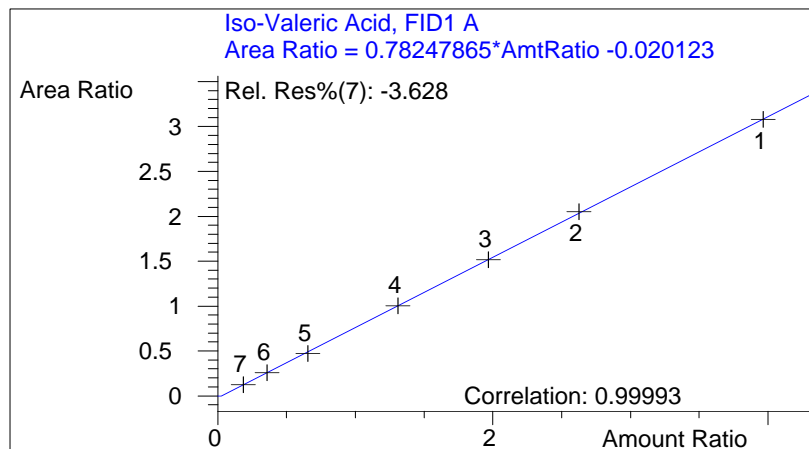


Figure 21. Calibration curve for Iso-Valeric Acid.

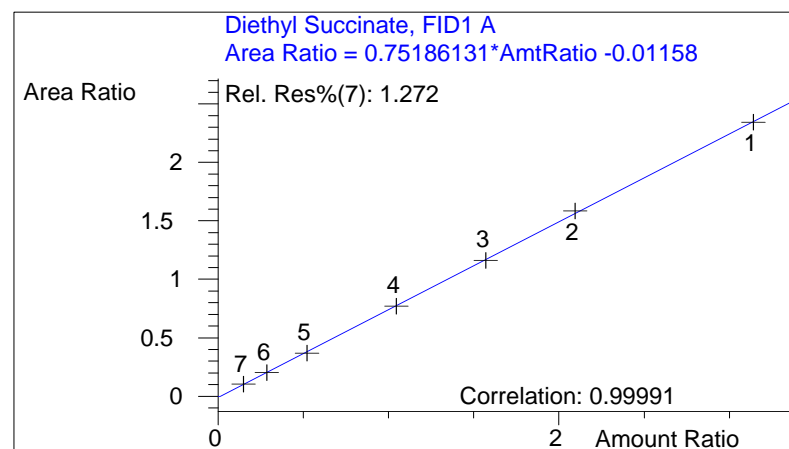


Figure 22. Calibration curve for Diethyl succinate.

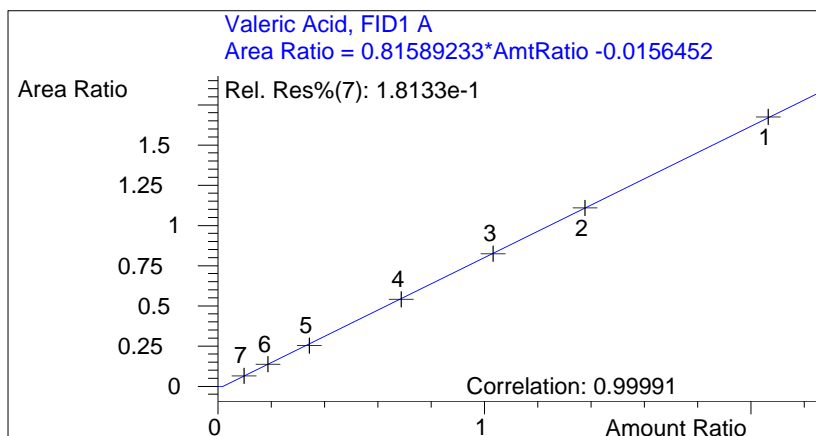


Figure 23. Calibration curve for Valeric Acid.

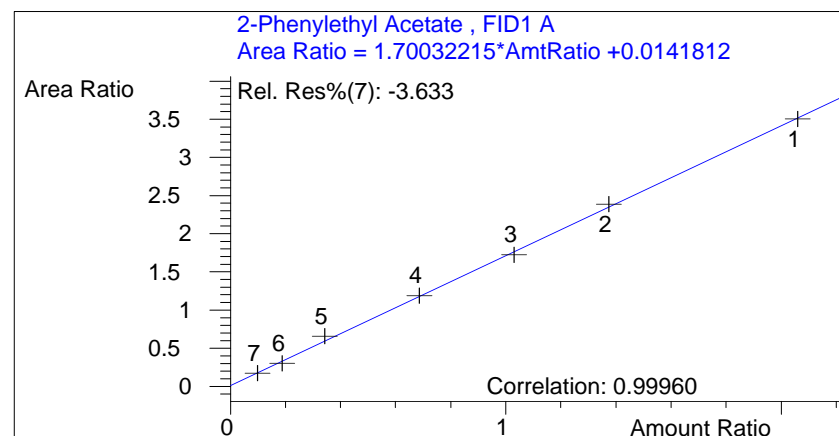


Figure 24. Calibration curve for 2-Phenylethyl Acetate.

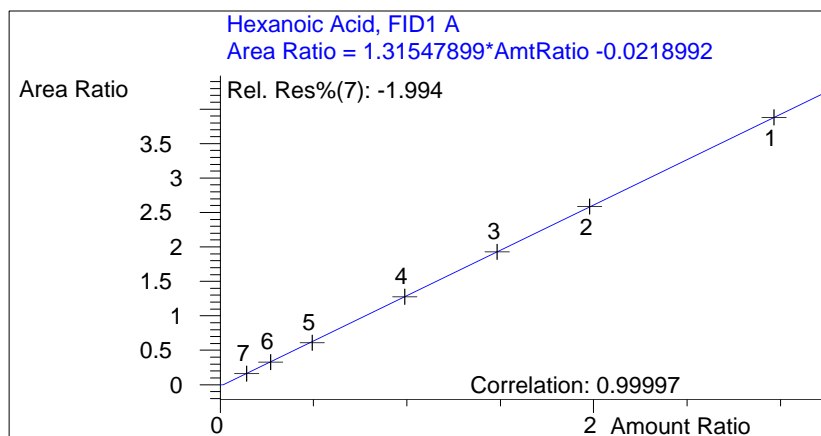


Figure 26. Calibration curve for 2-Phenylethanol.

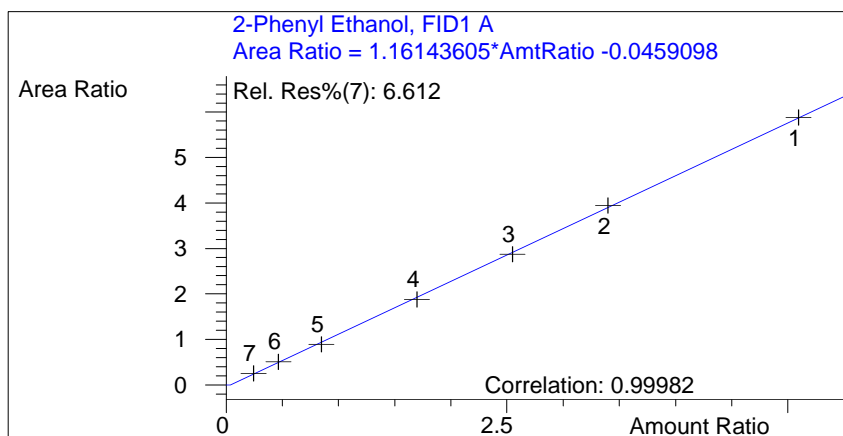


Figure 25. Calibration curve for Hexanoic Acid.

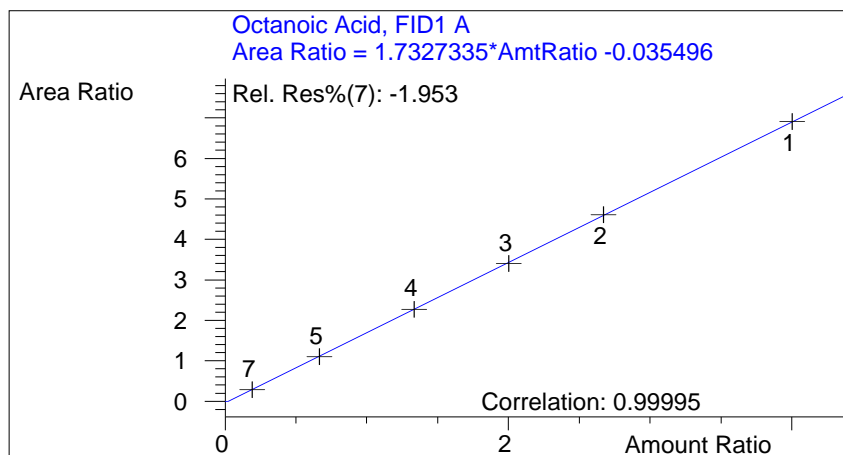


Figure 27. Calibration curve for Octanoic Acid.

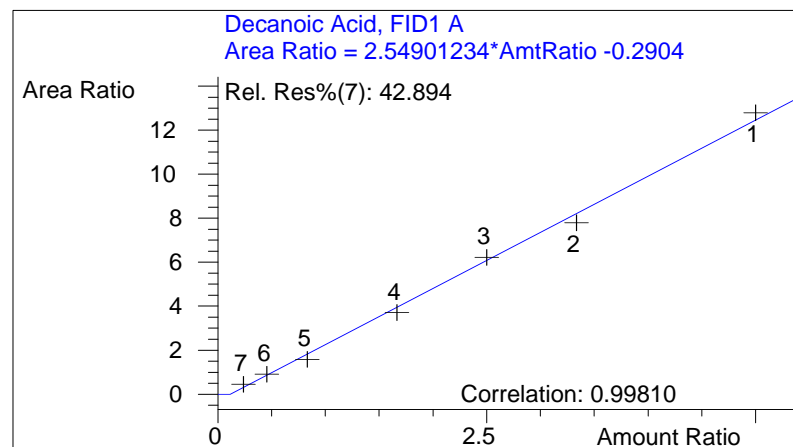


Figure 28. Calibration curve for Decanoic Acid.

LOD and LOQ were calculated as described in **STATISTICS**; the results are given below:

Table 5: Limit of detection and limit of quantification values in mg/L for each compound.

Compound	LOD	LOQ
Ethyl Acetate	0.104	0.348
Methanol	10.978	36.594
Ethyl Butyrate	0.016	0.055
Propanol	0.246	0.820
Isobutanol	0.048	0.160
Isoamyl Acetate	0.014	0.047
Butanol	0.060	0.200
Isoamyl Alcohol	0.018	0.061
Ethyl Hexanoate	0.022	0.072
Hexyl Acetate	0.021	0.069
Ethyl Lactate	0.517	1.723
Hexanol	0.016	0.054
Ethyl caprylate	0.017	0.058
Acetic acid	1.211	4.035
Propionic Acid	0.220	0.732
Iso-Butyric Acid	0.061	0.203
Butyric Acid	0.068	0.228
Ethyl Caprate	0.020	0.067
Iso-Valeric Acid	0.028	0.095
Diethyl Succinate	0.028	0.094
Valeric Acid	0.028	0.095
2-Phenylethyl Acetate	0.010	0.035
Hexanoic Acid	0.016	0.054
2-Phenylethanol	0.061	0.203
Octanoic Acid	0.038	0.125
Decanoic Acid	0.037	0.124

- **Recovery**

The recovery of the extraction process was investigated by injecting the ether extract of a wine as well as the extract of the same wine spiked with a mixture of all the abovementioned standards. This was done for red and white wine. Calculations as described in **STATISTICS**. Results are given in the table below:

Each extract was injected three times and the average of the three injections was calculated. The wines were spiked with a synthetic wine mixture at concentration level 5 in the calibration curve.

Table 6: Percentage recovery of analytes in white and red wine.

	White wine	Red wine
Compound	% Recovery	% Recovery
Ethyl Acetate	59.108	47.210
Methanol	74.504	54.170
Ethyl Butyrate	62.407	65.889
Propanol	44.393	35.698
Isobutanol	70.551	69.221
Isoamyl Acetate	86.377	62.624
Butanol	42.676	51.235
Isoamyl Alcohol	63.754	50.959
Ethyl Hexanoate	68.131	63.976
Hexyl Acetate	64.283	67.359
Ethyl Lactate	55.485	34.433
Hexanol	82.689	75.620
Ethyl caprylate	132.688	74.629
Acetic acid	50.582	42.416
Propionic Acid	43.174	31.834
Iso-Butyric Acid	62.230	64.173
Butyric Acid	59.014	84.513
Ethyl Caprate	76.906	83.374
Iso-Valeric Acid	92.518	91.945
Diethyl Succinate	61.081	60.423
Valeric Acid	71.026	72.318
2-Phenylethyl Acetate	82.967	88.652
Hexanoic Acid	96.575	86.460
2-Phenylethanol	63.097	38.248
Octanoic Acid	107.463	97.662
Decanoic Acid	105.184	107.746

As can be observed from the table, most recoveries are in the interval 60-110%. The exceptions are ethyl acetate, propanol, butanol, isoamyl alcohol, ethyl lactate, acetic acid and propionic acid. There is also a slight difference in recovery between the white wine matrix and the red wine matrix for methanol, isoamyl acetate, ethyl lactate, ethyl caprylate butyric acid and 2-phenyl ethanol.

- **Robustness**

The sample preparation can possibly be influenced by a number of factors, like the amounts of salt, ether and wine, the length of sonication, the pH of the wine, the temperature of the water in the ultrasonic bath, the ethanol concentration of the wine as well as the wine matrix (red or white). The influence of the variation of these parameters was evaluated comparing the concentrations obtained for the different analytes in question.

Amount of NaSO₄ used to dry extract.

A workable amount of NaSO₄ was chosen as 0.15 g, and different amounts between 0.05 and 0.25 g were used to determine whether this influences the concentrations obtained for the analytes. Each extract was injected three times and the average of the concentrations for the three injections was determined for each amount of salt. The results are summarised in Table 7.

No clear trend could be linked to the different amounts of salt used. Therefore, it is assumed that the amount of salt has no influence on the method.

Amount of Diethyl Ether used to extract the volatile compounds from the wine.

During sample preparation a variance in the amount of diethyl ether used will influence the concentration of the analytes injected. Normally one milliliter of diethyl ether is used and the maximum variance was estimated as 0.25 ml to either side. The average of the three injections for each extract is shown in Table 8.

Unacceptably large percentage standard deviations indicated that the amount of diethyl ether used for extraction definitely influences the results.

Amount of wine used for extraction.

A variance in the amount of wine as sample will affect the concentration of the analytes extracted from the wine and can lead to incorrect concentration readings from the calibration curve, as the curve was calibrated per 5ml of wine. The maximum variance in the amount was estimated as 0.5ml to either side. Each extract from a specific amount of wine, was injected three times and the average concentrations for each compound in the three injections are given in Table 9.

Although the percentage standard deviation was not as detrimental compared to the variances due to difference in ether amounts, variances due to incorrect sample amounts should be minimized by ensuring accurate measurements.

Table 7: Layout of the concentrations obtained for the analytes after extracts of the same wine was dried on different amounts of NaSO₄.

Compound	0.183 g	0.127 g	0.097 g	0.24 g	0.175 g	0.053 g	Average	STD	%RSD
Ethyl Acetate	200.665	222.193	213.738	194.129	194.289	207.294	205.385	11.207	5.457
Methanol	375.633	406.778	428.304	347.748	368.626	380.706	384.633	28.673	7.455
Ethyl Butyrate	11.756	11.674	12.220	12.287	11.658	11.976	11.928	0.277	2.321
Propanol	90.548	100.971	99.809	90.294	83.114	95.945	93.447	6.759	7.233
Isobutanol	54.867	60.346	59.676	54.212	52.148	57.785	56.506	3.267	5.782
Isoamyl Acetate	10.709	10.178	10.647	11.278	10.780	10.502	10.682	0.361	3.384
Butanol	10.580	11.361	11.289	10.408	10.168	10.971	10.796	0.487	4.508
Isoamyl Alcohol	245.945	250.818	252.514	245.069	245.195	246.984	247.754	3.152	1.272
Ethyl Hexanoate	17.364	16.180	17.005	18.485	17.615	16.900	17.258	0.774	4.487
Hexyl Acetate	13.145	12.267	12.879	14.030	13.288	12.796	13.067	0.589	4.504
Ethyl Lactate	258.947	271.977	264.814	255.363	255.678	255.568	260.391	6.733	2.586
Hexanol	16.832	16.135	16.694	17.551	16.937	16.579	16.788	0.466	2.774
Ethyl caprylate	3.410	3.167	3.368	3.702	3.464	3.366	3.413	0.174	5.094
Acetic acid	844.189	898.825	884.381	833.195	835.895	845.155	856.940	27.625	3.224
Propionic Acid	17.672	18.631	17.962	17.417	17.442	17.549	17.779	0.462	2.601
Iso-Butyric Acid	12.397	12.865	12.602	12.521	12.401	12.509	12.549	0.173	1.381
Butyric Acid	12.438	12.884	12.676	12.541	12.424	12.607	12.595	0.172	1.362
Ethyl Caprate	2.063	1.927	2.041	2.257	2.087	2.051	2.071	0.107	5.155
Iso-Valeric Acid	22.507	22.233	22.737	23.322	22.663	22.768	22.705	0.360	1.587
Diethyl Succinate	19.144	18.661	19.466	20.305	19.476	19.435	19.414	0.537	2.765
Valeric Acid	12.461	12.308	12.652	13.043	12.627	12.693	12.631	0.248	1.963
2-Phenylethyl Acetate	12.549	11.776	12.614	13.785	12.952	12.679	12.726	0.651	5.119
Hexanoic Acid	16.919	16.143	17.069	18.370	17.392	17.181	17.179	0.724	4.212
2-Phenylethanol	29.191	29.633	29.977	30.294	29.525	29.855	29.746	0.384	1.290
Octanoic Acid	26.049	24.347	26.376	29.181	27.198	26.604	26.626	1.578	5.925
Decanoic Acid	20.952	19.461	21.174	23.401	21.810	21.335	21.355	1.279	5.987

Table 8: Layout of concentrations obtained for the analytes after the same wine was extracted using 0.75 ml, 1 ml and 1.25 ml diethyl ether.

	0.75ml	1ml	1.25ml		
Compound	Average	Average	Average	STD	%RSD
Ethyl Acetate	126.034	209.3391	56.66615	76.442	58.496
Methanol	215.524	421.3532	62.80262	179.930	77.148
Ethyl Butyrate	0.301	nd	0.547264	0.174	41.051
Propanol	25.926	46.98845	75.7699	25.021	50.485
Isobutanol	38.925	63.53858	15.42713	24.058	61.221
Isoamyl Acetate	0.377	0.284718	0.622788	0.175	40.785
Butanol	2.001	2.948375	1.572458	0.704	32.391
Isoamyl Alcohol	234.165	304.0942	154.6702	74.763	32.368
Ethyl Hexanoate	0.588	0.472203	1.822335	0.748	77.891
Hexyl Acetate	nd	nd	nd	nd	nd
Ethyl Lactate	139.879	271.8085	32.73345	119.751	80.836
Hexanol	1.414	1.431769	1.239164	0.106	7.820
Ethyl caprylate	0.428	0.320231	1.779879	0.813	96.520
Acetic acid	371.429	695.8859	195.8203	253.698	60.254
Propionic Acid	70.566	123.7479	35.78199	44.302	57.762
Iso-Butyric Acid	1.157	1.745192	0.83182	0.463	37.191
Butyric Acid	0.912	1.208653	2.125284	0.632	44.675
Ethyl Caprate	0.185	nd	0.463783	0.197	60.826
Iso-Valeric Acid	2.072	2.631782	1.151639	0.747	38.290
Diethyl Succinate	34.796	37.5	7.750443	16.451	61.655
Valeric Acid	0.464	0.717605	nd	0.179	30.314
2-Phenylethyl Acetate	nd	nd	nd	nd	nd
Hexanoic Acid	1.313	1.129091	5.700782	2.588	95.342
2-Phenylethanol	35.320	46.36292	11.33872	17.906	57.748
Octanoic Acid	1.619	1.180298	6.793072	3.122	97.637
Decanoic Acid	1.372	1.286832	2.13551	0.467	29.254

nd = not detected

Length of sonication

The wine\ether mixture is sonicated for five minutes. Variance in the time of sonication may occur and the effect of these variances was investigated by sonicating the mixture for 4.5 min, 5 min and 5.5 min. Each extract was injected three times and the average of the concentrations for the three injections is given for each treatment.

Table 9: Layout of concentrations obtained for the analytes after different volumes of the same wine was extracted.

Compound	4.5ml wine	5ml wine	5.5ml wine	STD	%RSD
Ethyl Acetate	184.747	222.870	205.304	19.081	9.339
Methanol	86.883	91.822	95.312	4.236	4.637
Ethyl Butyrate	0.306	0.348	0.414	0.054	15.286
Propanol	120.276	133.431	129.324	6.731	5.272
Isobutanol	25.625	26.341	29.409	2.010	7.410
Isoamyl Acetate	6.734	8.211	9.773	1.520	18.445
Butanol	1.943	1.945	2.187	0.140	6.933
Isoamyl Alcohol	138.427	147.403	159.833	10.749	7.236
Ethyl Hexanoate	0.678	0.810	0.896	0.110	13.833
Hexyl Acetate	0.269	0.310	0.330	0.031	10.335
Ethyl Lactate	77.978	77.872	75.456	1.427	1.850
Hexanol	1.278	1.538	1.591	0.167	11.385
Ethyl caprylate	0.590	0.764	0.825	0.122	16.802
Acetic acid	303.521	292.098	286.305	8.760	2.980
Propionic Acid	20.207	21.645	19.841	0.953	4.636
Iso-Butyric Acid	1.180	1.194	1.172	0.011	0.946
Butyric Acid	1.227	1.279	1.104	0.090	7.475
Ethyl Caprate	0.326	0.402	0.443	0.059	15.171
Iso-Valeric Acid	0.859	0.951	0.892	0.047	5.174
Diethyl Succinate	0.432	0.494	0.511	0.041	8.651
Valeric Acid	0.440	0.453	0.417	0.018	4.127
2-Phenylethyl Acetate	0.058	0.100	0.115	0.029	32.343
Hexanoic Acid	2.442	3.001	3.073	0.345	12.169
2-Phenylethanol	7.025	8.143	7.890	0.586	7.630
Octanoic Acid	2.813	3.635	3.903	0.568	16.456
Decanoic Acid	1.978	2.227	2.328	0.180	8.288

Differences in the length of sonication influence the results and should therefore be avoided.

pH of the wine

Natural variances of wine pH regularly occur and the influence of this was investigated by adjusting the pH of the same wine to 3, 3.5 and 4 respectively. Each treatment was extracted and this extract was injected three times. The averages of the three injections for each treatment are given for the different pH values in Table 5.

The percentage standard deviations for all the compounds were considered to be acceptable and no pH adjustment of samples is needed.

Table 10: Layout of concentrations obtained for the analytes after the wine was sonicated for 4.5min, 5min or 5.5min.

	4.5 min	5min	5.5min		
Compound	Average	Average	Average	STD	%RSD
Ethyl Acetate	52.249	56.666	53.310	2.306	4.264
Methanol	70.816	62.803	69.788	4.360	6.431
Ethyl Butyrate	0.446	0.547	0.432	0.063	13.272
Propanol	88.376	75.770	83.667	6.370	7.711
Isobutanol	16.959	15.427	15.525	0.857	5.369
Isoamyl Acetate	0.507	0.623	0.469	0.080	15.056
Butanol	1.699	1.572	1.607	0.065	4.018
Isoamyl Alcohol	157.153	154.670	152.962	2.107	1.360
Ethyl Hexanoate	1.397	1.822	1.398	0.245	15.945
Hexyl Acetate	nd	nd	nd	nd	nd
Ethyl Lactate	34.740	32.733	33.926	1.009	2.986
Hexanol	1.186	1.239	1.200	0.027	2.268
Ethyl caprylate	1.275	1.780	1.286	0.289	19.944
Acetic acid	213.873	195.820	208.308	9.245	4.488
Propionic Acid	39.431	35.782	37.588	1.825	4.853
Iso-Butyric Acid	0.849	0.832	0.850	0.010	1.212
Butyric Acid	2.129	2.125	2.153	0.015	0.714
Ethyl Caprate	0.354	0.464	0.354	0.063	16.257
Iso-Valeric Acid	1.162	1.152	1.139	0.012	1.018
Diethyl Succinate	7.504	7.750	7.727	0.136	1.778
Valeric Acid	nd	nd	nd	nd	nd
2-Phenylethyl Acetate	nd	nd	nd	nd	nd
Hexanoic Acid	5.424	5.701	5.570	0.138	2.485
2-Phenylethanol	11.204	11.339	11.401	0.101	0.893
Octanoic Acid	6.418	6.793	6.507	0.196	2.980
Decanoic Acid	2.036	2.136	2.046	0.055	2.650

Temperature of the water in the ultrasonic bath.

The ultrasonic bath can take only ten samples at a time, therefore if more than ten samples are prepared, the ultrasonic bath is in use for a longer period of time and therefore the water in the bath may heat up. This can influence the extraction process. This was investigated by extracting the wines at three different temperatures of the water in the ultrasonic bath. Each extract was injected three times and the average concentrations of the analytes for these three runs are given for each temperature.

The temperature of the water should be kept constant to minimise variances in data due to different extraction temperatures.

Table 11: Layout of concentrations obtained for the analytes after the pH of the wine was adjusted to 3, 3.5, and 4.

	pH 3	pH 3.5	pH 4		
Compound	Average	Average	Average	STD	%RSD
Ethyl Acetate	153.4475	144.3999	153.3083	5.183926	3.447
Methanol	293.0674	287.5588	298.4494	5.445433	1.858
Ethyl Butyrate	nd	nd	nd	nd	nd
Propanol	36.05147	32.24963	40.2757	4.014887	11.093
Isobutanol	49.81305	46.98932	54.82992	3.971091	7.857
Isoamyl Acetate	0.273362	nd	nd	nd	nd
Butanol	2.434028	2.332557	2.586185	0.127655	5.208
Isoamyl Alcohol	270.6326	262.6133	270.1463	4.496092	1.679
Ethyl Hexanoate	0.45725	0.453327	0.42421	0.01805	4.057
Hexyl Acetate	nd	nd	nd	nd	nd
Ethyl Lactate	205.3762	189.8441	205.8442	9.105575	4.545
Hexanol	1.394217	1.38063	1.352887	0.021065	1.531
Ethyl caprylate	0.295528	0.289133	0.257317	0.020466	7.292
Acetic acid	518.9315	467.0814	475.8782	27.7471	5.694
Propionic Acid	93.93859	91.38681	97.91624	3.29056	3.485
Iso-Butyric Acid	1.622462	1.540788	1.556356	0.043365	2.756
Butyric Acid	1.113353	1.067559	1.068706	0.026114	2.411
Ethyl Caprate	nd	nd	nd	nd	nd
Iso-Valeric Acid	2.418142	2.312433	2.313997	0.060585	2.580
Diethyl Succinate	37.09645	36.5411	35.27573	0.933147	2.570
Valeric Acid	0.597285	0.567696	0.586505	0.014975	2.565
2-Phenylethyl Acetate	nd	nd	nd	nd	nd
Hexanoic Acid	1.230788	1.241468	1.172115	0.037342	3.074
2-Phenylethanol	42.97542	41.90176	41.32316	0.838401	1.993
Octanoic Acid	1.337465	1.360598	1.262596	0.051226	3.880
Decanoic Acid	1.320557	1.311885	1.292353	0.014446	1.104

Ethanol Concentration of the wine

The effect of various alcohol concentrations on the extraction efficiency was investigated by making up synthetic wine mixtures in wine simulant with 16%, 14%, 12% or 10% ethanol. These synthetic wines were extracted as normal and injected three times each. The averages of the concentrations of the compounds for each wine were determined and compared.

The ethanol concentration of the samples does not seem to influence the concentration of the analytes extracted. Therefore no adjustment of ethanol is needed during sample preparation.

Table 12: Layout of concentrations obtained for the analytes after the temperature of the water in the ultrasonic bath has been adjusted.

	14 °C	28 °C	41 °C		
Compound	Average	Average	Average	STD	%RSD
Ethyl Acetate	53.239	56.666	43.063	7.075	13.876
Methanol	71.119	62.803	64.162	4.461	6.756
Ethyl Butyrate	0.480	0.547	0.445	0.052	10.550
Propanol	62.787	75.770	109.541	24.135	29.184
Isobutanol	12.300	15.427	18.897	3.300	21.233
Isoamyl Acetate	0.573	0.623	0.481	0.072	12.831
Butanol	1.292	1.572	1.915	0.312	19.602
Isoamyl Alcohol	144.066	154.670	157.636	7.134	4.690
Ethyl Hexanoate	1.783	1.822	1.340	0.268	16.234
Hexyl Acetate	nd	nd	nd	nd	nd
Ethyl Lactate	29.512	32.733	39.518	5.108	15.058
Hexanol	1.352	1.239	1.186	0.085	6.737
Ethyl caprylate	1.740	1.780	1.294	0.270	16.809
Acetic acid	185.529	195.820	216.299	15.664	7.863
Propionic Acid	33.927	35.782	58.409	13.631	31.917
Iso-Butyric Acid	0.886	0.832	0.852	0.027	3.195
Butyric Acid	2.273	2.125	2.172	0.076	3.447
Ethyl Caprate	0.446	0.464	0.367	0.051	12.095
Iso-Valeric Acid	1.243	1.152	1.151	0.053	4.497
Diethyl Succinate	8.843	7.750	7.641	0.665	8.226
Valeric Acid	nd	nd	0.287	nd	nd
2-Phenylethyl Acetate	nd	nd	nd	nd	nd
Hexanoic Acid	6.822	5.701	5.381	0.756	12.674
2-Phenylethanol	12.456	11.339	11.426	0.622	5.294
Octanoic Acid	8.157	6.793	6.256	0.980	13.861
Decanoic Acid	2.352	2.136	2.031	0.164	7.533

Wine matrix

From the recovery experiment it was seen that the wine matrix influences the extraction process for the analytes, methanol, isoamyl acetate, ethyl lactate, ethyl caprylate butyric acid and 2-phenyl ethanol. All the other analytes are extracted in more or less the same amounts from white and red wine matrices.

Table 13: Layout of concentrations obtained for the analytes after the ethanol concentration of the synthetic wine has been adjusted.

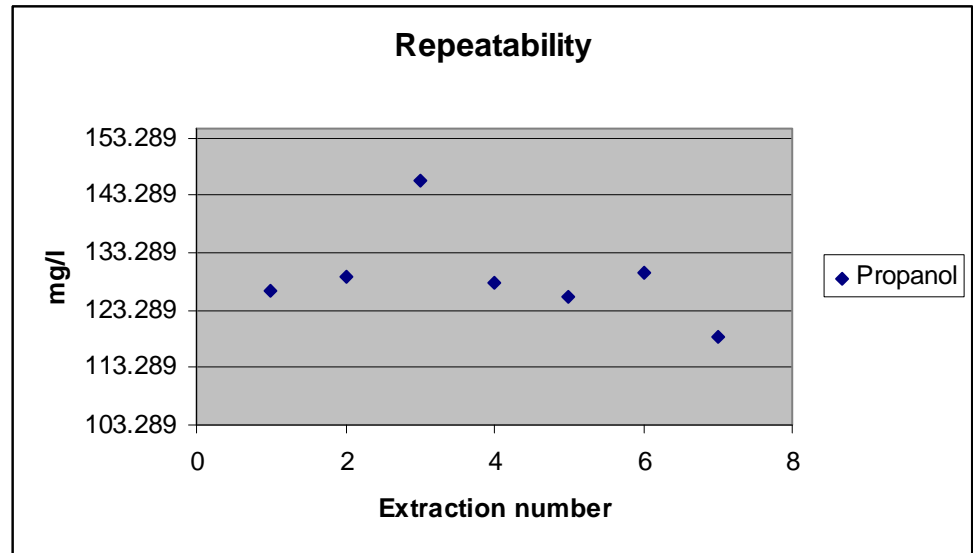
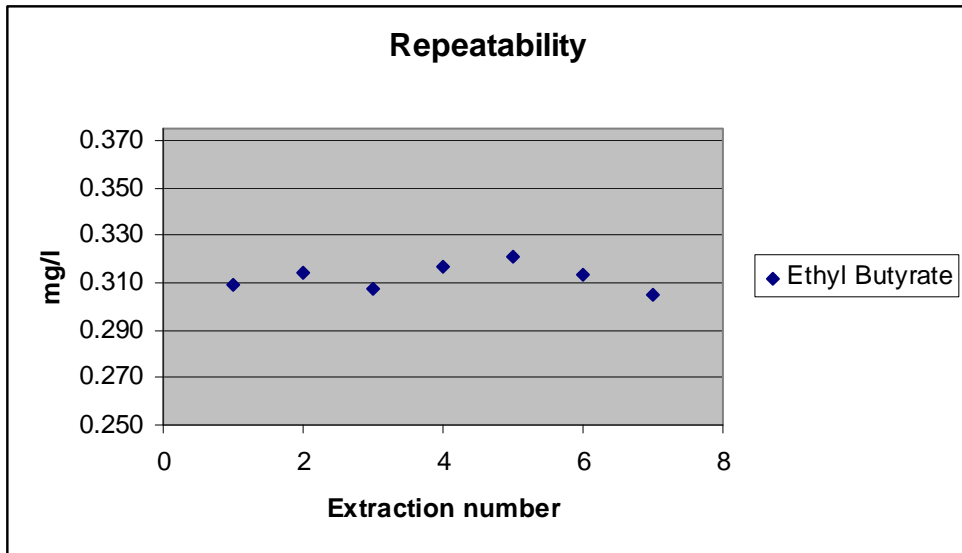
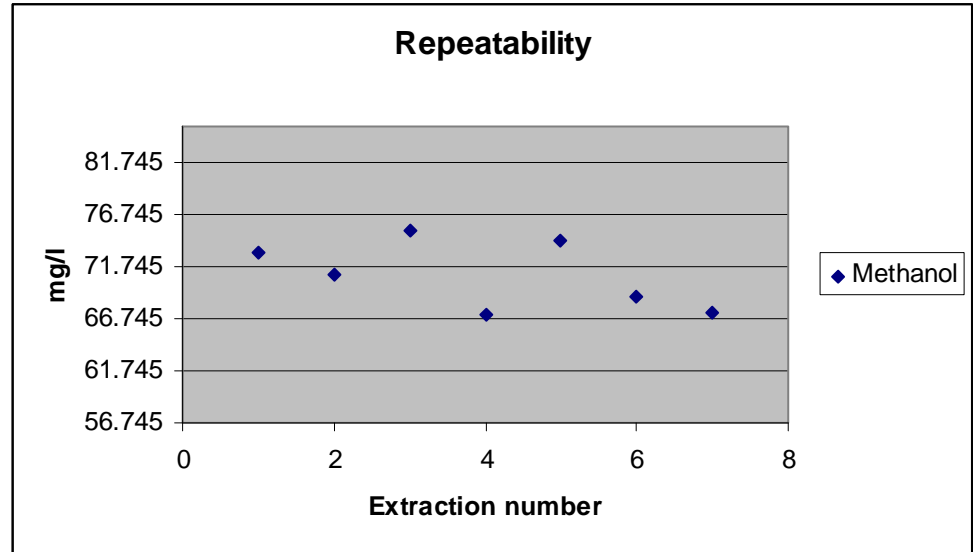
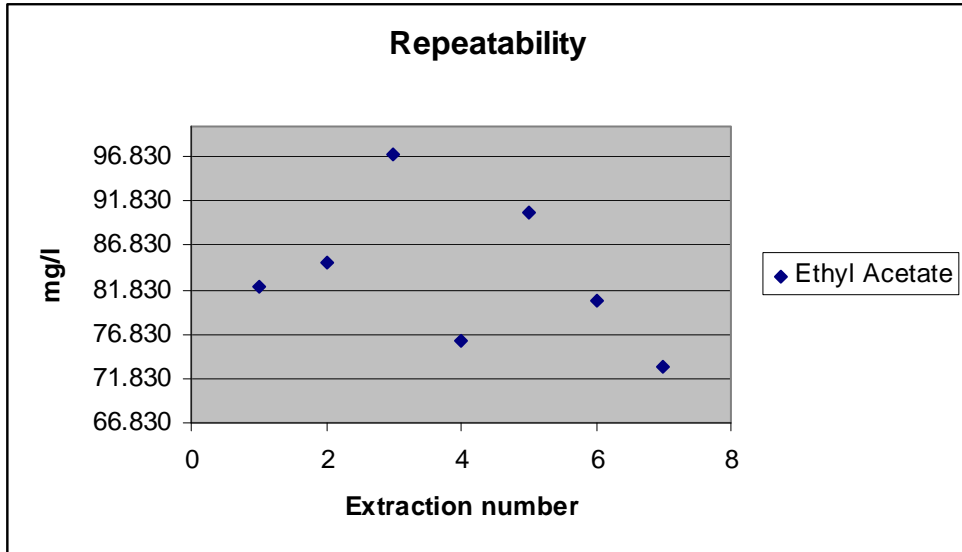
	16% Ethanol	14% Ethanol	12% Ethanol	10% Ethanol		
Compound	Average	Average	Average	Average	STD	%RSD
Ethyl Acetate	38.330	36.009	34.750	38.564	1.847	5.003
Methanol	113.924	89.760	88.818	89.423	12.301	12.883
Ethyl Butyrate	2.256	2.211	2.186	2.216	0.029	1.317
Propanol	17.149	15.546	14.314	16.034	1.175	7.454
Isobutanol	9.594	9.102	8.537	9.196	0.436	4.786
Isoamyl Acetate	2.131	2.074	2.044	1.975	0.065	3.167
Butanol	1.707	1.629	1.537	1.641	0.070	4.299
Isoamyl Alcohol	41.859	41.365	39.651	40.236	1.013	2.483
Ethyl Hexanoate	3.421	3.326	3.278	3.124	0.124	3.766
Hexyl Acetate	2.512	2.448	2.403	2.286	0.095	3.947
Ethyl Lactate	46.254	42.838	40.326	40.176	2.846	6.712
Hexanol	2.959	2.980	2.948	2.885	0.041	1.398
Ethyl caprylate	0.610	0.608	0.593	0.565	0.021	3.512
Acetic acid	166.895	146.392	143.168	139.888	12.166	8.160
Propionic Acid	3.078	2.959	2.714	2.630	0.209	7.332
Iso-Butyric Acid	1.868	1.891	1.815	1.840	0.033	1.763
Butyric Acid	1.824	1.886	1.822	1.854	0.030	1.638
Ethyl Caprate	0.391	0.395	0.385	0.371	0.010	2.637
Iso-Valeric Acid	3.569	3.699	3.626	3.638	0.053	1.468
Diethyl Succinate	3.032	3.150	3.100	3.116	0.050	1.607
Valeric Acid	1.938	2.050	2.020	2.034	0.050	2.468
2-Phenylethyl Acetate	2.163	2.242	2.238	2.158	0.046	2.094
Hexanoic Acid	2.896	3.096	3.105	3.057	0.097	3.204
2-Phenylethanol	4.548	4.587	4.430	4.476	0.070	1.562
Octanoic Acid	4.648	4.911	4.958	4.826	0.137	2.830
Decanoic Acid	4.441	4.607	4.616	4.534	0.081	1.784

PERFORMANCE

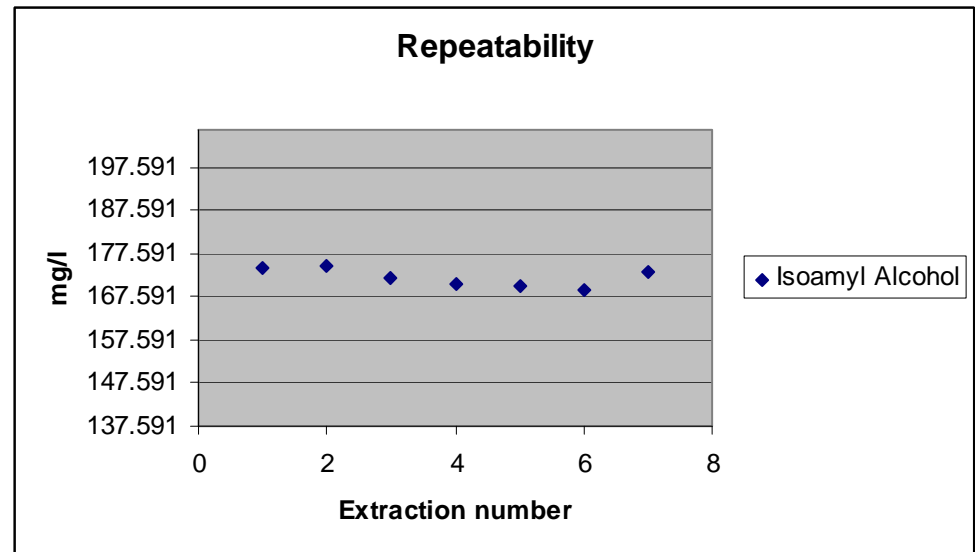
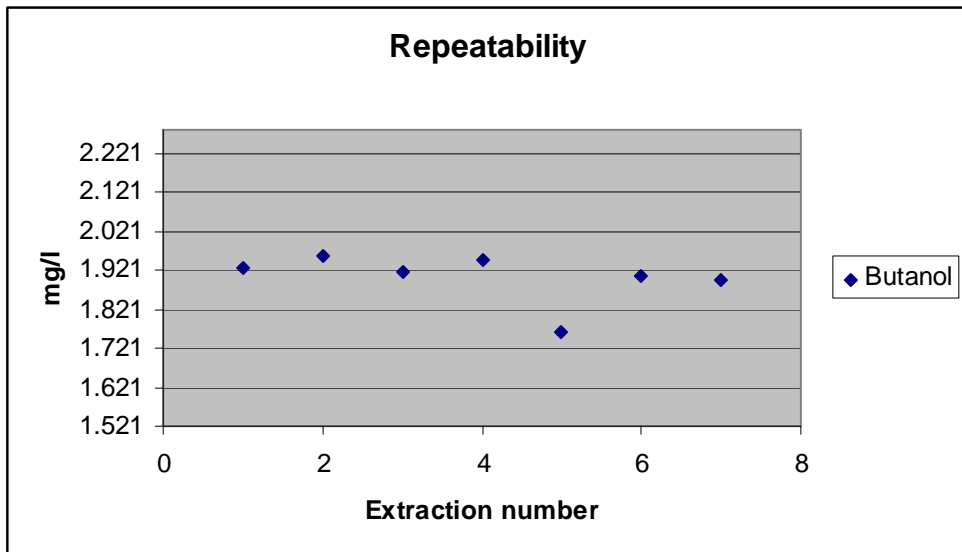
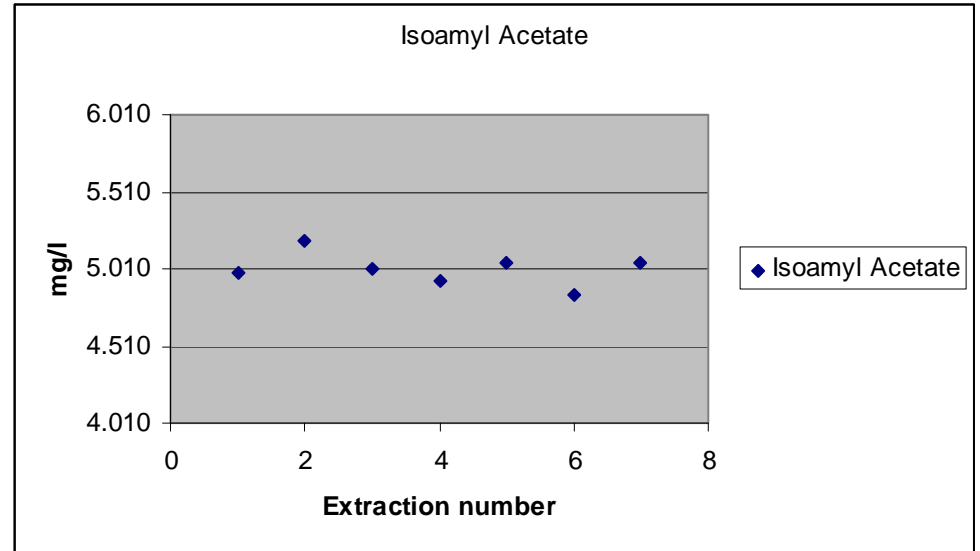
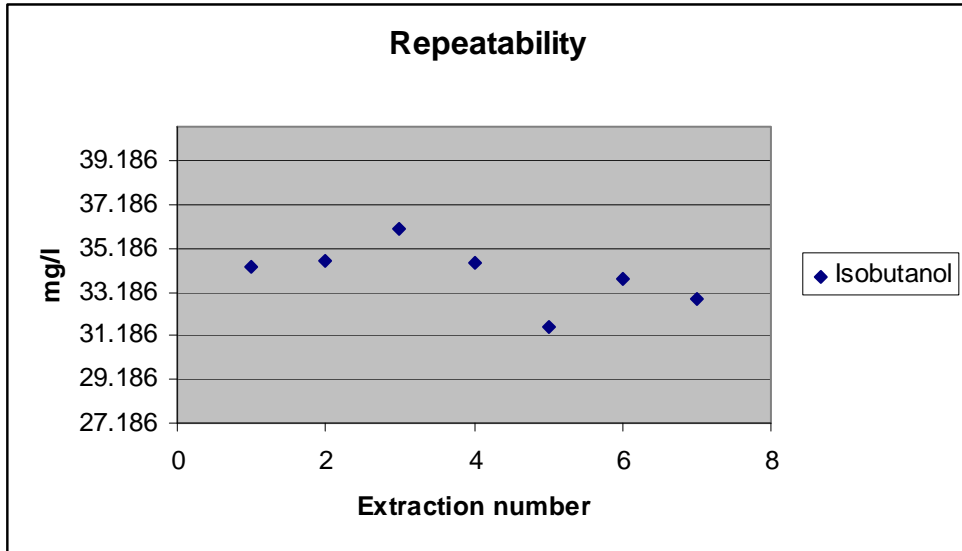
The repeatability data shows a variance of between 0.5 and 15% and therefore, it was decided to inject each extract three times for each sample and to determine the average of these three injections for each analyte.

Table 14. Averages of concentrations obtained for the analytes in 7 different extractions of the same wine.

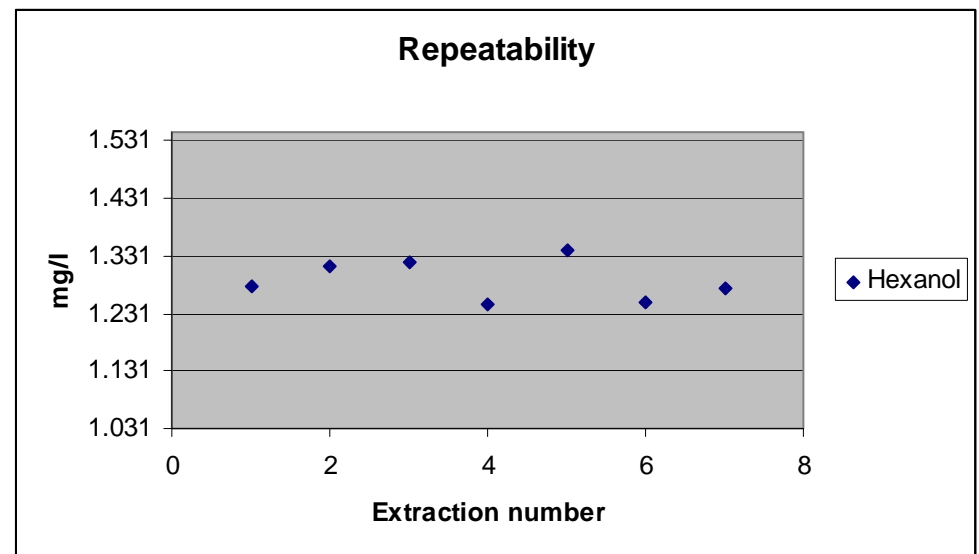
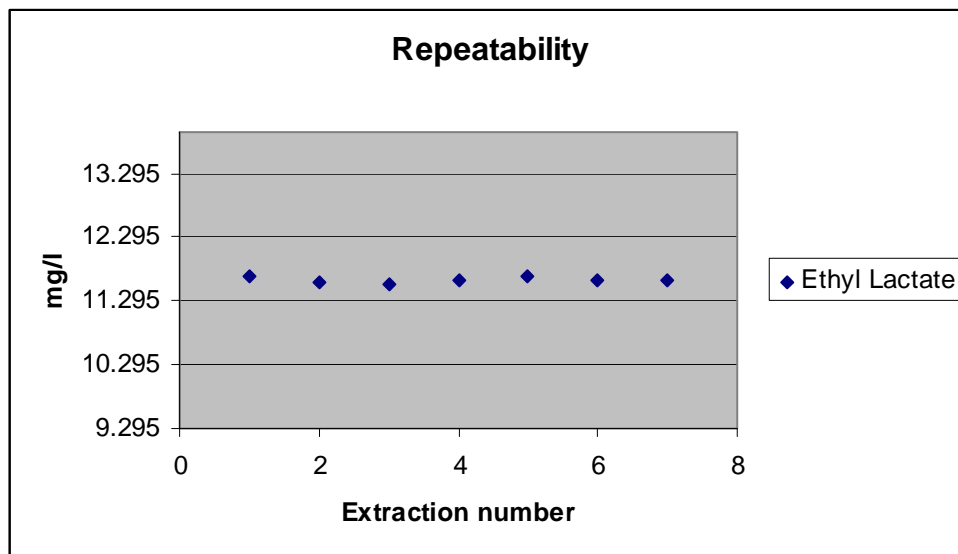
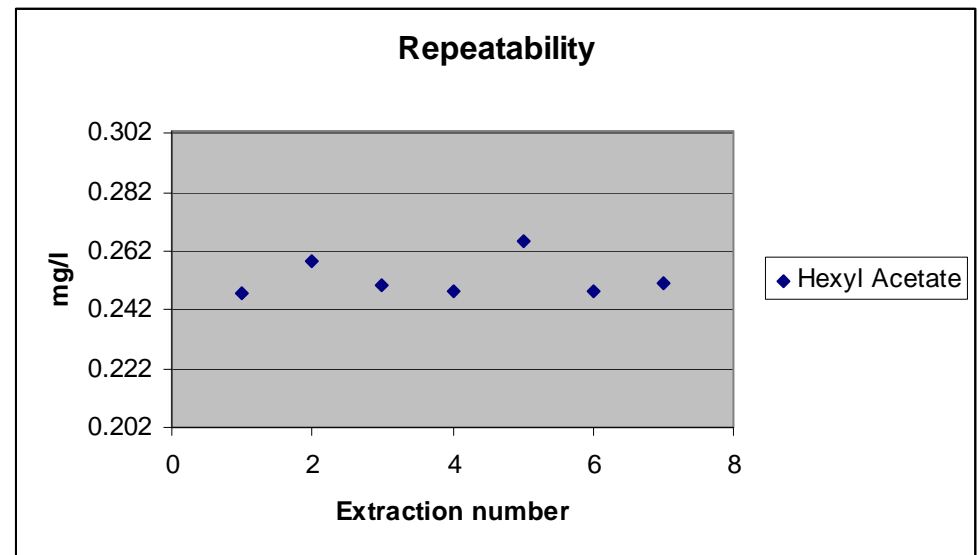
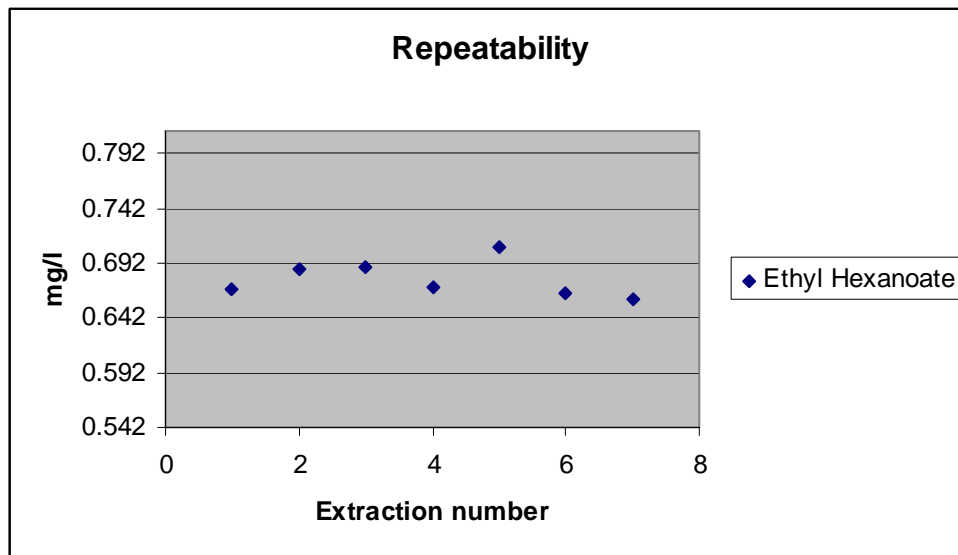
Compound	1	2	3	4	5	6	7	Average	STD	%RSD
Ethyl Acetate	82.196	84.996	97.062	76.055	90.620	80.629	73.202	83.537	8.249	9.875
Methanol	73.046	70.897	75.171	67.184	74.146	68.760	67.318	70.932	3.281	4.626
Ethyl Butyrate	0.309	0.315	0.307	0.316	0.321	0.314	0.305	0.312	0.006	1.810
Propanol	126.739	129.218	145.895	127.962	125.536	129.800	118.625	129.111	8.285	6.417
Isobutanol	34.388	34.619	36.078	34.537	31.585	33.786	32.885	33.983	1.429	4.206
Isoamyl Acetate	4.992	5.191	5.012	4.938	5.052	4.845	5.056	5.012	0.108	2.146
Butanol	1.925	1.956	1.918	1.947	1.761	1.907	1.897	1.902	0.066	3.446
Isoamyl Alcohol	174.189	174.765	171.960	170.457	170.009	169.372	173.168	171.988	2.123	1.235
Ethyl Hexanoate	0.668	0.687	0.688	0.670	0.707	0.664	0.660	0.678	0.017	2.488
Hexyl Acetate	0.248	0.259	0.250	0.249	0.265	0.248	0.251	0.253	0.007	2.609
Ethyl Lactate	11.693	11.580	11.542	11.609	11.682	11.603	11.619	11.618	0.054	0.461
Hexanol	1.277	1.312	1.319	1.247	1.341	1.252	1.273	1.289	0.036	2.765
Ethyl caprylate	0.495	0.520	0.529	0.502	0.512	0.471	0.491	0.503	0.019	3.853
Acetic acid	184.455	180.164	182.708	177.889	175.593	189.089	181.687	181.655	4.423	2.435
Propionic Acid	46.749	46.173	44.612	46.418	47.924	45.200	48.414	46.499	1.362	2.928
Iso-Butyric Acid	1.846	1.791	1.920	1.761	1.878	1.730	1.761	1.812	0.070	3.874
Butyric Acid	1.630	1.627	1.666	1.615	1.649	1.585	1.627	1.628	0.026	1.566
Ethyl Caprate	0.206	0.203	0.182	0.191	0.171	0.177	0.192	0.189	0.013	6.816
Iso-Valeric Acid	1.354	1.364	1.363	1.334	1.435	1.333	1.328	1.359	0.037	2.701
Diethyl Succinate	0.450	0.452	0.437	0.436	0.442	0.419	0.432	0.438	0.011	2.525
Valeric Acid	0.558	0.553	0.566	0.532	0.564	0.534	0.549	0.551	0.014	2.452
2-Phenylethyl Acetate	0.085	0.091	0.094	0.080	0.098	0.079	0.086	0.087	0.007	7.956
Hexanoic Acid	2.635	2.722	2.774	2.595	2.825	2.612	2.667	2.690	0.087	3.216
2-Phenylethanol	13.953	14.123	14.608	13.483	14.319	13.357	13.618	13.923	0.461	3.308
Octanoic Acid	2.680	2.812	2.901	2.694	2.842	2.680	2.708	2.760	0.091	3.287
Decanoic Acid	1.672	1.699	1.721	1.671	1.695	1.668	1.678	1.686	0.020	1.158



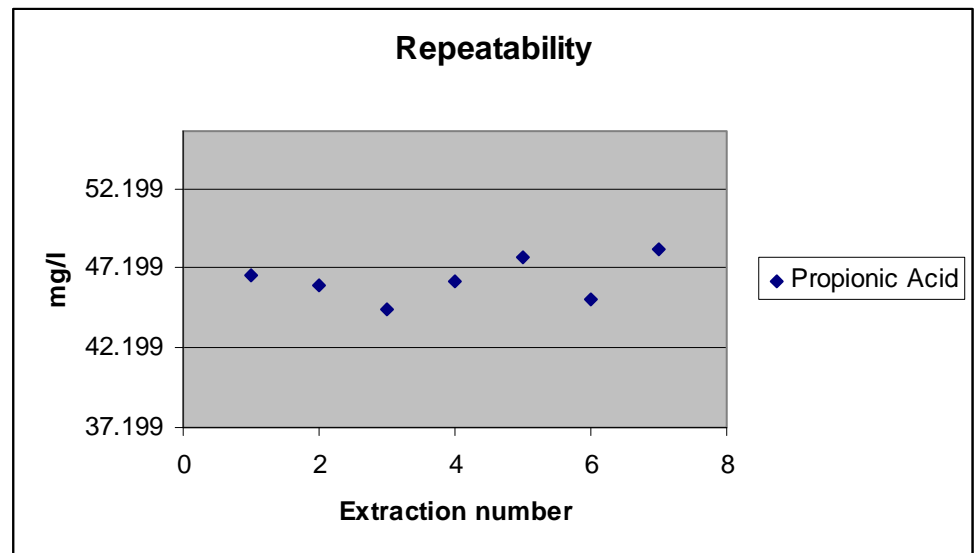
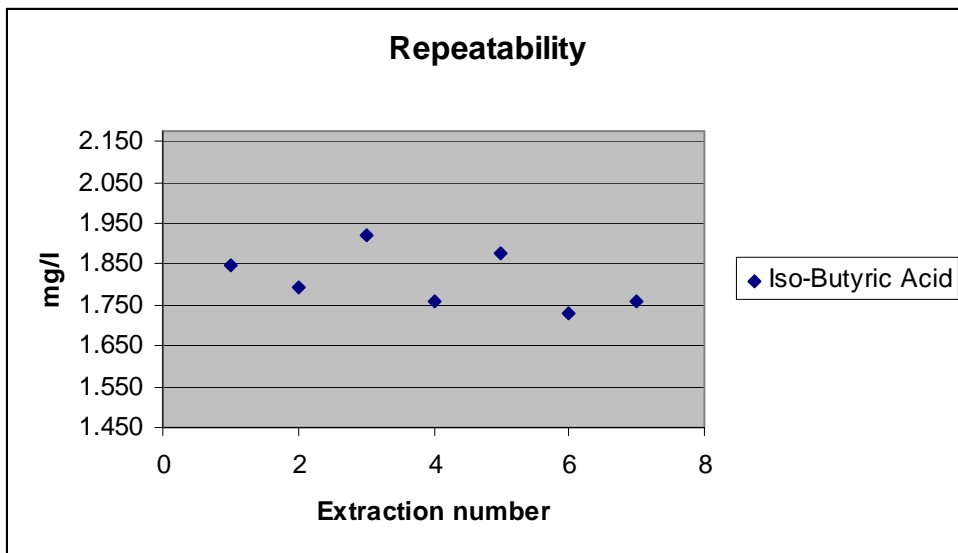
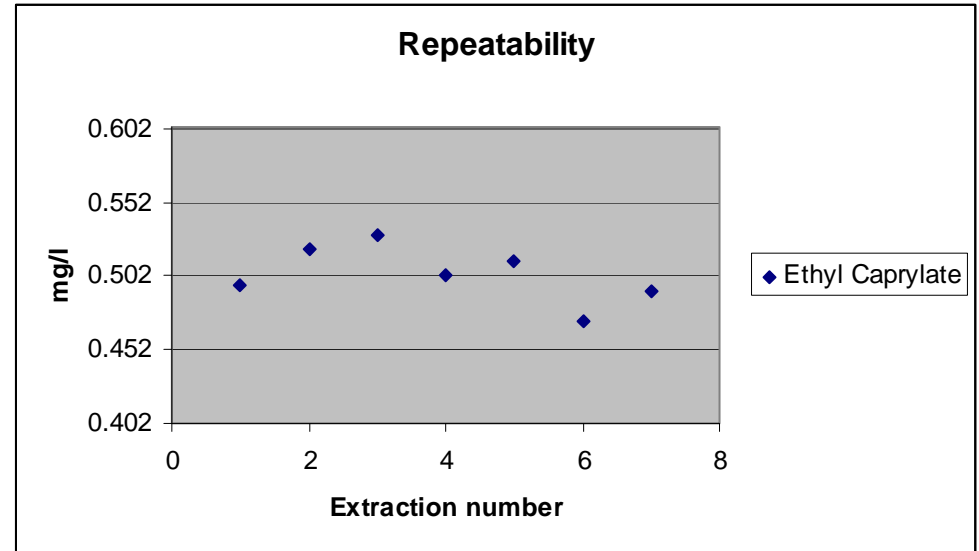
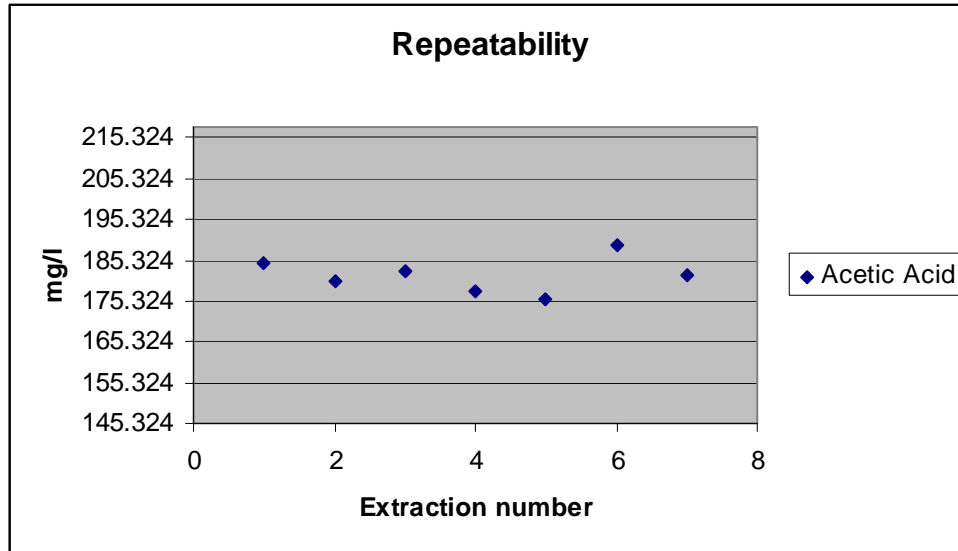
Figures 29 – 32. Repeatability data for Ethyl Acetate, Methanol, Ethyl butyrate and Propanol.



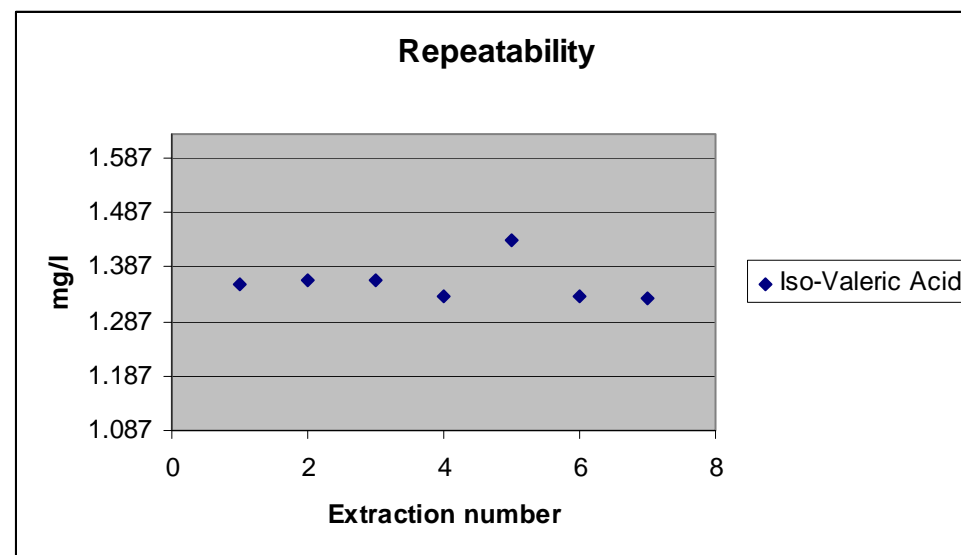
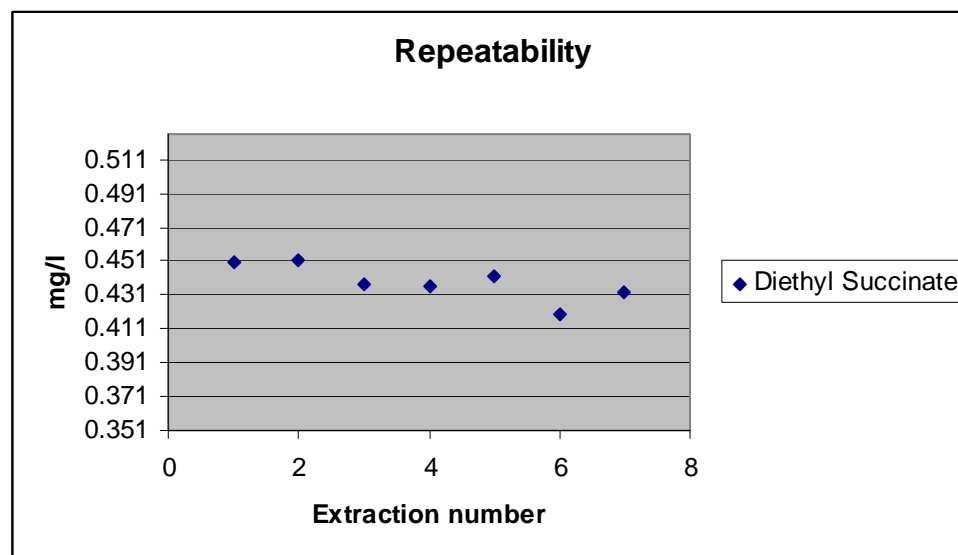
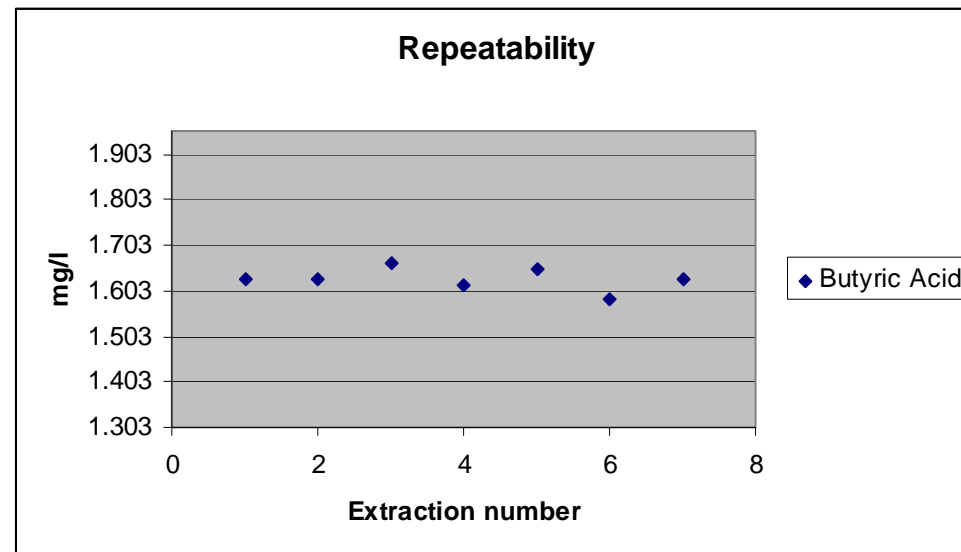
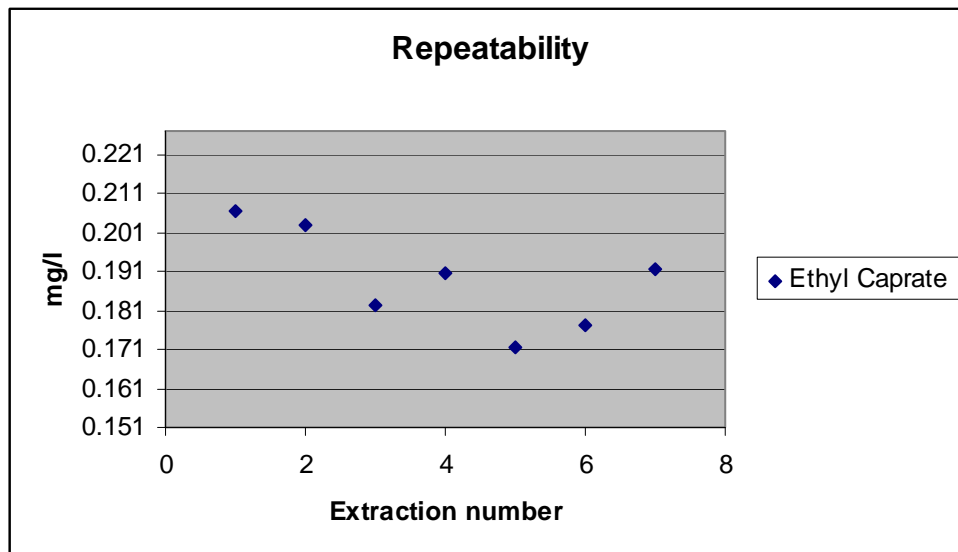
Figures 33 - 36. Repeatability data for Isobutanol, Isoamyl Acetate, Butanol and Isoamyl Alcohol.



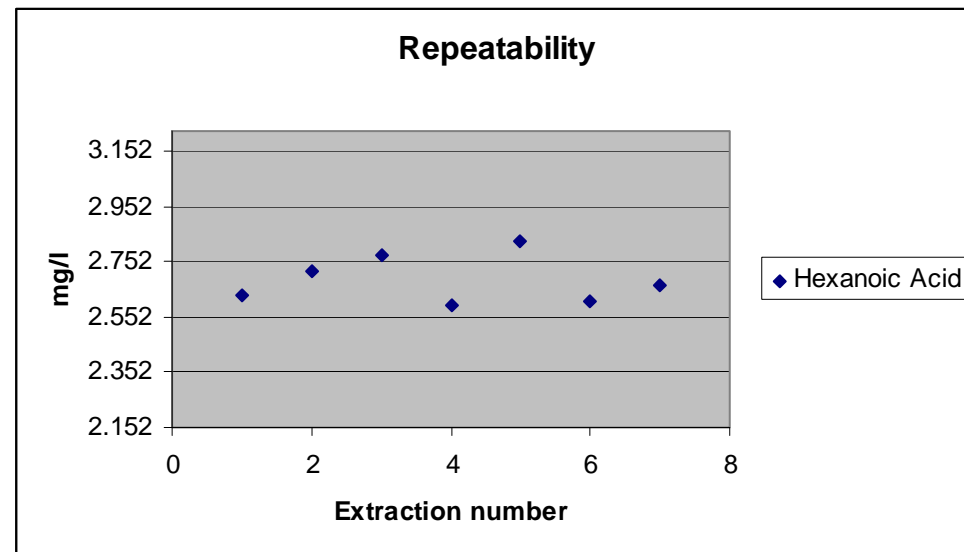
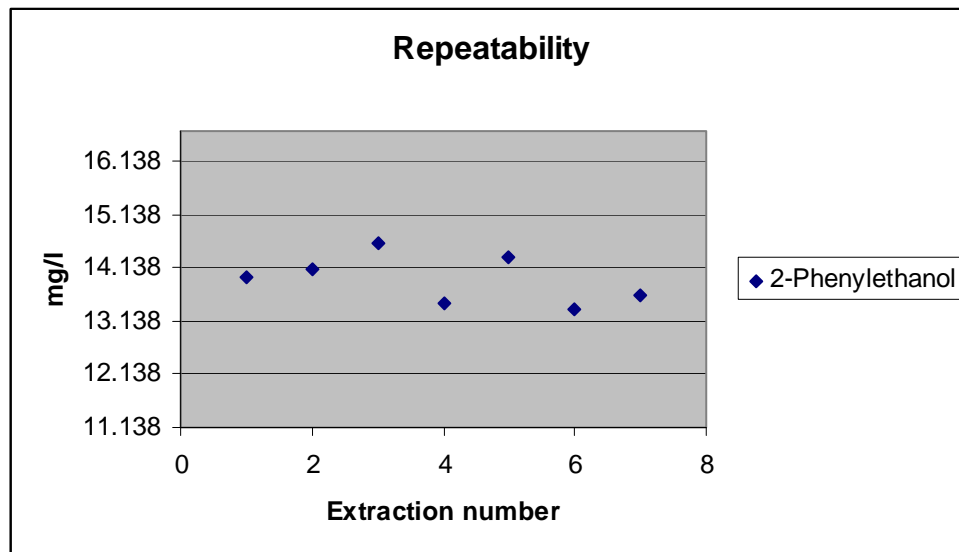
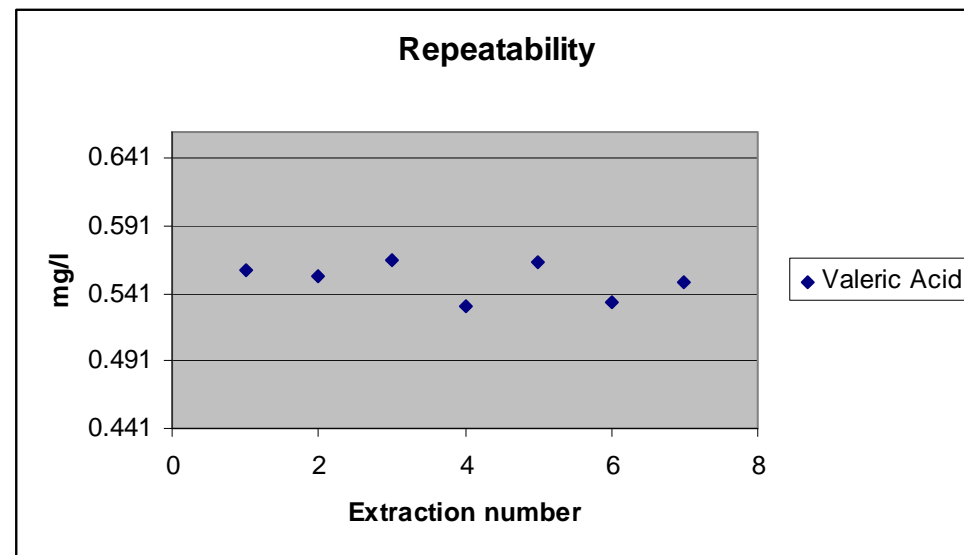
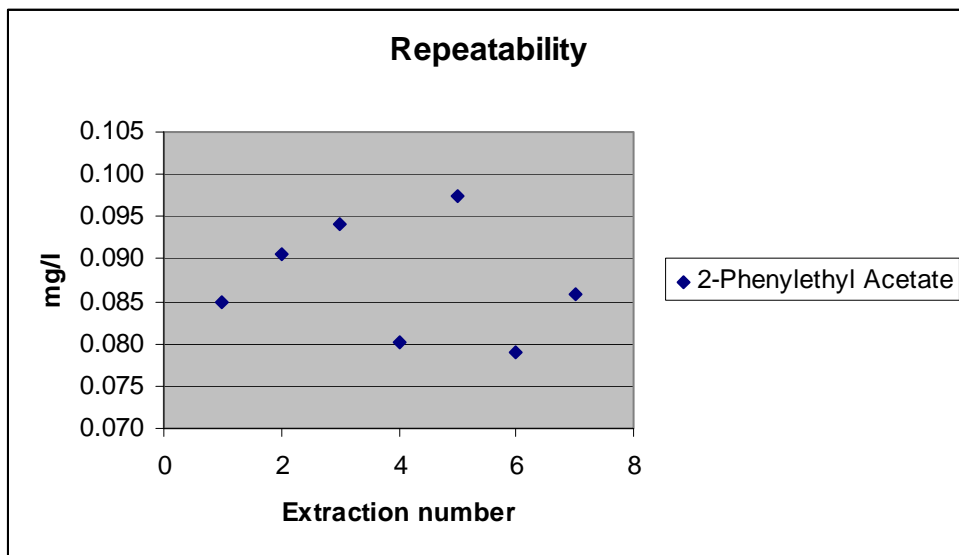
Figures 37 - 40. Repeatability data for Ethyl Hexanoate, Hexyl Acetate, Ethyl Lactate and Hexanol.



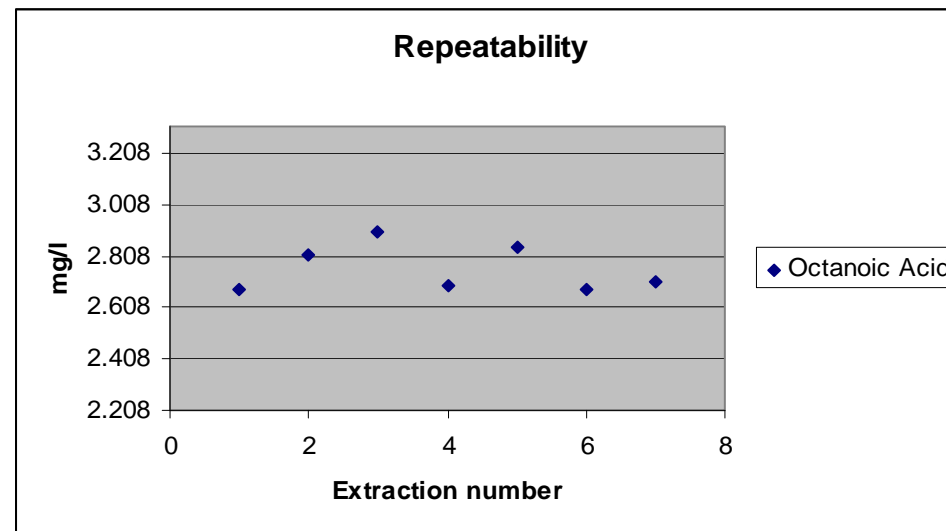
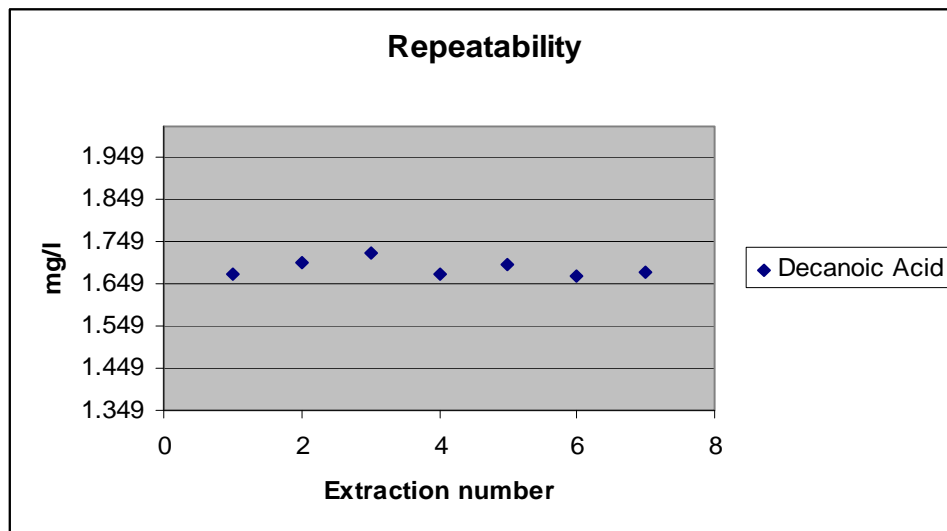
Figures 41 - 44. Repeatability data for Acetic Acid, Ethyl Caprylate, Iso-Butyric Acid and Propionic Acid.



Figures 45 - 48. Repeatability data for Ethyl Caprate, Butyric Acid, Diethyl Succinate and Iso-Valeric Acid.



Figures 49 - 52. Repeatability data for 2-Phenylethyl Acetate, Valeric Acid, 2-Phenylethanol and Hexanoic Acid.

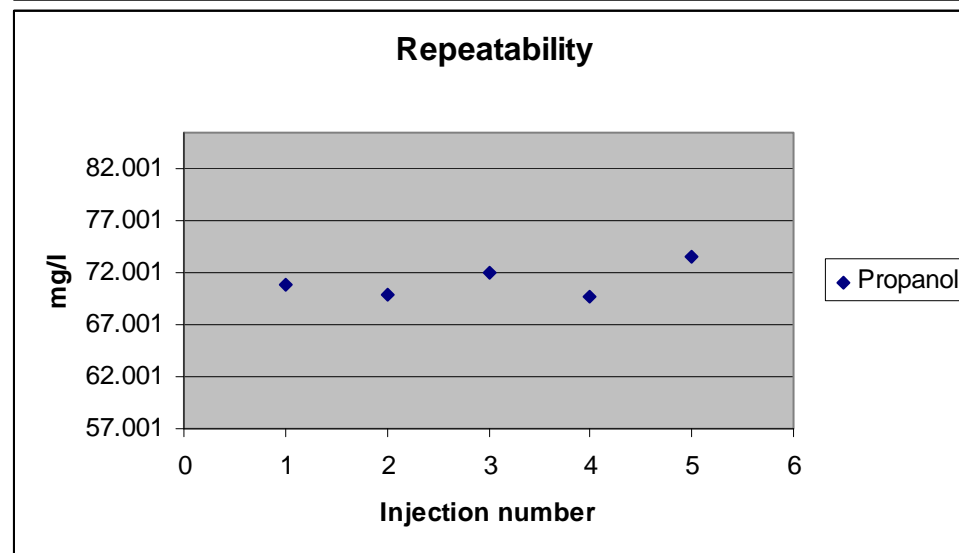
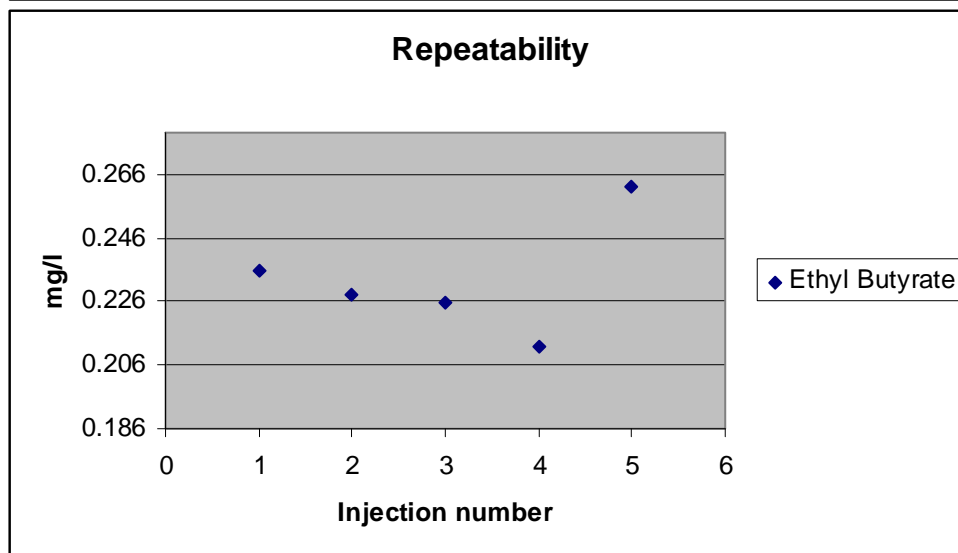
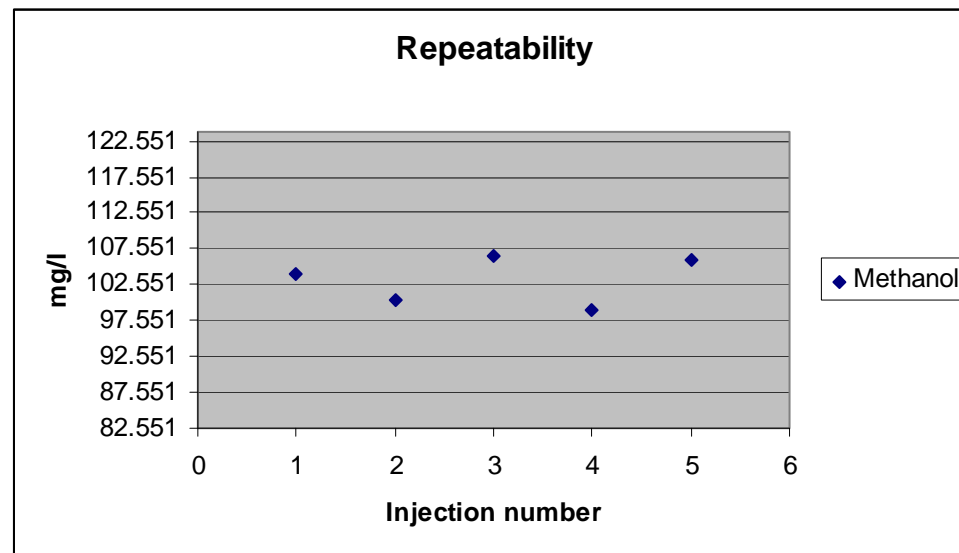
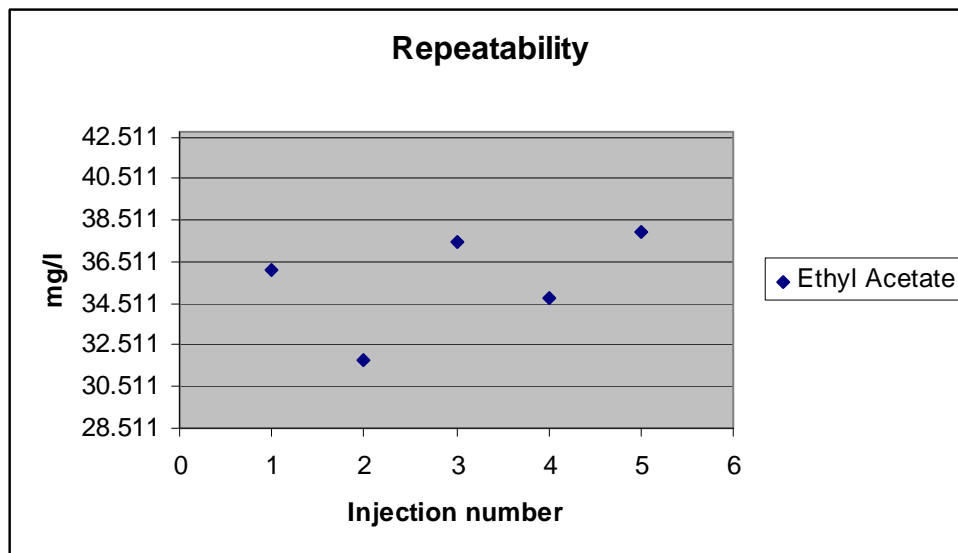


Figures 53 - 56. Repeatability data for Decanoic Acid and Octanoic Acid.

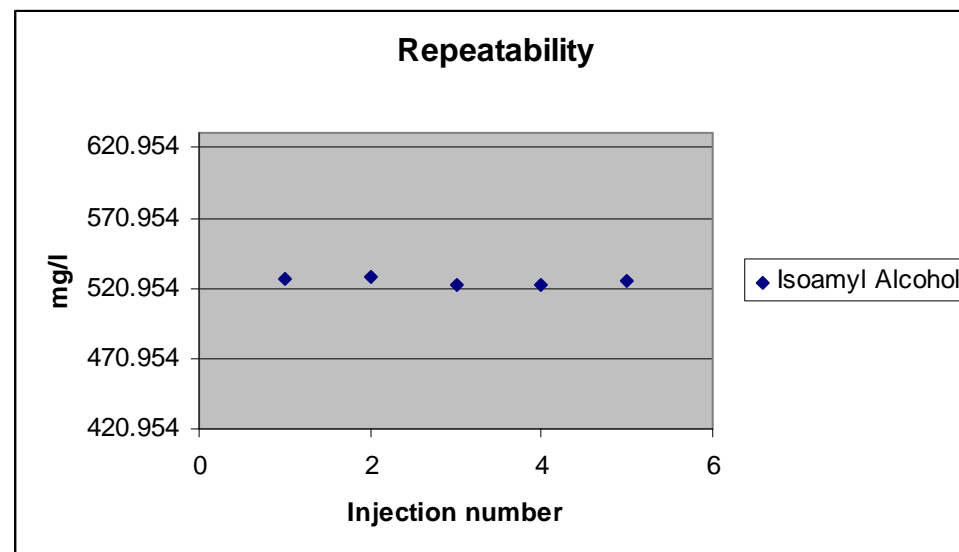
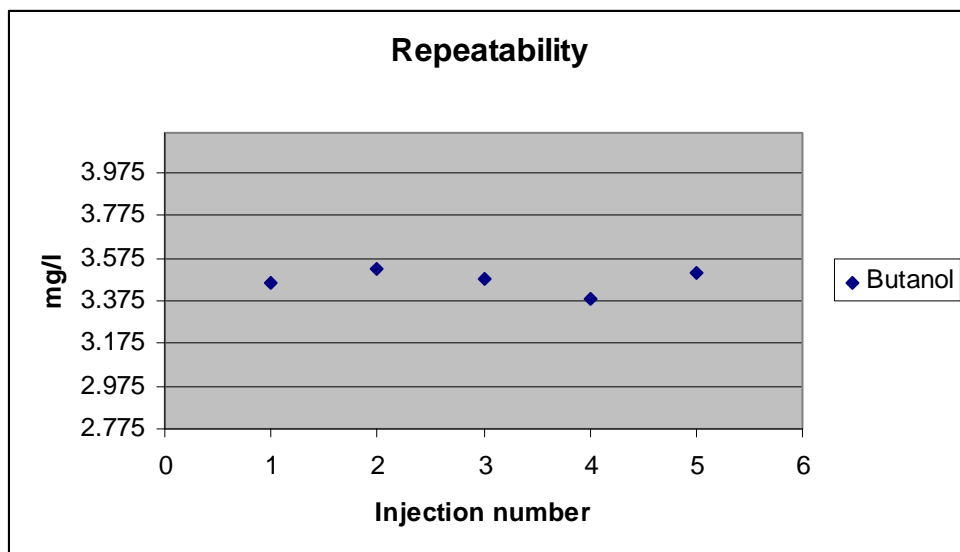
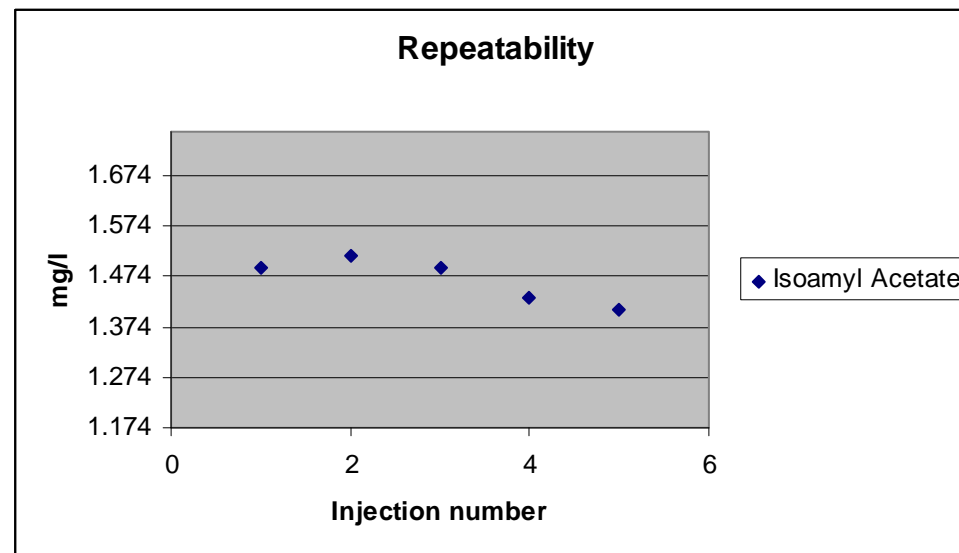
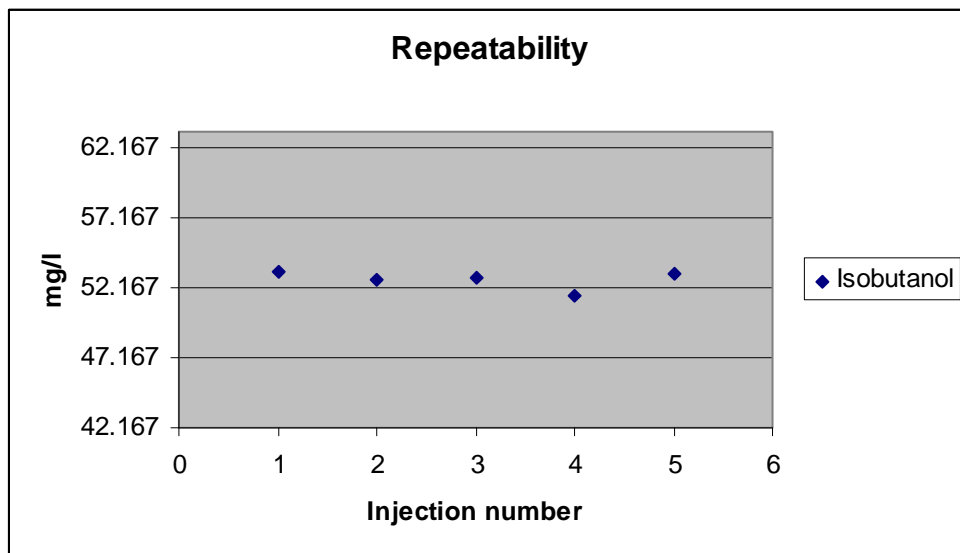
Table 15. Layout of the averages of the concentrations obtained for each analyte after injecting the same extract consecutively for five times.

Compound	1	2	3	4	5	Average	STD	%RSD
Ethyl Acetate	36.07664	31.82894	37.50097	34.80686	37.98166	35.639	6.924	2.468
Methanol	103.9816	100.5109	106.4382	98.97222	106.0435	103.189	3.225	3.327
Ethyl Butyrate	0.235425	0.228219	0.225775	0.211483	0.262169	0.233	8.025	0.019
Propanol	70.89167	69.96135	72.00772	69.74086	73.65504	71.251	2.266	1.614
Isobutanol	53.25962	52.72182	52.79322	51.56768	53.20259	52.709	1.293	0.681
Isoamyl Acetate	1.490095	1.514268	1.491007	1.43398	1.41033	1.468	2.977	0.044
Butanol	3.457814	3.524348	3.475241	3.38377	3.5049	3.469	1.564	0.054
Isoamyl Alcohol	528.2907	529.5953	523.234	524.001	525.8397	526.192	0.518	2.723
Ethyl Hexanoate	0.368404	0.323228	0.325986	0.31002	0.327638	0.331	6.645	0.022
Hexyl Acetate	nd	nd	nd	nd	nd	nd	nd	Nd
Ethyl Lactate	40.8192	41.79259	41.12145	40.85046	41.00633	41.118	0.964	0.396
Hexanol	1.884328	1.915591	1.892433	1.86544	1.84385	1.880	1.446	0.027
Ethyl caprylate	nd	nd	nd	nd	nd	nd	nd	nd
Acetic acid	189.9809	191.2981	164.4094	167.8501	165.432	175.794	7.746	13.617
Propionic Acid	50.15046	53.847	49.53107	53.26755	52.53119	51.865	3.699	1.919
Iso-Butyric Acid	1.8056	2.081216	1.928013	2.14819	1.98132	1.989	6.711	0.133
Butyric Acid	1.219421	1.264819	1.26944	1.25194	1.23123	1.247	1.726	0.022
Ethyl Caprate	nd	nd	nd	nd	nd	nd	nd	nd
Iso-Valeric Acid	3.834515	3.855418	3.510364	3.80114	3.71567	3.743	3.760	0.141
Diethyl Succinate	3.878717	3.945852	3.82004	3.94023	3.90569	3.898	1.319	0.051
Valeric Acid	0.349169	0.338932	0.353736	0.338377	0.351165	0.346	2.064	0.007
2-Phenylethyl Acetate	0.134971	0.122017	0.135076	0.132792	0.129238	0.131	4.173	0.005
Hexanoic Acid	1.716155	1.646868	1.690059	1.67589	1.56734	1.659	3.444	0.057
2-Phenylethanol	114.1835	117.5684	117.2358	116.7973	114.5622	116.069	1.360	1.578
Octanoic Acid	1.591552	1.598404	1.538519	1.56666	1.52635	1.564	2.026	0.032
Decanoic Acid	1.455932	1.321328	1.316905	1.3068	1.30318	1.341	4.830	0.065

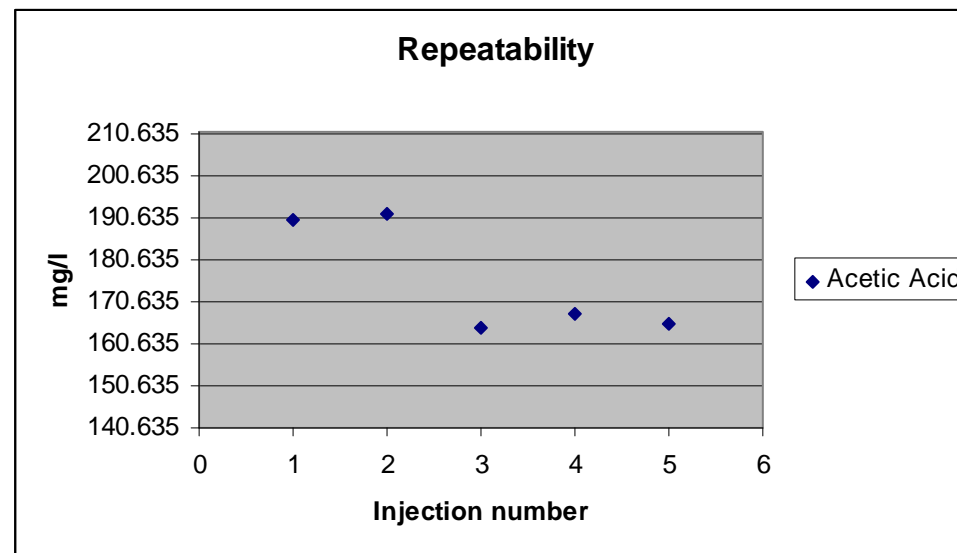
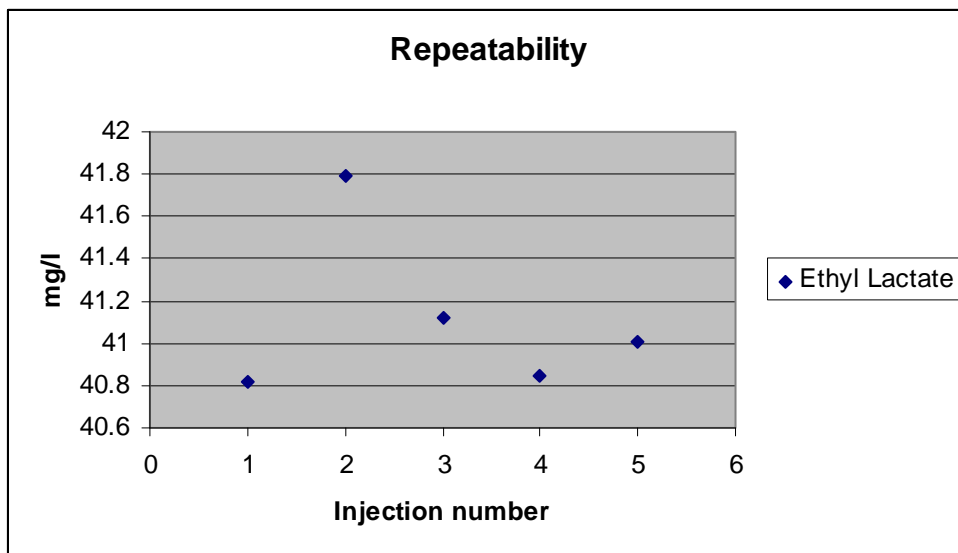
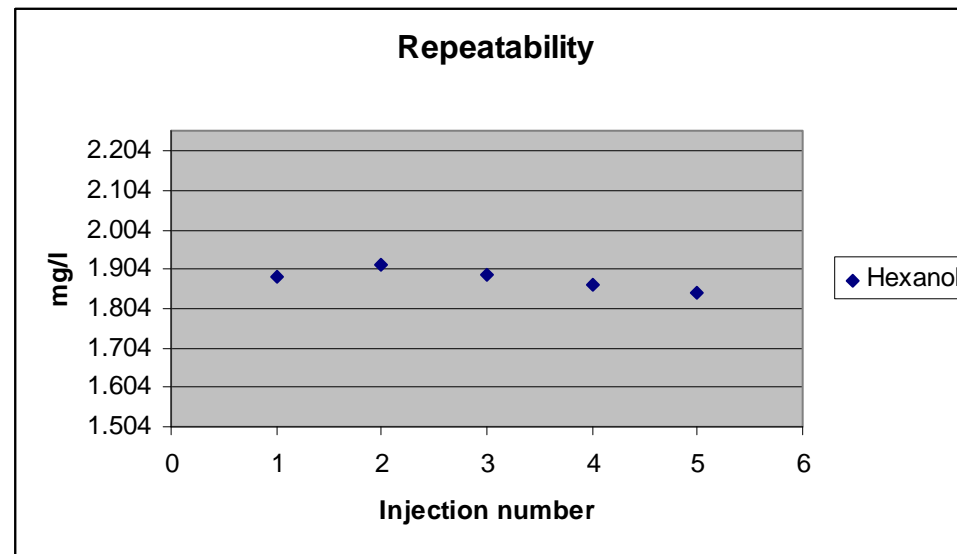
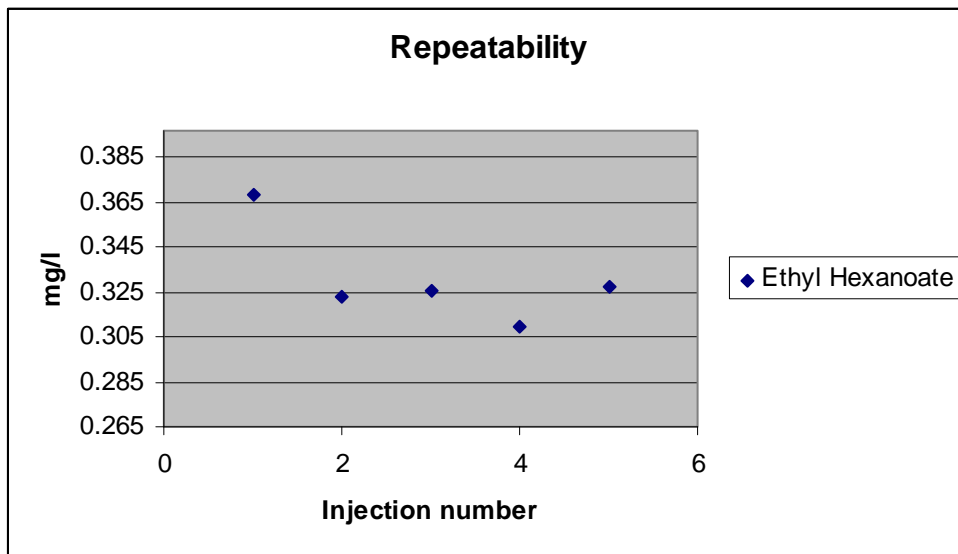
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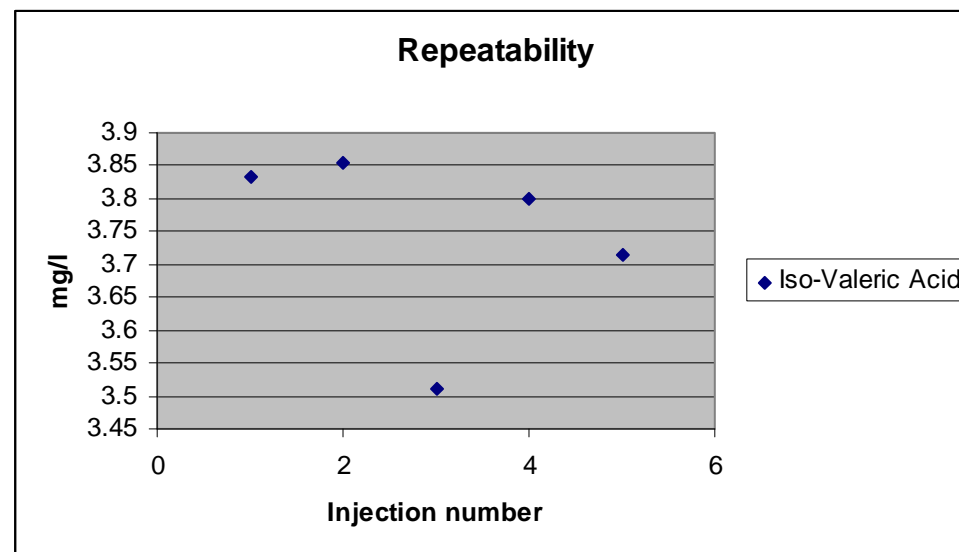
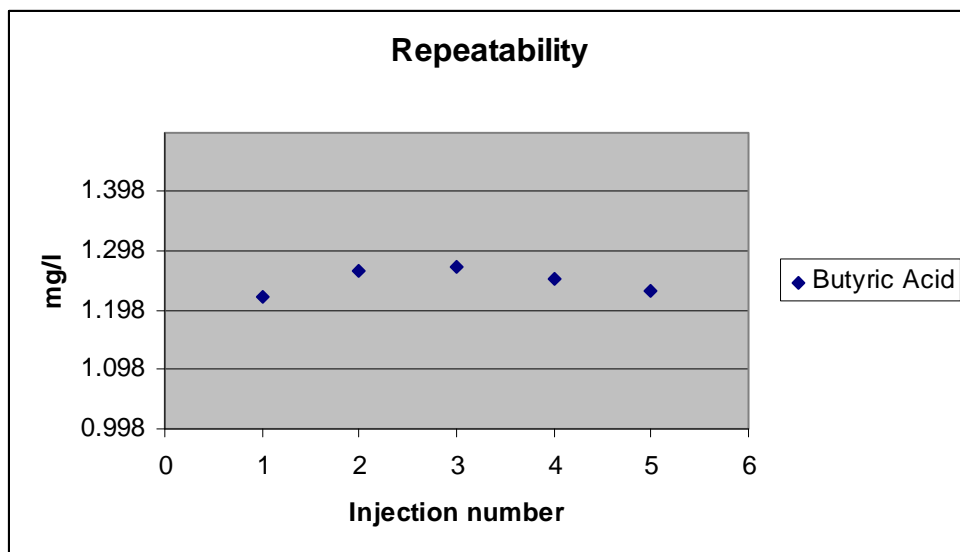
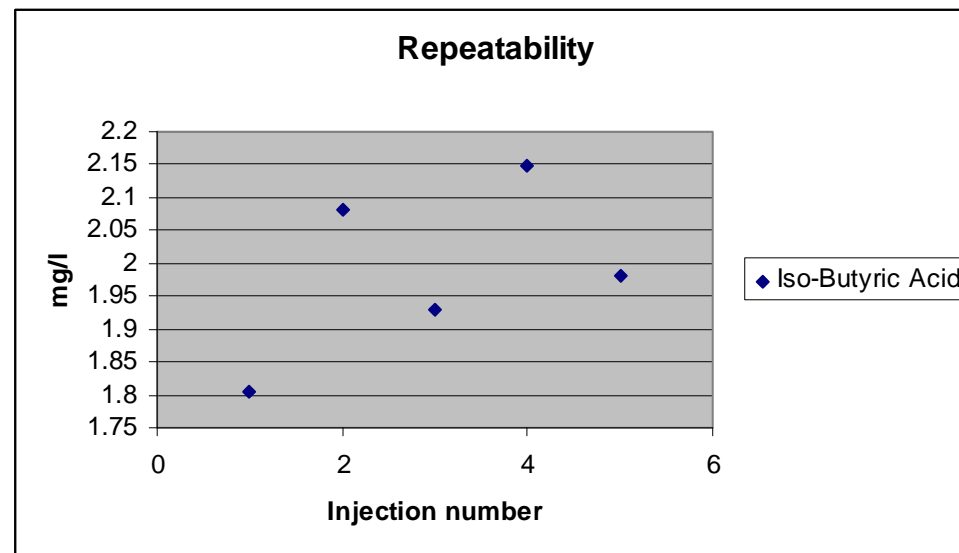
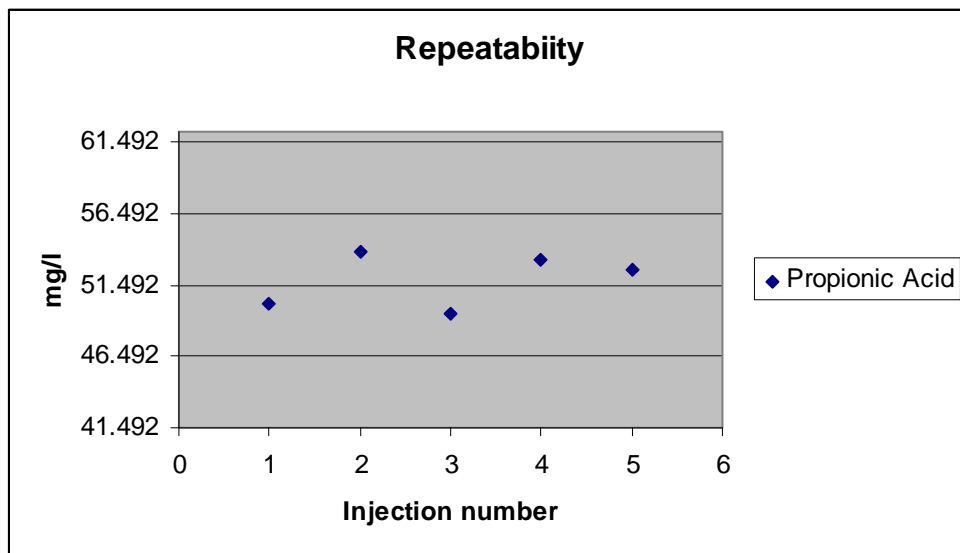
Figures 57 – 60. Repeatability data for Ethyl Acetate, Methanol, Ethyl butyrate and Propanol.



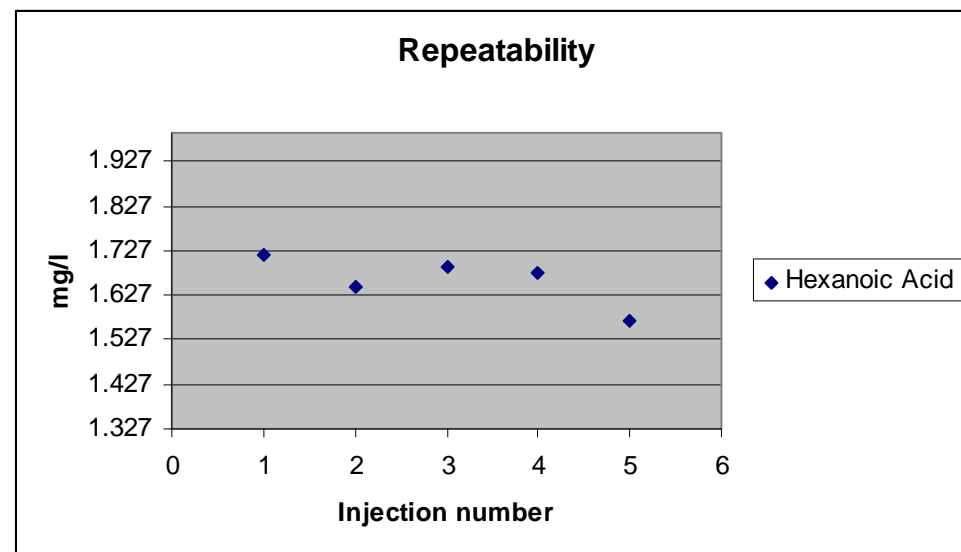
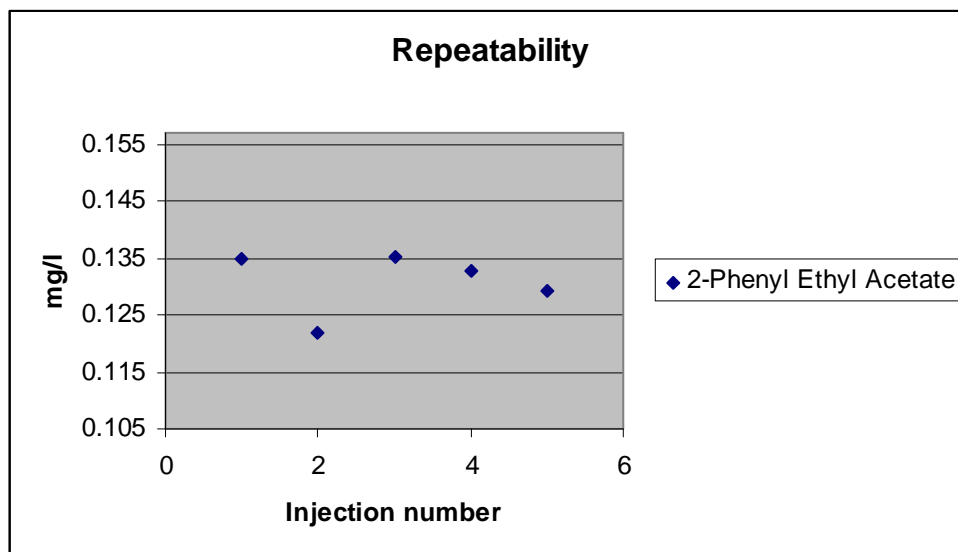
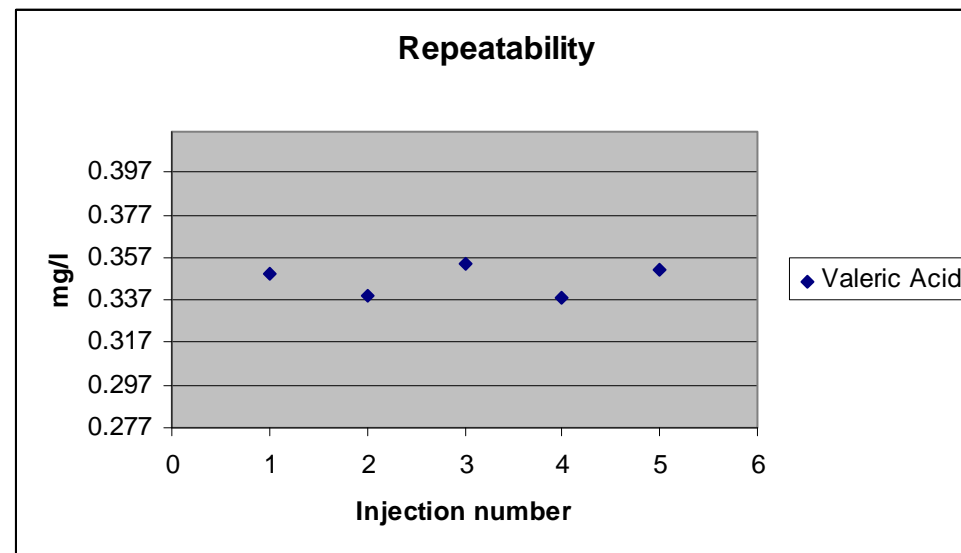
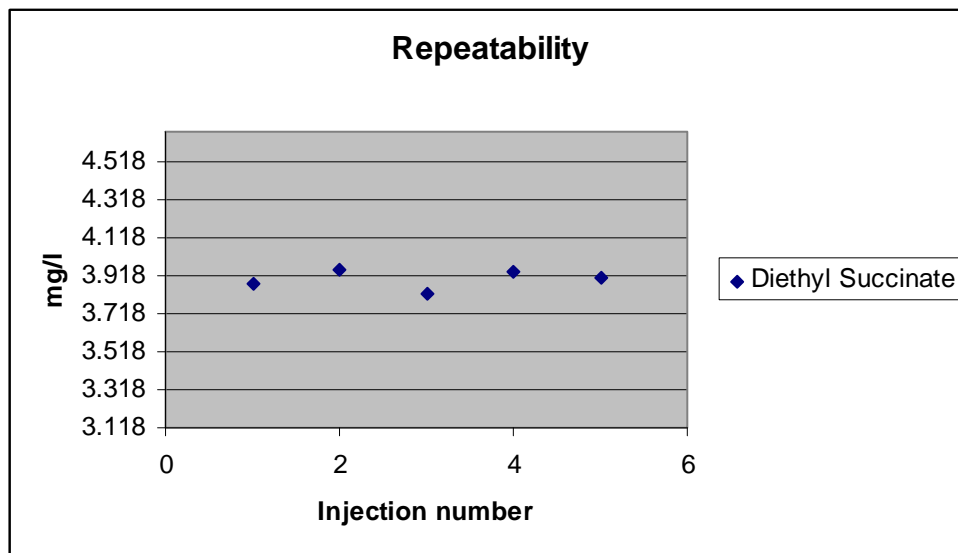
Figures 61 – 64. Repeatability data for Isobutanol, Isoamyl Acetate, Butanol Isoamyl Alcohol.



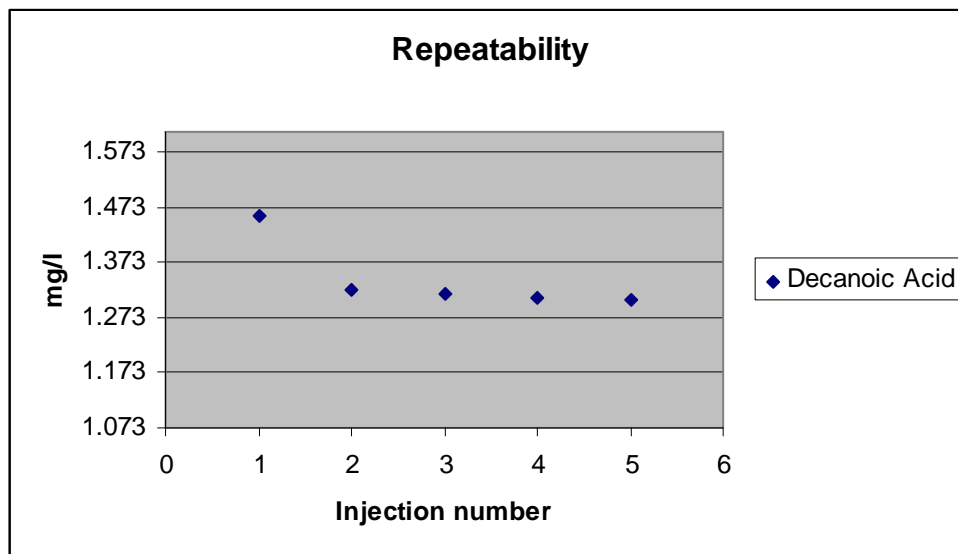
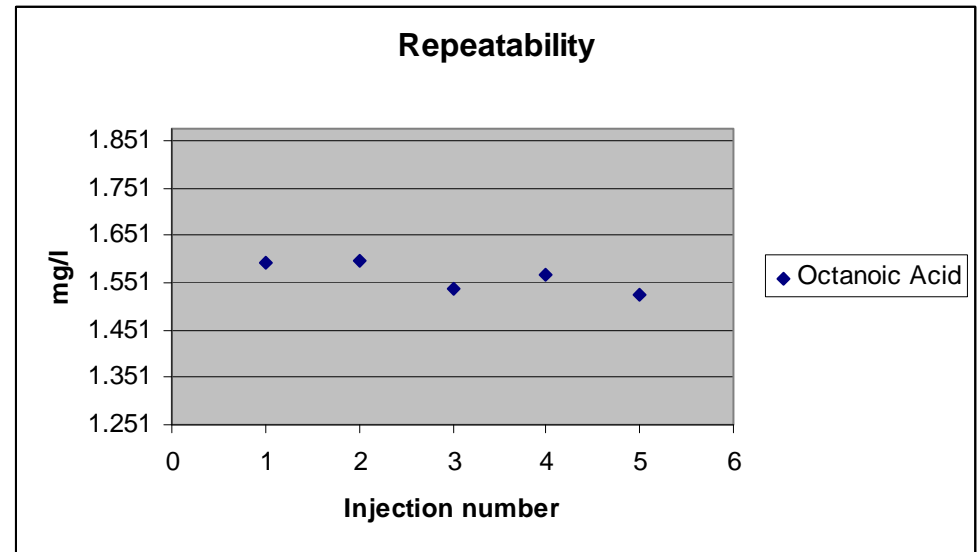
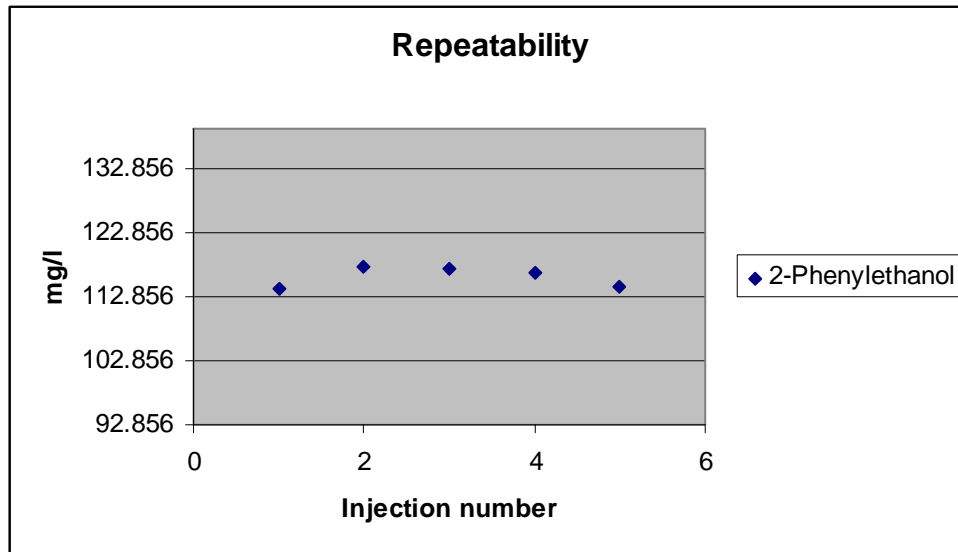
Figures 65 – 68. Repeatability data for Ethyl Hexanoate, Hexanol, Ethyl Lactate and Acetic Acid.



Figures 69 – 72. Repeatability data for Propionic Acid, Iso-Butyric Acid, Butyric Acid and Iso-Valeric Acid.



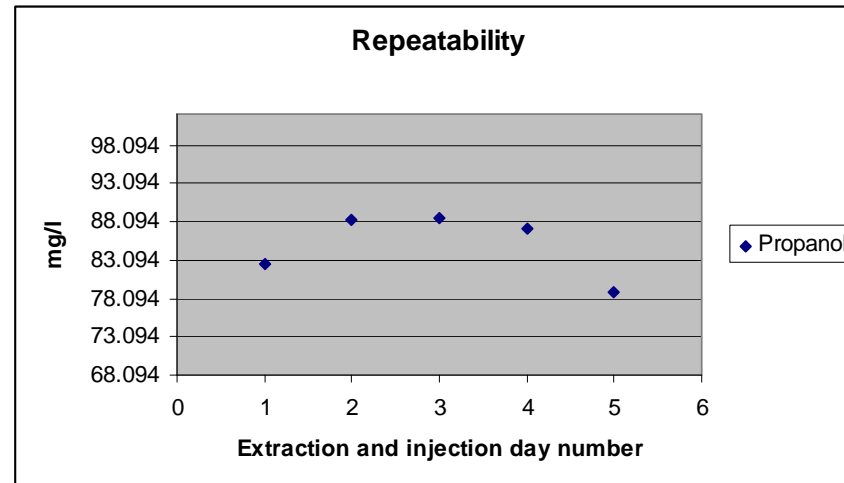
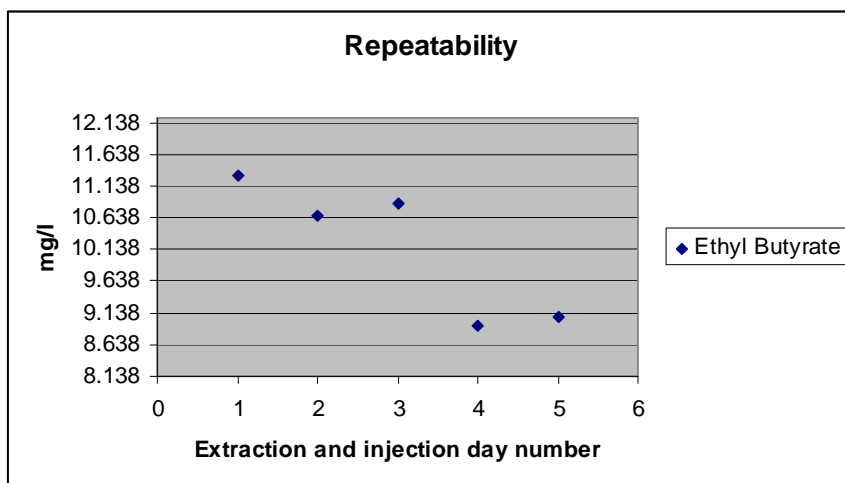
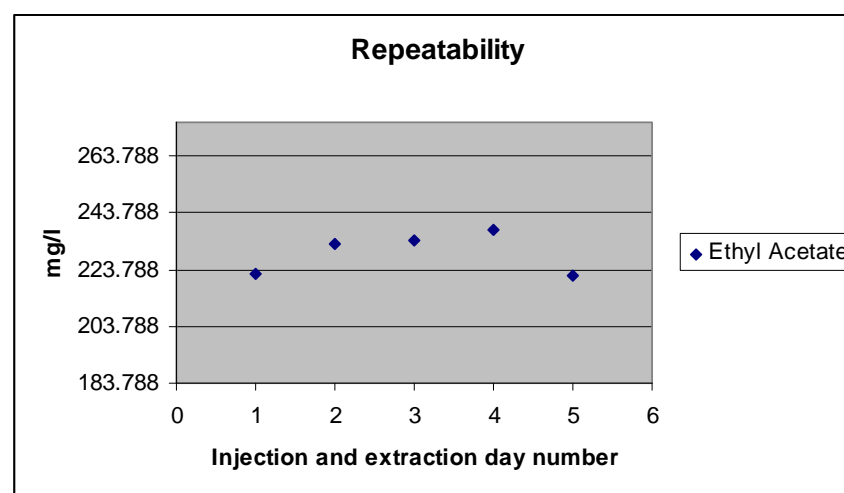
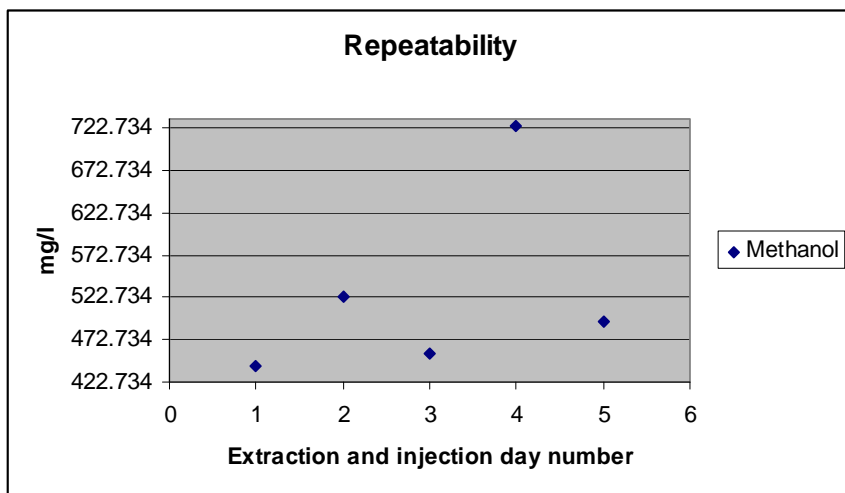
Figures 73 – 76. Repeatability data for Diethyl Succinate, Valeric Acid, 2-Phenyl Ethyl Acetate and Hexanoic Acid.



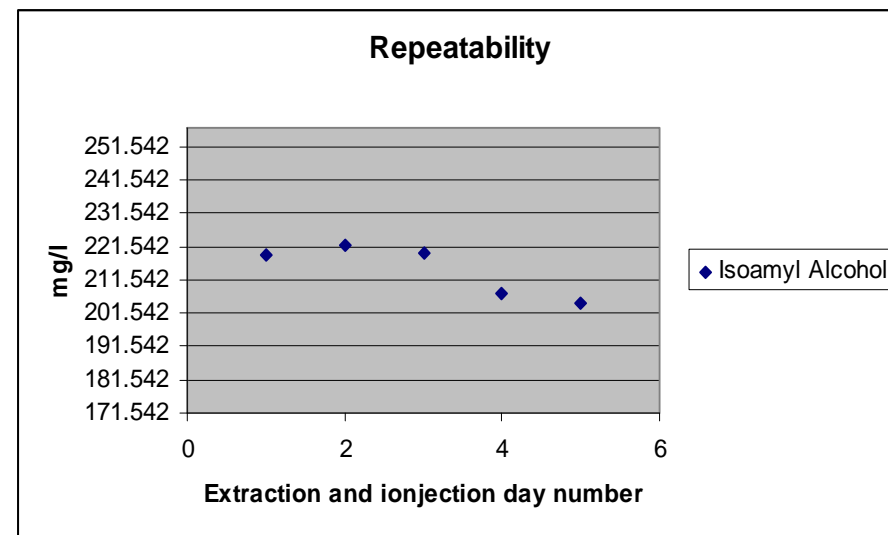
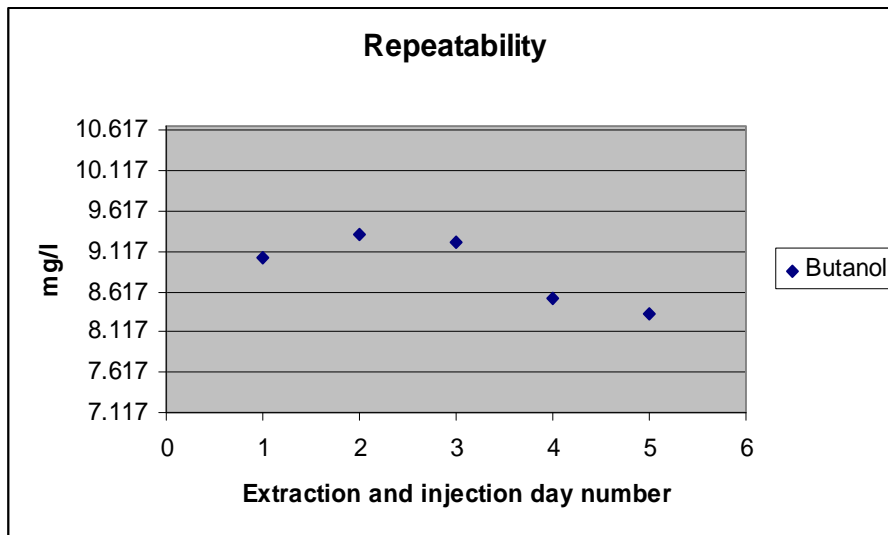
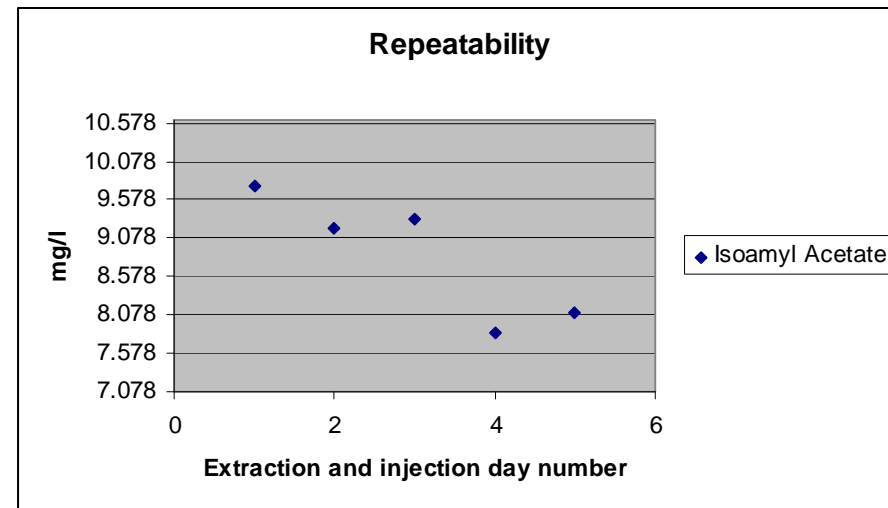
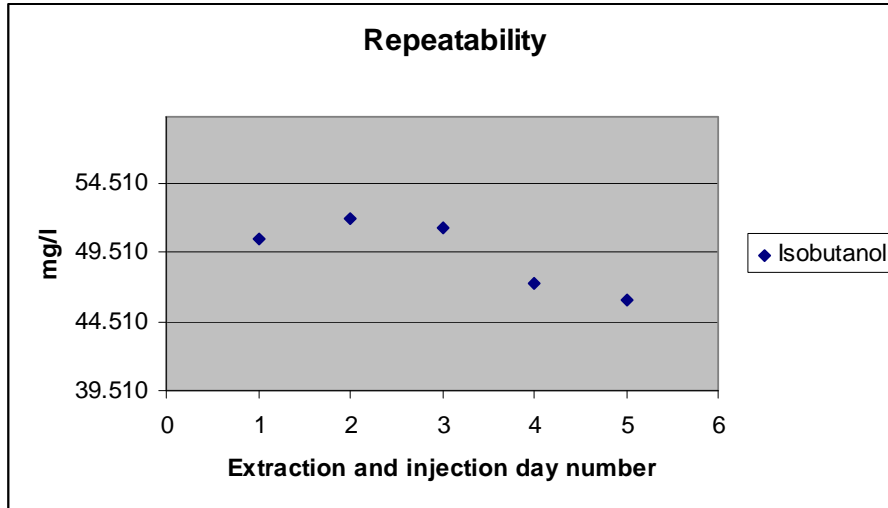
Figures 77 – 80. Repeatability data for 2-Phenylethanol, Octanoic Acid and Decanoic Acid.

Table 16. Averages of the concentrations obtained for each analyte after injecting five different extracts of the same synthetic wine on five different days.

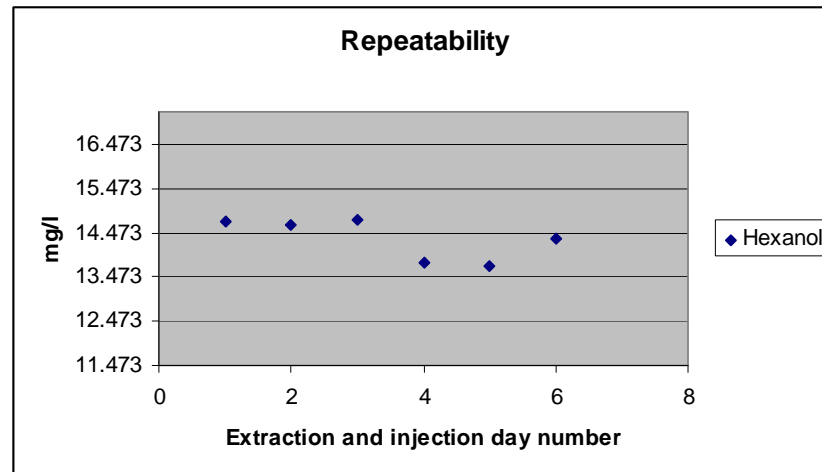
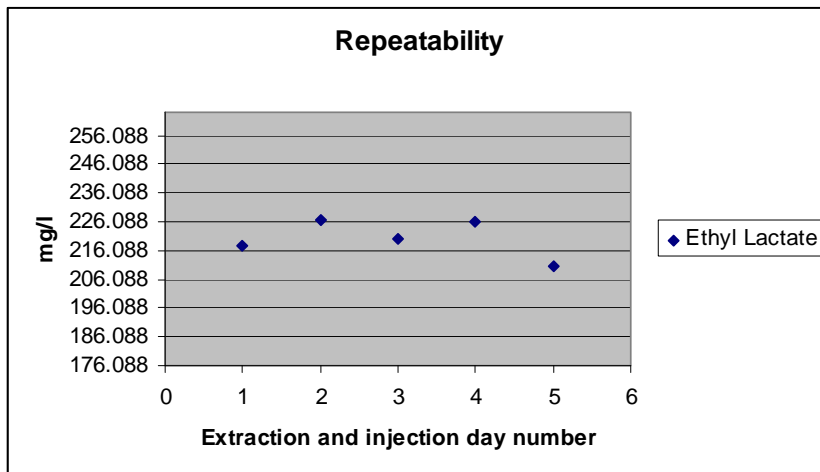
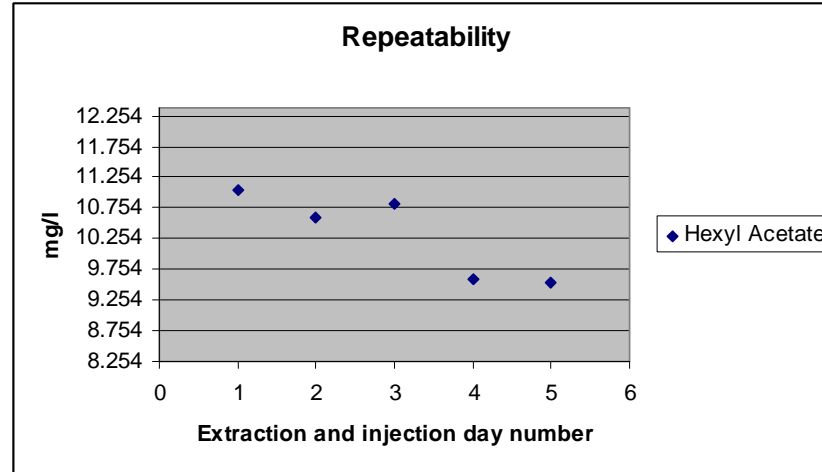
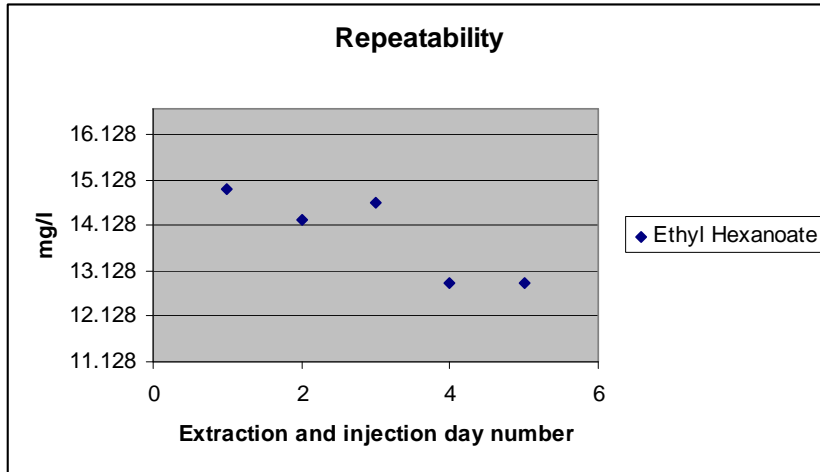
Compound	Day 1	Day 2	Day 3	Day 4	Day 5	Average	STD	%RSD
Ethyl Acetate	222.101	232.928	234.378	237.738	221.529	229.735	7.440	3.239
Methanol	441.189	523.830	456.980	725.179	494.911	528.418	114.642	21.695
Ethyl Butyrate	11.312	10.656	10.869	8.942	9.085	10.173	1.086	10.672
Propanol	82.583	88.418	88.502	87.270	78.811	85.117	4.277	5.025
Isobutanol	50.408	51.977	51.312	47.237	46.004	49.388	2.623	5.312
Isoamyl Acetate	9.752	9.214	9.332	7.831	8.109	8.848	0.832	9.399
Butanol	9.045	9.333	9.221	8.538	8.340	8.896	0.435	4.890
Isoamyl Alcohol	218.806	221.726	219.494	207.289	204.825	214.428	7.767	3.622
Ethyl Hexanoate	14.931	14.260	14.614	12.875	12.870	13.910	0.976	7.020
Hexyl Acetate	11.031	10.599	10.828	9.588	9.544	10.318	0.703	6.818
Ethyl Lactate	217.656	226.502	219.817	225.974	210.601	220.110	6.553	2.977
Hexanol	14.746	14.662	14.764	13.809	13.724	14.341	0.527	3.673
Ethyl caprylate	2.688	2.527	2.587	2.264	2.252	2.463	0.196	7.966
Acetic acid	735.398	749.061	717.352	742.875	706.731	730.283	17.743	2.430
Propionic Acid	13.999	14.411	14.399	14.949	14.169	14.386	0.359	2.494
Iso-Butyric Acid	9.983	10.267	10.189	10.273	9.974	10.137	0.149	1.467
Butyric Acid	9.846	10.377	10.281	10.451	10.082	10.208	0.245	2.398
Ethyl Caprate	1.572	1.345	1.363	1.188	1.190	1.332	0.158	11.849
Iso-Valeric Acid	18.968	19.205	19.144	18.564	18.322	18.841	0.383	2.032
Diethyl Succinate	15.614	15.755	15.685	14.983	14.865	15.380	0.422	2.742
Valeric Acid	10.346	10.502	10.495	10.144	10.014	10.300	0.217	2.103
2-Phenylethyl Acetate	10.561	10.588	10.657	9.687	9.736	10.246	0.489	4.775
Hexanoic Acid	14.518	14.709	14.796	13.744	13.731	14.299	0.523	3.656
2-Phenylethanol	23.360	23.706	23.318	22.968	22.572	23.185	0.431	1.859
Octanoic Acid	21.689	22.149	22.373	20.171	20.179	21.312	1.067	5.006
Decanoic Acid	17.169	17.334	17.439	15.726	15.526	16.639	0.932	5.604



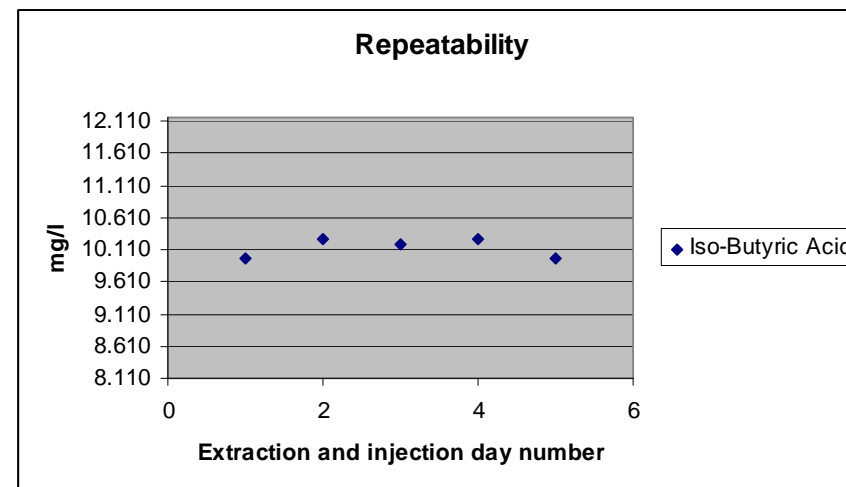
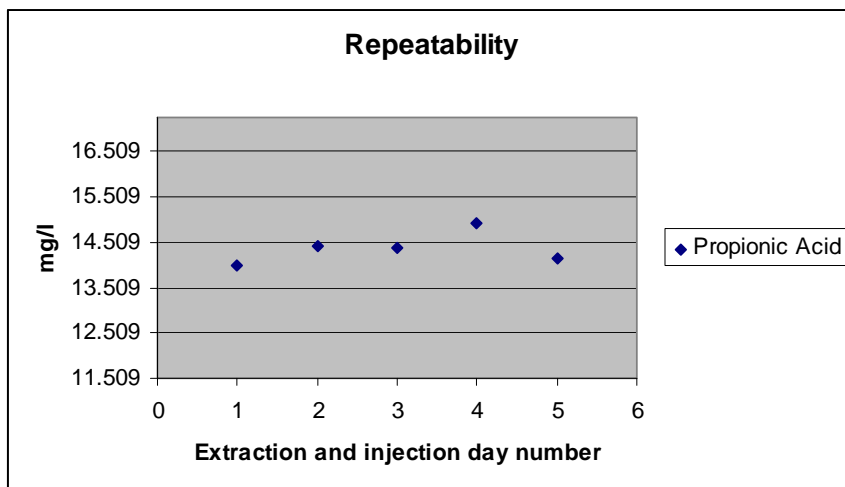
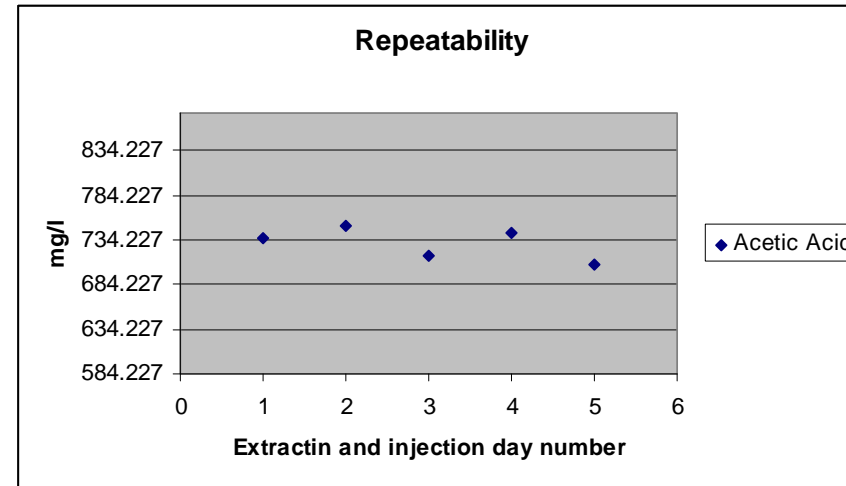
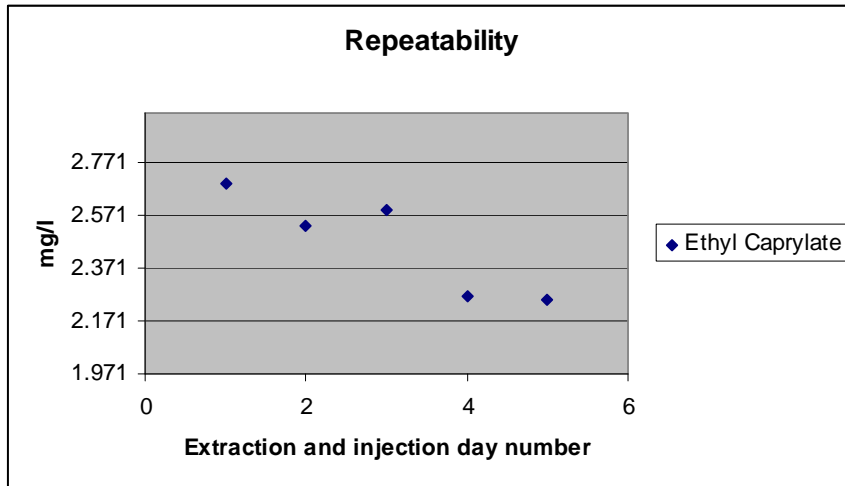
Figures 81 – 84. Repeatability data for five different extractions injected on five different days for Ethyl Acetate, Methanol, Ethyl Butyrate and Propanol.



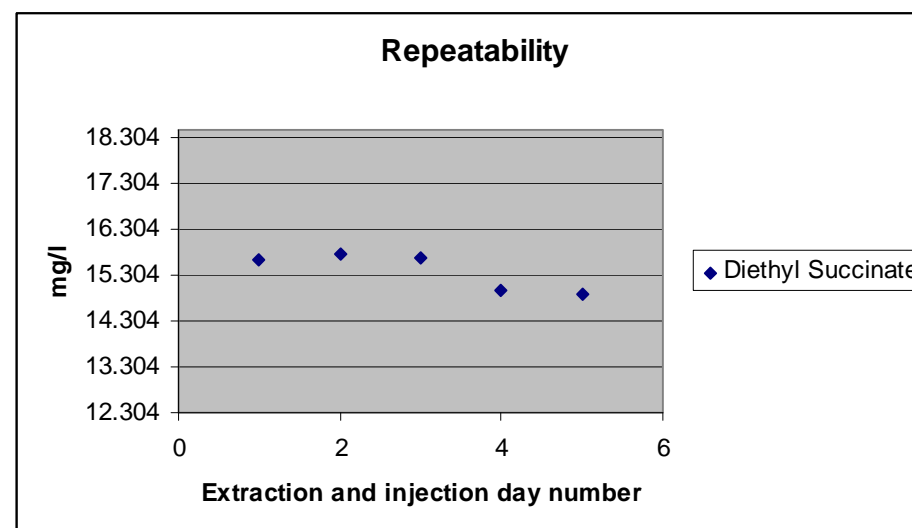
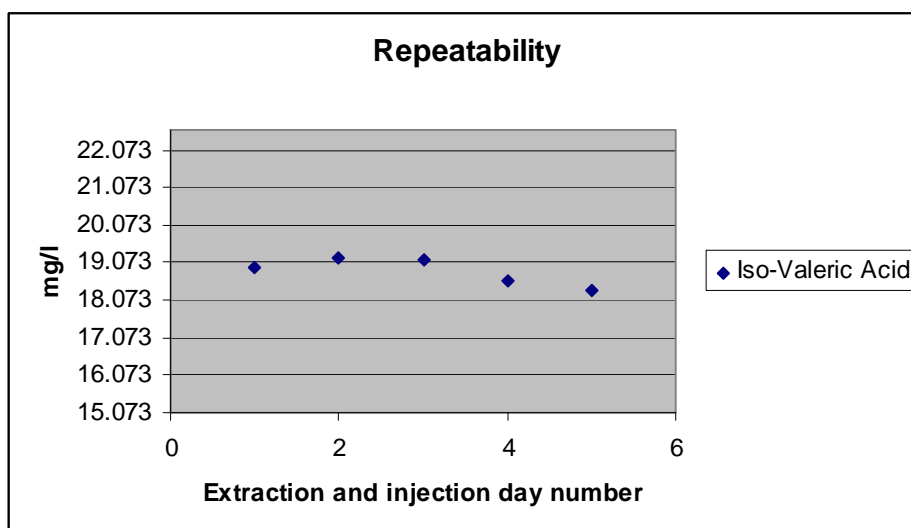
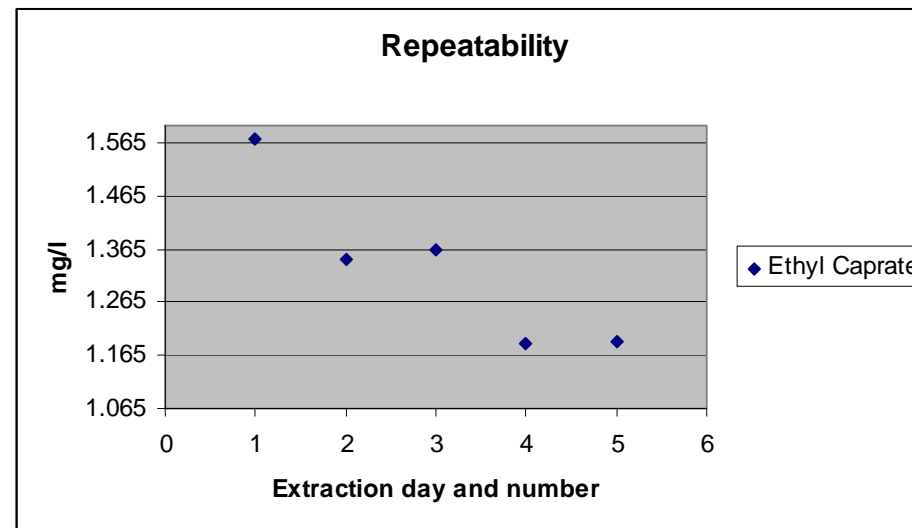
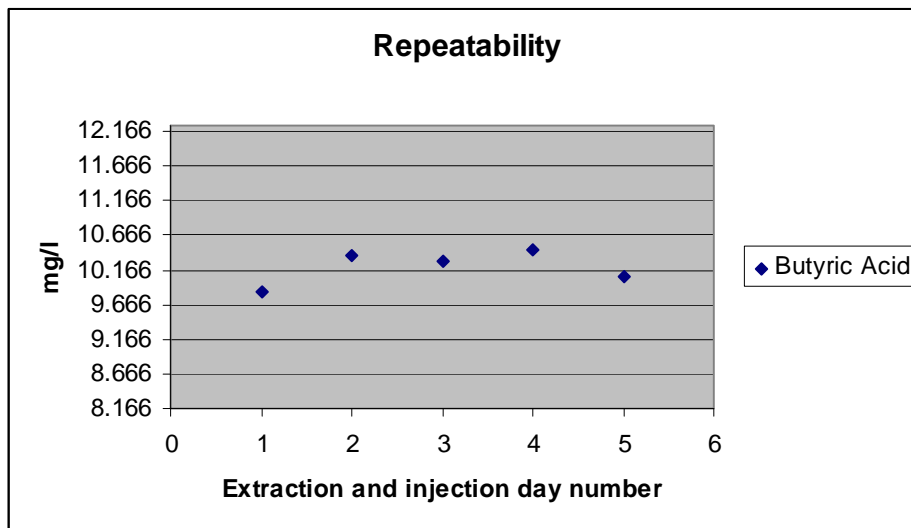
Figures 85 – 88. Repeatability data for five different extractions injected on five different days for Isobutanol, Isoamyl Acetate, Butanol and Isoamyl Alcohol.



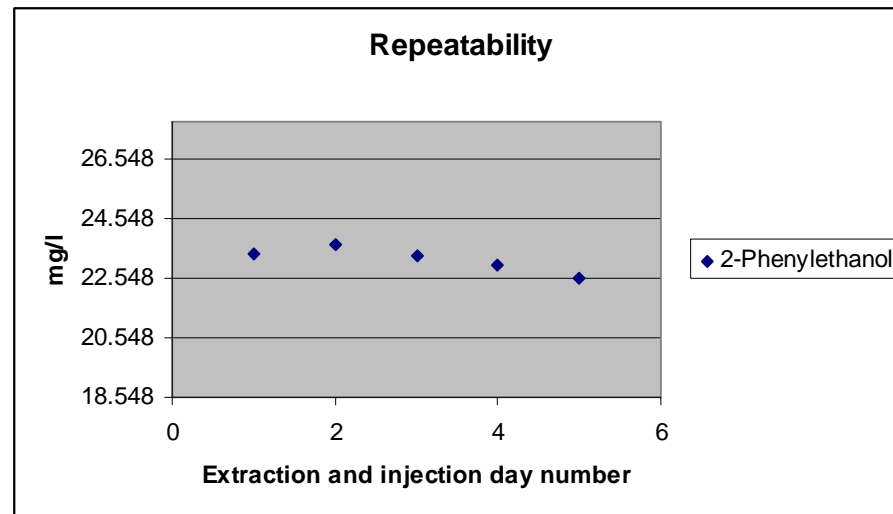
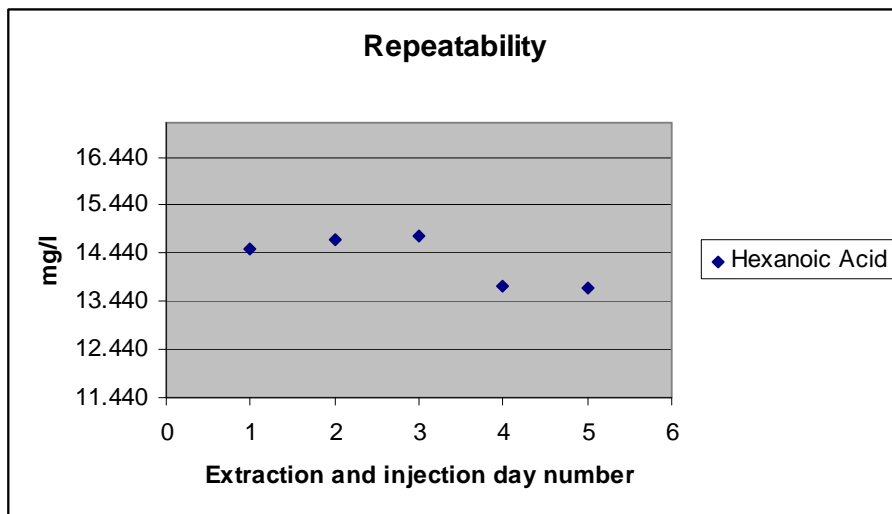
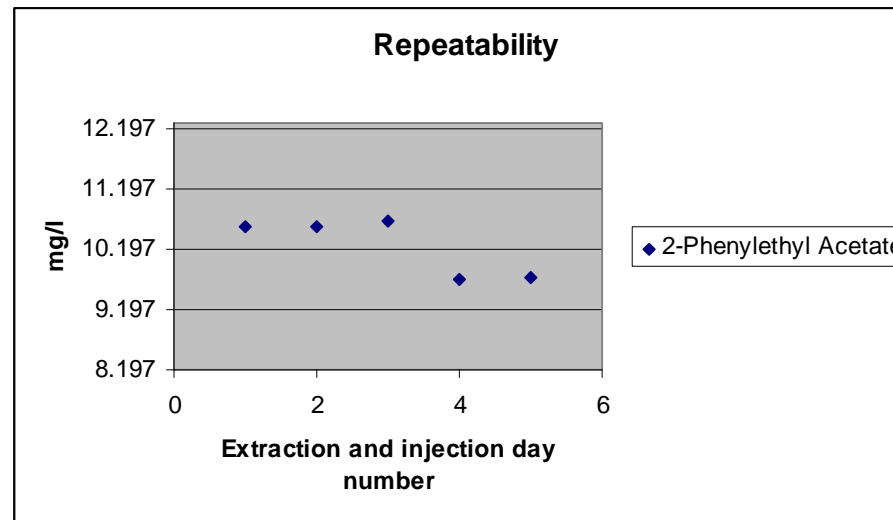
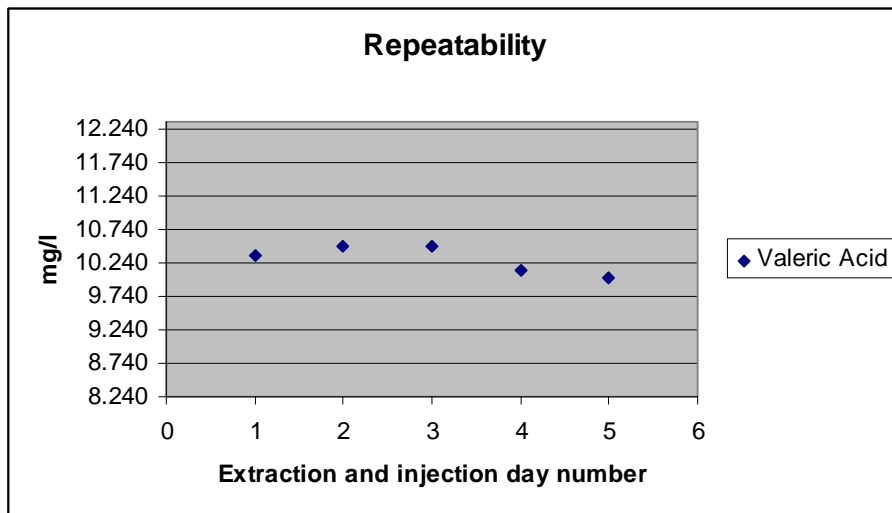
Figures 89 – 92. Repeatability data for five different extractions injected on five different days for Ethyl Hexanoate, Hexyl Acetate, Ethyl Lactate and Hexanol.



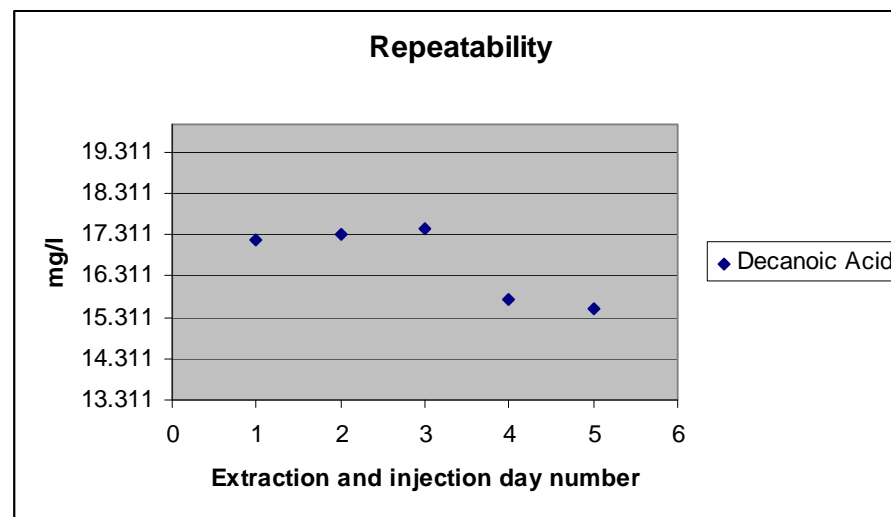
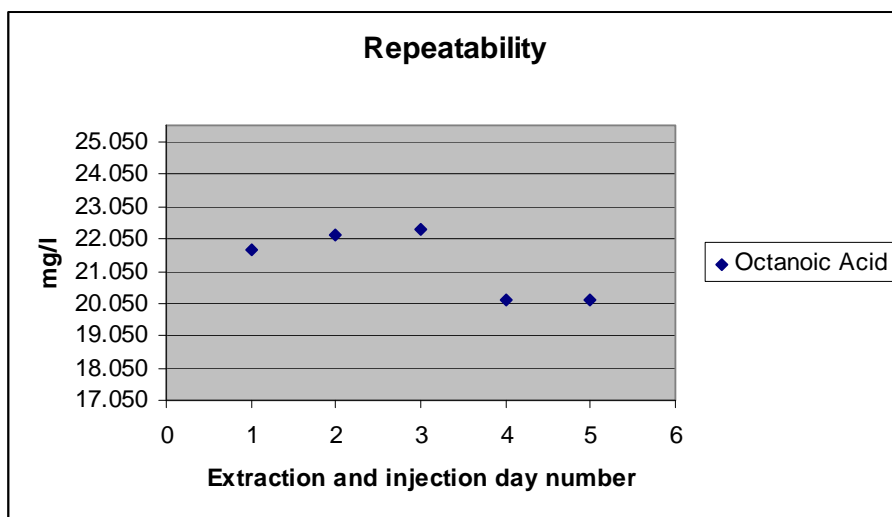
Figures 93 – 96. Repeatability data for five different extractions injected on five different days for Ethyl Caprylate, Acetic Acid, Propionic Acid and Iso-Butyric Acid.



Figures 97 – 100. Repeatability data for five different extractions injected on five different days for Butyric Acid, Ethyl caprate, Iso-Valeric Acid and Diethyl Succinate.



Figures 101 – 104. Repeatability data for five different extractions injected on five different days for Valeric Acid, 2-Phenylethyl Acetate, Hexanoic Acid and 2-Phenylethanol.



Figures 105 – 106. Repeatability data for five different extractions injected on five different days for Octanoic Acid, and Decanoic Acid.

The repeatability for methanol varied more than 20% on day 4.

ROUTINE QC

After each sample run, a post run of 5 minutes at oven temperature 240°C, with a gas flow of 6ml/min cleans the column. After every 30 samples the column is thermally and chemically cleaned by injecting hexane at an oven temperature of 220°C. Once a day, a mixture of all the standards is injected to check if the calibration curve is still valid.

CONCLUSIONS

The proposed method was found suitable for the analysis of volatile compounds in wine. Slight matrix effects were observed between red and white wines. The following factors in the protocol have a significant influence on the results and should be closely adhered to: amount of diethyl ether, sample volume, length of sonication and the temperature of the water bath. The amount of NaSO₄ salt, ethanol concentration and pH of the sample did not influence the extraction efficiency significantly. No trends were observed between analysis of the same extract or wine on the same day or over time. Large variations in concentrations were observed during methanol analysis over time.

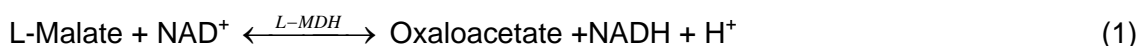
Addendum B

Enzymatic assays
Reactions and calculations

ADDENDUM B

L-MALIC ACID (R-BIOPHARM CAT. NO. 10 139 068 035)

The method is based upon the oxidation of L-malic acid (L-malate) to oxaloacetate by nicotinamide-adenine dinucleotide (NAD⁺) in the presence of L-malate dehydrogenase (L-MDH) (1). This reversible reaction favours the production of L-malate. By removing the oxaloacetate from the system, the reaction system causes the equilibrium to shift towards the production of oxaloacetate. This can be achieved by the conversion of oxaloacetate to L-aspartate in a reaction catalysed by glutamate-oxaloacetate transaminase (GOT) in the presence of L-glutamate (2). The resulting stimulation of oxaloacetate production as indicated in reaction 1, causes the production of NADH. The amount of NADH formed is stoichiometric to the amount of L-malate present in the sample and is measured by its light absorbance at 334, 340 or 365 nm. The maximum absorbance of NADH occurs at 340nm.



The L-Malic content of a sample is quantified by measuring the absorbance of NADH after the removal of oxaloacetate (A_1) and after the completion of the oxidation reaction of L-Malate in the presence of an excess L-MDH (A_2). Absorbance values should ideally be lower than 1.000. The absorbance difference (ΔA) is calculated as indicated in equation 3. An absorbance difference of at least 0.100 absorbance units are considered acceptable to produce precise results. The abovementioned specifications in terms of absorbance values and absorbance differences is applicable to all further mentioned enzymatic assays

$$\Delta A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}} \quad (3)$$

The concentration of L-malic acid is calculated as follows:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \quad (4)$$

Where V = final volume (ml)

MW = molecular weight: for L-Malic acid = 134.09 g/mol

ε = extinction coefficient of NADH: at 340nm = 6.3 (l × mmol⁻¹ × cm⁻¹)

d = light path (cm)

v = sample volume (ml)

L-LACTIC ACID (R-BIOPHARM CAT. NO. 10 139 084 035)

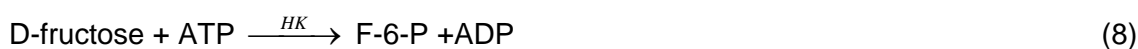
L-lactic acid is determined by the oxidation of L-lactate to pyruvate by NAD^+ in the presence of L-lactate dehydrogenase (L-LDH) (5). This equilibrium of this reaction is in favour of the production of L-lactate. By removing the pyruvate from the system, the reaction system causes the equilibrium to shift towards the production of pyruvate. To determine L-lactic acid, pyruvate is converted to 2-oxoglutarate in a reaction catalysed by glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate (6). The resulting stimulation of pyruvate production as indicated in reaction 5, causes the production of NADH. The amount of NADH formed is stoichiometric to the amount of L-lactate present in the sample and is measured by its maximum light absorbance at 340nm.



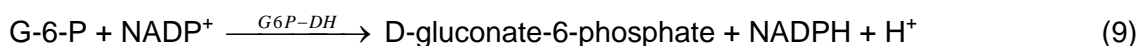
The L-lactic acid content of a sample is quantified by determining the absorbance of NADH after the removal of pyruvate (A_1) and after the completion of the oxidation reaction of L-lactate in the presence of an excess L-LDH (A_2). The absorbance difference (ΔA) is calculated as indicated in equation 3. The concentration of L-lactic acid is calculated as indicated in equation 4 where the molecular weight of L-lactic acid is 90.1 g/mol.

D-GLUCOSE/D-FRUCTOSE (R-BIOPHARM CAT. NO. 10 139 106 035)

The enzymatic determination of D-glucose and D-fructose is accomplished in a three step enzymatic reaction. First, D-glucose and D-fructose are simultaneously phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to G-6-P (D-glucose-6-phosphate) and F-6-P (D-fructose-6-phosphate) respectively (7,8).



In a subsequent reaction catalysed by the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate (NADP^+) to D-gluconate-6-phosphate (9).



The amount of NADPH formed is stoichiometric to the amount of D-glucose present in the sample and is measured by its maximum light absorbance at 340nm.

Following this reaction, F-6-P is converted to G-6-P by the enzyme phosphoglucose isomerase (PGI) (10).

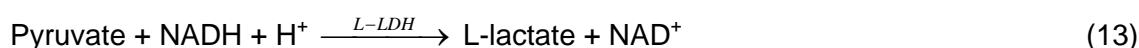


The reaction shown in equation 9 is repeated and in this case the amount of NADPH formed is stoichiometric to the amount of D-fructose present in the sample and is measured by its maximum light absorbance at 340nm.

The quantification of D-glucose is calculated from the absorbance difference before and after the simultaneous induction of reactions 7-9. The quantification of D-fructose is calculated from the absorbance difference before and after reaction 10 takes place. Calculations are based on equation 3 and 4 where the molecular weight of D-glucose and D-fructose is 180.16 g/mol.

GLYCEROL (R-BIOPHARM CAT. NO. 10 148 270 035)

Glycerol is phosphorylated by ATP in the presence of glycerokinase (GK) to L-glycerol-3-phosphate (11). The ADP formed during this reaction is reconverted to ATP by phosphoenolpyruvate in the presence of pyruvate kinase (PK) (12). This reaction results in the formation of pyruvate. In a subsequent reaction, pyruvate is reduced by NADH to L-lactate with the simultaneous formation of NAD^+ (13). The decrease in NADH in this last reaction is stoichiometric to the amount of glycerol present in the sample and is measured by its maximum light absorbance at 340nm.



The glycerol content of a sample is quantified by measuring the absorbance of NADH after the formation of pyruvate (A_1) and after the completion of the oxidation reaction of pyruvate in the presence of an excess L-MDH (A_2). The absorbance difference (ΔA) is calculated as indicated in equation 14. The concentration of glycerol is calculated as indicated in equation 4 where the molecular weight of glycerol is 92.1 g/mol.

$$\Delta A = (A_1 - A_2)_{\text{sample}} - (A_1 - A_2)_{\text{blank}} \quad (14)$$