

The evaluation of Fourier transform infrared spectroscopy (FT-IR) for the determination of total phenolics and total anthocyanins concentrations of grapes

by

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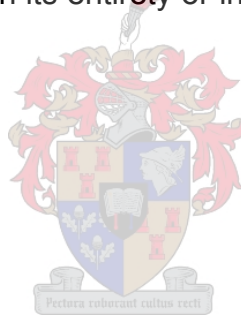
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DECLARATION

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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Date

SUMMARY

The assessments of grape and wine quality are complex issues and the wine industry needs more objective analysis of grape and wine quality. The standard quality assessment protocol for grading grapes at most wine cellars in South Africa is based on viticultural practices and the determination of chemical parameters such as °Brix, pH and titratable acidity (TA). Grape juice indices calculated by *formulae* such as °Brix/pH, TA/pH, °Brix/TA, °Brix x (pH)² have been used in the past but these approaches have had limited success. It was shown that the total anthocyanins and total phenolics of red grapes correlate with wine quality and provide additional objective measures of grape quality. Most methods for the quantification of total anthocyanins and total phenolics are complex and time consuming and therefore not easily implemented in the routine laboratory environment. Fourier transform infrared spectroscopy (FT-IR) is widely used in South African laboratories for the routine quantification of wine and grape parameters but the commercial calibration models supplied for the quantification of grape total anthocyanins and phenolics are not satisfactory. The focus of this study was to develop new FT-IR calibration models for the quantification of total anthocyanins and phenolics of grapes and to use the generated data during a preliminary evaluation of the implementation of these parameters as part of the grape quality control protocol at a commercial winery in South Africa.

The potential of Fourier transform infrared spectroscopy (FT-IR) for the rapid quantification of total anthocyanins and total phenolics in red grapes was investigated and evaluated for prediction accuracy with independent validation sets. The design of calibration sets aimed at capturing most of the variation due to vintage (2004 and 2005), cultivar (Cabernet Sauvignon, Merlot, Pinotage, and Shiraz) and sugar concentration. Best prediction accuracies were obtained for calibration sets using grapes from a single vintage or cultivar or approximately the same sugar concentration. The highest prediction accuracies were obtained for total anthocyanins calibration sets of grapes with sugar concentrations $\geq 23.5^\circ\text{Brix}$ ($SEP = 0.13 \text{ mg/g}$; R^2 validation set = 0.77) and for total phenolics calibration sets of grapes with sugar concentrations $< 23.5^\circ\text{Brix}$ ($SEP = 0.13 \text{ OD}_{280}/\text{g}$; R^2 validation set = 0.74). Strong correlations were found between the spectral data and the total anthocyanins ($SEP = 0.12 \text{ mg/g}$; R^2 validation set = 0.84) and total phenolics concentration data ($SEP = 0.10 \text{ OD}_{280}/\text{g}$; R^2 validation set = 0.76) for 2005 Merlot calibration sets indicating that the FT-IR spectra captured most of the variation. Overall the RPD (ratio of the standard deviation of the reference data to the standard error of prediction) values of all calibration models were below 3 indicating that calibration models are fit for screening purposes. Spectroscopic absorbance at 280 nm is not specific enough for the quantification of total phenolics and the use of an alternative reference method such as high performance liquid chromatography (HPLC) will be considered in the future. Principal component analysis (PCA) revealed that the major sources of variation in the FT-IR spectra of grapes could be ascribed to vintage and grape sugar concentration

and this had an effect on the accuracy of the analytical data generated when using FT-IR spectroscopy. This report is the first to our knowledge where FT-IR has been used for the quantification of total anthocyanins and phenolics of grapes.

The evaluation of the reference laboratory protocol for the quantification of total anthocyanins and total phenolics in grapes were evaluated in Chapter 4 and emphasized the importance of meticulous laboratory practices to obtain reliable reference data for calibration purposes.

This large scale investigation of the total anthocyanins and phenolics concentrations in grapes is the first of its kind in South Africa and a quantitative database containing analytical data of the anthocyanins and total phenolics concentrations of 692 grape samples representing a wide range of grape maturities of *Vitis vinifera* cultivars Cabernet Sauvignon, Merlot, Pinotage and Shiraz from the 2004 and 2005 vintages was established based on the reference values. The data were used in a preliminary investigation of the implementation of total anthocyanins and total phenolics concentrations as part of grape quality classification at a commercial South African winery (Chapter 5). The results showed that the total anthocyanins and total phenolics concentration in grapes increased with increasing grape maturity (measured as sugar concentration). ANOVA and post-hoc analysis (Bonferroni testing) revealed significant differences between the total anthocyanins and total phenolics concentrations of the four *Vitis vinifera* cultivars investigated. Grapes harvested earlier in the season had significantly higher ($p \leq 0.05$) total anthocyanins and total phenolics concentrations than grapes harvested later in the season. This implies that grapes harvested earlier in the harvest season could produce wines with higher quality. Grapes from regularly irrigated vineyards had lower total anthocyanins and total phenolics concentrations compared to dryland vineyards. The current grape grading system in use at the industrial cellar did not correlate well with the total anthocyanins and total phenolics concentrations of grapes which highlighted the need for the inclusion of more objective measures during grape grading. The information captured in the database can be used as a starting point to establish profiles of the typical anthocyanins and total phenolics of South African grapes and data from more vintages should be included and continually updated. These findings highlight the important contribution of the results obtained in this preliminary study for the incorporation of total anthocyanins and phenolics concentrations as objective parameters of grape quality. Finally multivariate data analysis of the FT-IR spectra revealed important information regarding factors (both physical and chemical) that contribute to the variation of the spectra. The main variation between the 2004 and 2005 samples can probably be interpreted in terms of the water content of the samples.

OPSOMMING

Die bepaling van druif en wynkwaliteit is kompleks en die wynindustrie benodig meer objektiewe analysis om druif en wynkwaliteit te bepaal. Die standaard druifkwaliteit graderings protokol by meeste wynkelders in Suid-Afrika is gebaseer op wingerdkundige praktyke en die bepaling van chemiese parameters soos °Brix, pH en titreerbare suur (TS). Druifsap indekse bereken deur formules soos °Brix/pH, TS/pH, °Brix/TS en °Brix x (pH)² is al in die verlede gebruik, maar hierdie metodes het beperkte sukses getoon. Dit is bewys dat die totale antosianiene en totale fenole konsentrasies van rooi druifkorrels korreleer met wynkwaliteit en dat dit twee addisionele objektiewe meetings van druifkwaliteit is. Meeste metodes vir die kwantifisering van totale antosianiene en totale fenole is egter kompleks en tydrowend en kan daarom nie maklik implementeer word in die roetine laboratorium omgewing nie. Fourier transform infrarooi spektroskopie (FT-IR) word redelik algemeen gebruik in Suid-Afrikaanse laboratoria vir die kwantifisering van wyn en druif parameters. Die kommersiële kalibrasie modelle vir die kwantifisering van totale antosianiene en totale fenole van druifkorrels is nie bevredigend nie. Die fokus van hierdie studie was om nuwe FT-IR kalibrasie modelle te ontwikkel vir die kwantifisering van totale antosianiene en totale fenole van druifkorrels en om die gegenereerde data te gebruik tydens 'n voorlopige evaluasie van die implementering van hierdie parameters as deel van die druifkwaliteits kontrole protokol by 'n kommersiële wynkelder in Suid-Afrika.

Die potensiaal van Fourier transform infrarooi spektroskopie (FT-IR) as 'n vinnige metode vir die kwantifisering van totale antosianiene en totale fenole in rooi druifkorrels is ondersoek. Die prediksie akkuraatheid van kalibrasie modelle is bepaal met onafhanklike validasie stelsel. Kalibrasie stelsel is ontwerp om die meeste variasie toegeskryf aan oesjaar (2004 en 2005), kultivar (Cabernet Sauvignon, Merlot, Pinotage, en Shiraz) en suikerkonsentrasie in te sluit. Die beste prediksie akkuraatheid is verkry vir kalibrasiestelsel wat druifkorrels ingesluit het van 'n enkele oesjaar of kultivar of druifkorrels van ongeveer dieselfde suikerkonsentrasie. Die hoogste prediksie akkuraatheid is verkry vir totale antosianiene kalibrasiestelsel wat druifkorrels ingesluit het met totale suikerkonsentrasies $\geq 23.5^\circ\text{Brix}$ ($SEP = 0.13 \text{ mg/g}$; R^2 validasiestelsel = 0.77) en vir totale fenole kalibrasiestelsel wat druifkorrels ingesluit het met suikerkonsentrasies $< 23.5^\circ\text{Brix}$ ($SEP = 0.13 \text{ OD}_{280/\text{g}}$; R^2 validasiestelsel = 0.74). Goeie korrelasies is gevind tussen die spektrale data en die totale antosianiene ($SEP = 0.12 \text{ mg/g}$; R^2 validasiestelsel = 0.84) en totale fenole konsentrasies data ($SEP = 0.10 \text{ OD}_{280/\text{g}}$; R^2 validasiestelsel = 0.76) vir 2005 Merlot kalibrasiestelsel wat aandui dat die FT-IR spektra meeste van die variasie ingesluit het. Oor die algemeen was die ratios van die standaard afwykings van die verwysingsdata tot die standaard foute van prediksies (RPD) waardes van al die kalibrasiestelsel minder as 3 wat aandui dat die kalibrasie modelle geskik is vir sifting. Spektroskopiese absorpsie by 280 nm is nie spesifiek genoeg vir die kwantifisering van totale fenole nie en die gebruik van 'n alternatiewe verwysingsmetode soos hoë druk vloeistof chromatografie sal oorweeg word in die toekoms. Hoofkomponent

analise het getoon dat die belangrikste bron van variasie binne FT-IR spektra van druiwe die oesjaar en suikerkonsentrasies van druiwe was en dat dit moontlik 'n effek kon hê op die akkuraatheid van die analitiese data gegengereer toe FT-IR spektroskopie gebruik is. Hierdie verslag is die eerste waarvan ons weet waar FT-IR gebruik is vir die kwantifisering van totale antosianiene en totale fenole konsentrasies van druiwe.

Die evaluasie van die verwysingsmetode laboratorium protokol vir die kwantifisering van die totale antosianiene en totale fenole konsentrasies in druiwe is geëvalueer soos beskryf in Hoofstuk 4 en het die belangrikheid van akkurate laboratorium praktyke benadruk om sodoende betroubare data vir kalibrasie doeleindes te verkry.

Hierdie grootskaalse ondersoek van die totale antosianiene en totale fenole konsentrasies in druiwe is die eerste van sy soort in Suid-Afrika. 'n Kwantitatiewe databasis is geskep wat die analitiese data van die totale antosianiene en totale fenole konsentrasies van 692 monsters van die *Vitis vinifera* kultivars Cabernet Sauvignon, Merlot, Pinotage en Shiraz bevat van 'n wye reeks rypheidsvlakke van die 2004 en 2005 oesjare. Die data is gebruik in 'n voorlopige ondersoek van die implementering van totale antosianiene en totale fenole konsentrasies as deel van druifkwaliteits klassifikasie by 'n kommersiële wynkelder in Suid-Afrika (Hoofstuk 5). Die resultate het getoon dat die totale antosianiene en totale fenole konsentrasies in druiwe toegeneem het met toenemende rypheid van druiwe (gemeet as suikerkonsentrasie). ANOVA en post-hoc toetse (Bonferroni toetsing) het getoon dat daar beduidende verskille tussen die totale antosianiene en totale fenole konsentrasies van die vier *Vitis vinifera* kultivars ondersoek is. Druiwe wat vroeër in die parseisoen geoes is het beduidend hoër ($p \leq 0.05$) totale antosianiene en totale fenole konsentrasies gehad as druiwe wat later in die parseisoen geoes is. Dit impliseer dat druiwe wat vroeër in die parseisoen geoes word wyne van hoër kwaliteit kan lewer. Druiwe van wingerde wat gereeld besproei is het laer totale antosianiene en totale fenole konsentrasies in vergelyking met druiwe vanaf droëland wingerde gehad. Die huidige druifgraderingsstelsel in gebruik by 'n industriële kelder het nie gekorreleer met die totale antosianiene en totale fenole konsentrasies van druiwe nie. Dit het die behoefte beklemtoon vir die insluiting van meer objektiewe meetings gedurende druifgradering. Die informasie ingesluit in die databasis kan gebruik word as 'n vertrekpunt om die tipiese totale antosianiene en totale fenole profiele van Suid-Afrikaanse druiwe te bepaal en data van meer oesjare moet ingesluit word en deurlopend opdateer word. Hierdie bevindinge het die belang beklemtoon vir die insluiting van totale antosianiene en totale fenole konsentrasies as objektiewe parameters van druifkwaliteit. Uiteindelik het meervariante data analise van die FT-IR spektra belangrike inligting getoon aangaande faktore (beide fisies en chemies) wat bygedra het tot die variasie van die spektra. Die belangrikste variasie tussen die 2004 en 2005 monsters kan moontlik toegeskryf word aan die waterinhoud van die monsters.

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The Almighty, who renders the significant insignificant and the insignificant significant.

This thesis is dedicated to my husband and family for their love and continuous support.
You bless my life in countless ways.

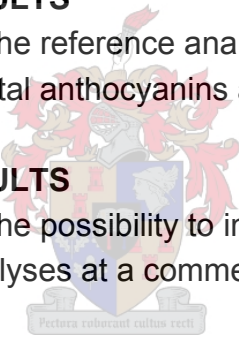
Hierdie tesis word opgedra aan my man en familie vir hul liefde en volgehoue
ondersteuning.

Julle seën my lewe op ontelbare wyses.



PREFACE

This thesis is presented as a compilation of six chapters. Each chapter is introduced separately and Chapter 3 will be submitted for publication.

- Chapter 1** **GENERAL INTRODUCTION AND PROJECT AIMS**
- Chapter 2** **LITERATURE REVIEW**
The importance of phenolic compounds associated with the colour of red grapes and wine
- Chapter 3** **RESEARCH RESULTS**
The evaluation of Fourier transform infrared spectroscopy (FT-IR) for the quantification of total anthocyanins and total phenolics in grapes
- Chapter 4** **RESEARCH RESULTS**
The evaluation of the reference analysis method protocol for the determination of total anthocyanins and total phenolics in grapes
- Chapter 5** **RESEARCH RESULTS**
The evaluation of the possibility to implement grape total anthocyanins and total phenolics analyses at a commercial South African winery – a preliminary study
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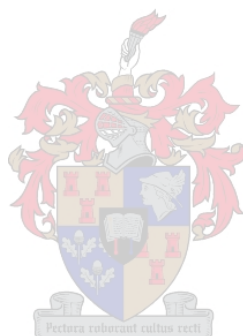
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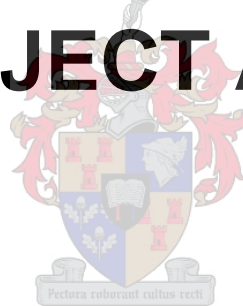
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GENERAL INTRODUCTION AND PROJECT AIMS



GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The opening of the international markets to the South African economy in 1994 placed pressure on the local wine industry to change from a production driven to a market orientated industry. In such an environment consumers demand different wine styles of consistent quality. To fulfil this consumer requirement objective measurements of grape quality in order to produce wines of consistent quality are of utmost importance. There are a number of possibilities available to the producer to assess the quality of grapes. An assessment of vineyard characteristics on standardised scorecards might include: fruit yield to pruning weight, balance between crop load and canopy capacity, uniformity of ripening, vine vigour, grape sunburn, sanitary state and observable berry characteristics (size, seeds, colour, taste) (Dry *et al.*, 2004; Francis *et al.*, 2004). Chemical measurements, such as total soluble solids, pH and titratable acidity are also used to assess grape quality. Grape juice indices such as °Brix/pH, TA/pH, °Brix/TA, °Brix x (pH)² (Du Plessis and Van Rooyen, 1982) have been developed but has had limited success. Due to the need for improved grape quality indicators research has focussed on the phenolic compounds in the case of red grapes.

Phenolic compounds are associated with sensory sensations such as colour, flavour, astringency, bitterness and hardness of grapes and wine (Somers, 1971; Noble, 1994; Gawel, 1998). Phenolic compounds are located primarily in the skins and seeds of grape berries. Grape and wine phenolic composition is influenced by numerous factors including grape cultivar, vineyard location, viticultural practices and winemaking techniques. Phenolic compounds are divided into flavonoid and non-flavonoid phenols based on their chemical structure. Tannins, types of flavonoid species, are associated with the astringency and mouth-feel sensory sensation of red wine (Herderich and Smith, 2005). It is widely speculated that wine tannins are correlated to red wine quality (Herderich *et al.*, 2004). Anthocyanins, another group of flavonoids, are associated with the red colour of grapes and wine (Singleton and Esau, 1969). Wine colour is a very important aspect of its perceived quality. Research has shown that red wine colour intensity is related to the colour intensity of the grapes used for its production. Deeper coloured red grapes normally produce deeper coloured red wines (Iland, 1987; Francis *et al.*, 1999) with more intense aroma and better wine quality (Marais and October, 2005). Therefore the anthocyanin concentration of grapes is an important determining factor of the final wine quality. Anthocyanins are localised primarily in grape berry skins and the reported average anthocyanin content of *Vitis vinifera* grapes range from 0.30 mg/g to 7.50 mg/g berry (Bridle and Timberlake, 1997).

The addition of total anthocyanins and total phenolics concentrations analyses as part of grape quality classification is important for several reasons. Most viticulturists and winemakers assess the phenolic development of grapes only by means of tasting. The inclusion of more objective chemical analyses as part of grape quality classification protocols will result in more reliable and consistent grape quality classification and ensure better streaming of grape musts inside the cellar to ensure that grape musts of more or less the same quality are grouped together (Damberg *et al.*, 2003). Ultimately compensation schemes will be based on more objective and accurate grape quality classification parameters (Damberg *et al.*, 2003).

Phenolic compounds have complex chemical structures and recent advances in analytical techniques have made it possible to detect and measure individual phenolic compounds accurately and precisely (Waterhouse *et al.*, 1999; Waterhouse and Kennedy, 2004). Several chromatographic and spectroscopic techniques exist for the identification and quantification of grape and wine phenolics. Due to the relatively low concentrations and structural diversity of grape and wine phenolics most of these methods require sophisticated instrumentation and generate large quantities of chemical waste that are expensive and hazardous to the environment. However, the results obtained using these techniques provide valuable information regarding grape and wine phenolic compounds. Many of the techniques are time consuming, limiting the number of analyses that can be carried out on a daily basis for routine laboratory analyses. To be able to implement total anthocyanins and total phenolics concentrations measurements within vineyard and winery quality control procedures, there is a need for robust and selective analytical techniques. Near infrared spectroscopy (NIR) has proven to be a rapid, accurate and robust analytical technique for the quantification of grape and wine phenolic compounds (Damberg *et al.*, 2003; Cozzolino *et al.*, 2004). With a reduction in analyses time more time will be available to analyse higher amounts of sample numbers. Fourier transform infrared spectroscopy (FT-IR) is an analytical technique that requires minimum sample preparation, minimises analytical time and saves costs related to chemicals. The development of purpose built FT-IR spectrometers enabled the development of an array of applications fit for routine analyses in the wine industry including analyses for parameters such as sugar, pH, alcohol, total acidity, glucose/fructose, malic acid, lactic acid, acetic acid, tartaric acid, CO₂, glycerol, gluconic acid, sucrose, free amino nitrogen and ethyl carbamate (Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003; Patz *et al.*, 2004). However, the total anthocyanins and total phenolics commercial calibration models supplied by manufacturers are not satisfactory.

1.2 PROJECT AIMS AND RESEARCH STRATEGIES

No published information exists about either the determination of the total anthocyanins or the total phenolics concentrations of grapes as part of routine analyses at South African wineries. This study forms part of a larger survey to study the total anthocyanins and total

phenolics profile and concentration of South African grapes and to evaluate their inclusion as part of grape quality control. The first phase of the project involved the evaluation of Fourier transform infrared spectroscopy (FT-IR) for the routine analysis of total anthocyanins and total phenolics concentrations in grapes. The second part of this project was to establish a database containing information about the total anthocyanins and total phenolics concentration of grapes produced in South Africa. The last phase of the project involved an evaluation of the possibility to implement total anthocyanins and total phenolics concentrations analyses as part of the grape quality classification system of a commercial South African winery. The individual aims of all these phases are outlined below.

1.2.1 THE EVALUATION OF THE REFERENCE ANALYSIS METHOD PROTOCOL FOR THE DETERMINATION OF TOTAL ANTHOCYANINS AND TOTAL PHENOLICS IN GRAPES

The aims of this phase were to:

- 1) Evaluate the length of the extraction steps described in the reference analysis method protocol
- 2) Determine the repeatability of the reference analysis method
- 3) Determine the standard error of laboratory (SEL)

1.2.2 THE EVALUATION OF FOURIER TRANSFORM INFRARED SPECTROSCOPY (FT-IR) FOR THE ROUTINE ANALYSIS OF TOTAL ANTHOCYANINS AND TOTAL PHENOLICS CONCENTRATIONS IN GRAPES

The aims of this phase were to:

- 4) Evaluate Fourier transform infrared spectroscopy (FT-IR) as a technique for the routine quantification of total anthocyanins and total phenolics concentrations in red grapes.
- 5) Develop total anthocyanins and total phenolics calibration models for the Foss WineScan FT 120 spectrometer (Foss Analytical, Denmark).
- 6) Determine the influences of calibration set design on the prediction accuracy of calibration models.

1.2.3 THE ESTABLISHMENT OF A DATABASE

The aims of this phase were to establish a database containing:

- 1) Analytical data including the total anthocyanins and total phenolics concentrations of South African grapes.
- 2) FT-IR spectra of South African grapes.
- 3) Additional information regarding the vintage, origin, irrigation treatment and viticultural grape grading.

1.2.4 THE EVALUATION OF THE POSSIBILITY TO IMPLEMENT TOTAL ANTHOCYANINS AND TOTAL PHENOLICS CONCENTRATIONS ANALYSES AS PART OF GRAPE QUALITY CLASSIFICATION AT A COMMERCIAL WINERY

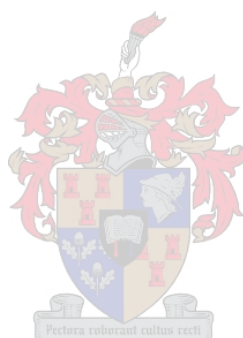
The aims of this phase were to investigate:

- 1) The distribution of total anthocyanins and total phenolics concentrations in grapes from different maturity levels.
- 2) The distribution of total anthocyanins and total phenolics concentrations in different grape cultivars.
- 3) The influence of irrigation treatments on total anthocyanins and total phenolics concentrations in grapes.
- 4) The influence of geographic origin on total anthocyanins and total phenolics concentrations in grapes.
- 5) The relationships between the total anthocyanins and total phenolics concentrations of grapes and grape chemical parameters such as sugar, pH and titratable acidity.
- 6) The current grape grading according to viticultural practices with the total anthocyanins and total phenolics concentrations of the grapes.

1.3 LITERATURE CITED

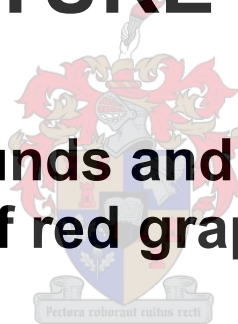
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LITERATURE REVIEW

**Phenolic compounds and their relevance to
the colour of red grapes and wine**



LITERATURE REVIEW

2.1 INTRODUCTION

Wine colour is the first sensory impression perceived in the glass during tasting (Bakker and Arnold, 1993; Almela *et al.*, 1995). The depth of red wine colour is accepted as being correlated to quality; deeper coloured red wines are normally considered of higher quality than their lighter coloured counterparts (Gishen *et al.*, 2002). As a result, chemical compounds related to wine colour are of great importance and are continually investigated. Anthocyanins, a group of phenolic compounds, are primarily responsible for the red colour of grapes and wines (Singleton and Esau, 1969a). The anthocyanin concentrations of grapes, musts and young red wines are highly correlated with wine colour intensity (Somers and Evans, 1974; Jackson *et al.*, 1978; Iland, 1987; Francis *et al.*, 1999; Marais *et al.*, 2001; Marais and October, 2005). The anthocyanin concentrations of grapes can be used to predict subsequent red wine colour (Iland, 1987).

Due to the search for more objective measurements of grape quality, the combination of grape colour with established indicators of grape quality (such as °Brix, pH and titratable acidity) to grade grapes has recently gained interest (Gishen *et al.*, 2002; Damberg *et al.*, 2003). The use of analytical methods instead of organoleptic methods for quality control is essential due to their performance and objectivity (Berente *et al.*, 2000). Recently many advances have been made regarding analytical instrumentation for the identification and quantification of chemical compounds related to grape and wine colour. These advances will aid in the quest to unravel the basic chemistry of red wine colour, one of the major challenges for wine chemists (Waterhouse and Kennedy, 2004).

Grape and wine phenolics are extensively reviewed subjects. This review presents a brief overview of the basic properties of phenolic compounds with the focus on anthocyanins, the phenolic compounds responsible for the red colour of grapes and wines. Viticultural and winemaking factors that influence anthocyanin concentrations in grapes and wines are summarized. Given the interest of the wine industry in evaluating rapid measurements of anthocyanin quantification, a summary of the available analytical techniques for the quantification of anthocyanins is provided.

2.2 PHENOLIC COMPOUNDS IN RED GRAPES AND WINE

Phenolic compounds are chemical constituents of grapes and wine that contribute to sensorial properties such as colour, flavour, astringency and bitterness (Somers, 1971; Noble, 1994; Gawel, 1998). In *Vitis vinifera* grapes approximately 40% of the phenolic compounds are located in the skins and 60% in the seeds of grape berries (Table 1) (Singleton and Esau, 1969b). Many factors influence the phenolic concentration of grapes

and wines including grape variety, terroir, ripening conditions, viticultural practices and winemaking techniques.

Table 1. The distribution of total phenolics concentrations in *Vitis vinifera* grapes (adapted from Singleton and Esau, 1969b).

Berry tissue	Total phenolics concentration (GAE) ^a
Skin	1.86
Pulp	0.04
Juice	0.21
Seeds	3.53
Total	5.64

^aExpressed as mg/g gallic acid equivalents

Phenolic compounds are cyclic benzene compounds with a minimum of one hydroxyl group associated directly with the ring structure. Based on their structure two groups namely flavonoid and non-flavonoid phenols are distinguished (Figure 1) (Bowyer, 2002). Flavonoid phenols are subdivided into anthocyanins, flavanols, flavonols and tannins (Allen, 1998; Kennedy *et al.*, 2002). Non-flavonoid phenols consist primarily of phenolic acids and their esters (Singleton and Noble, 1976). The differences between the two groups are the number and orientation of phenolic sub-units within the molecules (Bowyer, 2002).

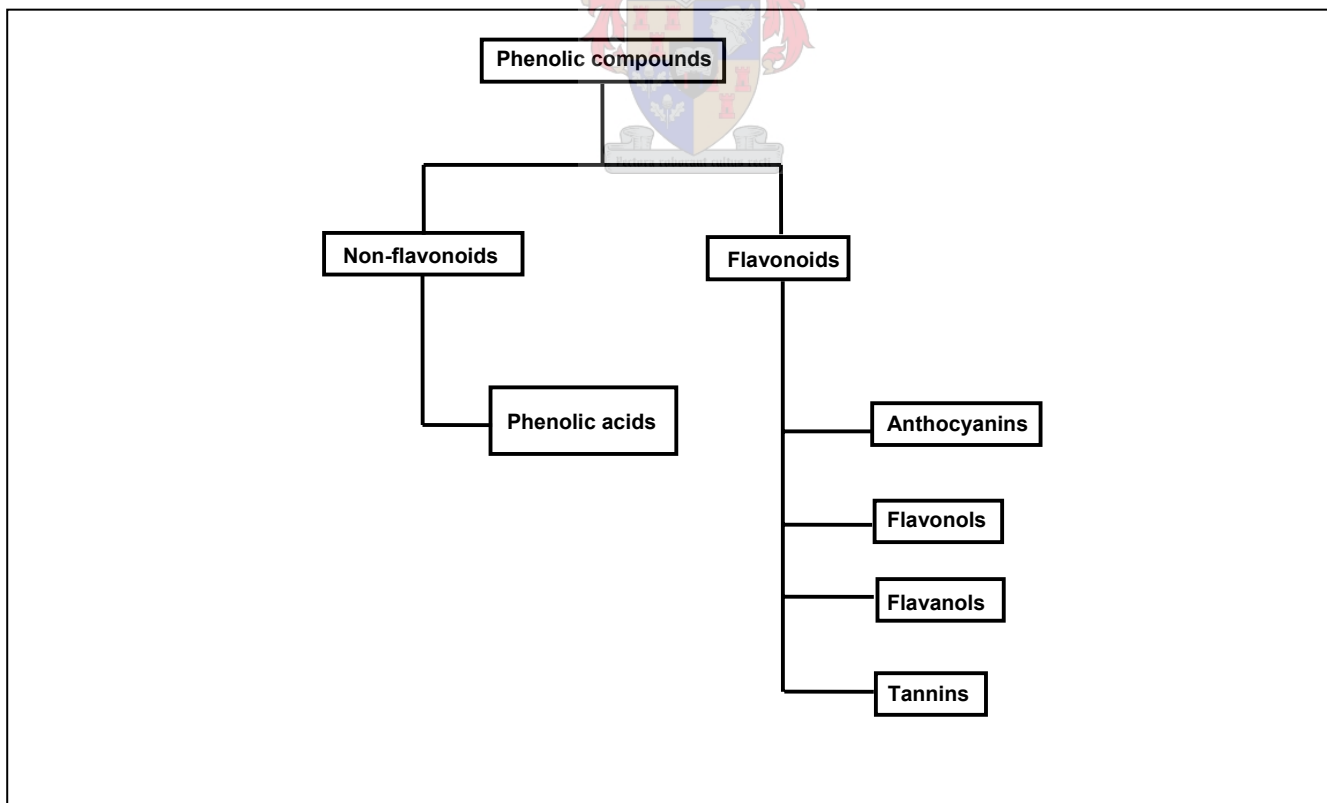


Figure 1. The division of phenolic compounds based on their structure.

2.2.1 FLAVONOIDS

2.2.1.1 Anthocyanins

Anthocyanins, pigments responsible for the red colour of grapes and wines, are located primarily in the lower epidermis (Asen, 1975) of grape skins (Singleton and Esau, 1969a). Anthocyanins are glycosylated derivatives of the 3,5,7,3'-tetrahydroxyflavylium cation also known as anthocyanidin (Figure 2a) (Košir *et al.*, 2004). The sugar compound increases the chemical stability of the anthocyanidin, therefore anthocyanins are the chemical form most prevalent in grapes and wine (Allen, 1998). Individual anthocyanins differ with respect to the number of hydroxyl groups, the type and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to the sugars in the molecule (Favretto and Flamini, 2000; Kong *et al.*, 2003). Anthocyanins of *Vitis vinifera* grapes are fundamentally 3-monoglucosides of the five aglycones malvidin, peonidin, petunidin, cyanidin and delphinidin (Figure 2b) (Wulf and Nagel, 1978). Malvidin-3-glucoside quantitatively is the most important anthocyanin of red grapes and wine (Mazza and Miniati, 1993b). The anthocyanin concentration of *Vitis vinifera* red grapes ranges from 0.30 to 7.50 mg/g berry (Table 2) and varies greatly between cultivar, season, and environment (Bridle and Timberlake, 1997). The average anthocyanins concentration in a *Vitis vinifera* red wine is approximately 150 mg/L (Singleton and Noble, 1976).

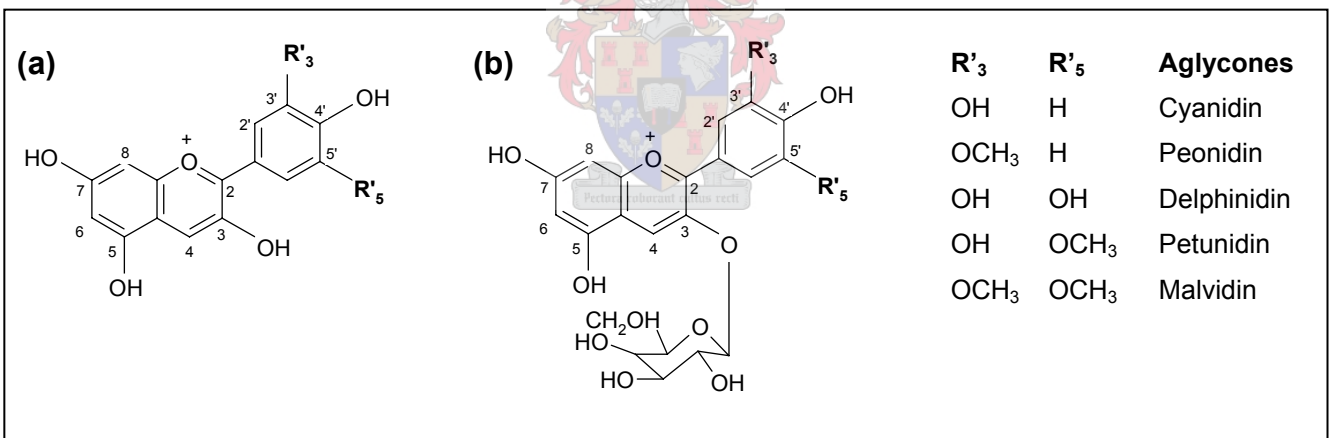


Figure 2. The structure of (a) anthocyanidins and (b) anthocyanin 3-monoglucosides in grapes and wine (adapted from Ribéreau-Gayon *et al.*, 2000).

2.2.1.2 Flavanols

Monomeric flavan-3-ols contribute significantly to the sensory sensation of bitterness occasionally perceived in red wines (Singleton and Trousdale, 1992). Flavan-3-ols are located in the seeds and skins of grape berries (Thorngate and Singleton, 1994) but are absent in the grape pulp (Ricardo da Silva *et al.*, 1992). Their concentration in white wines ranges from 10 to 50 mg/L and may reach 200 mg/L in red wines (Table 2) (Singleton and Esau, 1969b). Flavan-3-ols form dimers, trimers, oligomers and larger polymeric groups through numerous interflavan (C4-C6/C4-C8) linkages. The procyanidins (R=H) (2-8 units),

prodelphinidins (R=OH) (2-8 units) or condensed tannins (>8 units) are the products formed during these polymerization reactions (Haslam, 1998a; Zoecklein *et al.*, 1995a). The structures of procyanidins include (+)-catechin (Figure 3a) and its diastereoisomer (-)-epicatechin (Figure 3b) and the structures of prodelphinidins include (-)-epigallocatechin and (-)-gallocatechin. A single esterified derivative (-)-epicatechin-3-O-gallate has also been reported in grapes (Prieur *et al.*, 1994; Souquet *et al.*, 1996). Flavan-3,4-diols or leucoanthocyanidins differ from flavan-3-ols by an additional hydroxyl attached at carbon 4. Flavan-3,4-diols are subject to the same chemical reactions as the flavan-3-ols. Leucoanthocyanins are glycosylated leucoanthocyanidins (Zoecklein *et al.*, 1995a).

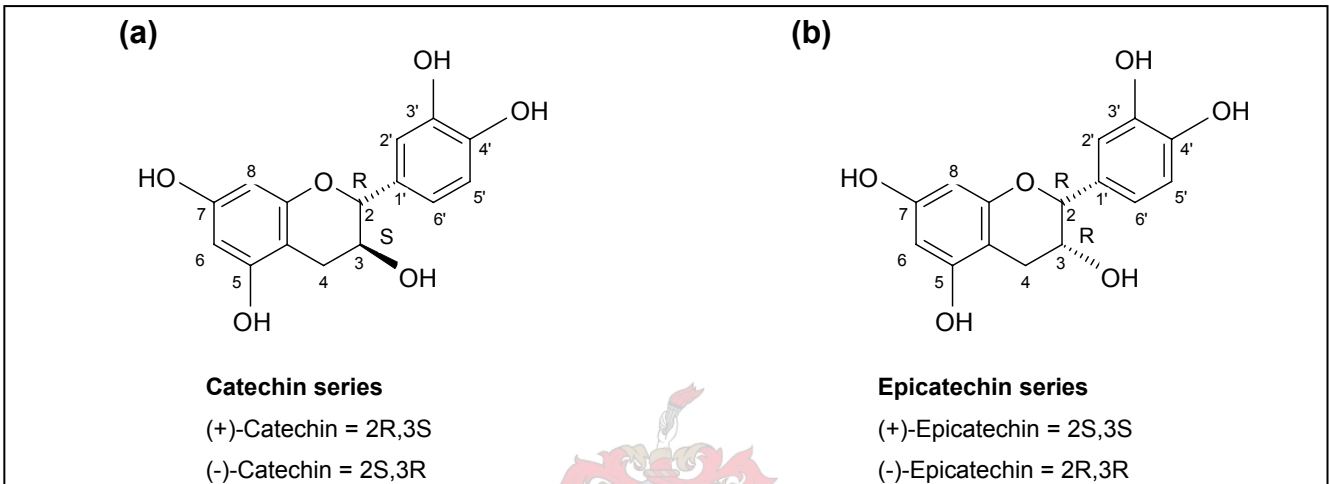


Figure 3. Structure of (a) catechin and (b) epicatechin flavan-3-ol precursors of procyanidins and tannins (adapted from Ribéreau-Gayon *et al.*, 2000).

Table 2. Concentrations of various phenolic compounds in *Vitis vinifera* red grapes and wine.

Phenolic compounds	Group	Grapes or wine	Concentration	Reference
Flavonoids	Anthocyanins	Grapes	0.30 to 7.50 mg/ g berry	Bridle and Timberlake (1997)
		Wine	150 mg/L	Singleton and Noble (1969)
	Flavanols	Wine	200 mg/L	Singleton and Esau (1969b)
	Flavonols	Grapes	0.02 to 0.9 mg/ g berry	Macheix <i>et al.</i> (1990)
Non-flavonoids	Tannins (condensed)	Wine	30 mg/L	Singleton (1988)
		Wine	750 mg GAE/L	Singleton and Noble (1976)
	Phenolic acids	Grapes	190 mg/L	Singleton <i>et al.</i> (1986)
		Wine	100-200 mg/L	Ribéreau-Gayon <i>et al.</i> (2000)
	Stilbenes	Wine	2.0-13.7 mg/L	Lamuella-Raventós <i>et al.</i> (1995)
			2.3-53.5 mg/L	Ribero de Lima <i>et al.</i> (1999)
			3.0-8.1 mg/L	Baptista <i>et al.</i> (2001)
		9.6-27.9 mg/L	Netzel <i>et al.</i> (2003)	

2.2.1.3 Flavonols

Flavonols are light yellow pigments (De Freitas and Glories, 1999) synthesized in the upper epidermis of plant organs (Beggs *et al.*, 1987). Flavonols are primarily present in grape berry skins (Moskowitz and Hrazdina, 1981) but low concentrations are also present in the leaves (Hmamouchi *et al.*, 1996) and stems of grapevines (Souquet *et al.*, 2000). Kaempferol, quercetin and myricetin are the most important flavonols present in grapes (Figure 4) (Cheynier and Rigaud, 1986; Price *et al.*, 1995). The majority of flavonols are quercetins and their concentrations in grape skins range from 0.01 to 0.1 mg/g berry (Table 2) (Macheix *et al.*, 1990). Quercetins elicit a bitter taste with weak astringency (Dadic and Belleau, 1973). Quercetin glycosides act as UV screening compounds, helping to protect the plant tissue from damage (Smith and Markham, 1998). The concentration of flavonol glycosides and aglycones in red wines is approximately 30 mg/L (Singleton, 1988).

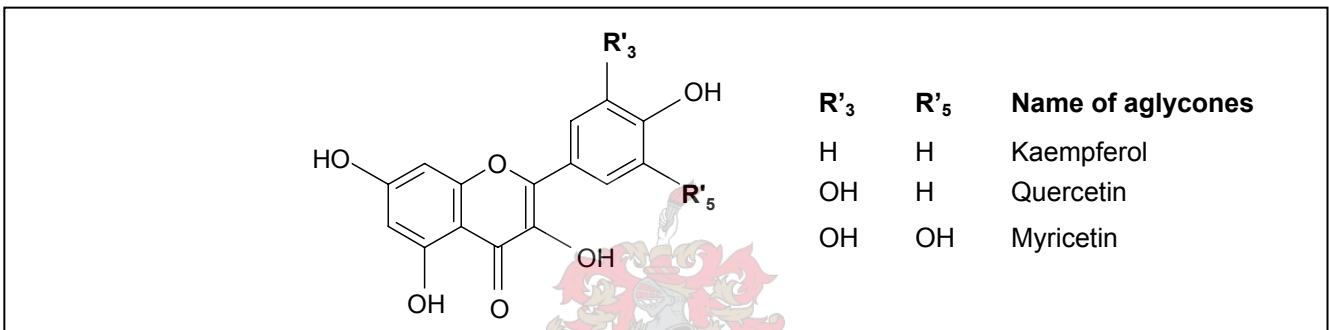


Figure 4. The structure of flavonols present in grapes and wine (adapted from Ribéreau-Gayon *et al.*, 2000).

2.2.1.4 Tannins

Tannins or proanthocyanidins are polymeric flavonoid compounds present in grape skins (Souquet *et al.*, 1996), grape seeds (Prieur *et al.*, 1994), and the stems (Souquet *et al.*, 2000) of *Vitis vinifera* grapevines. Proanthocyanidins are extracted during red winemaking and affect the colour, astringency, stability, and ageing characteristics of wines (Haslam and Lilley, 1988; Sun *et al.*, 1999). The structure of a proanthocyanidin polymer is characterized by the nature of its constitutive extension and terminal flavan-3-ol units and its degree of polymerisation (DP). The DP refers to the average number of units in the polymer (Prieur *et al.*, 1994). The three-dimensional shape of tannins determines its reactivity as well as the way in which tannins interact with wine proteins (protein precipitation), salivary proteins (astringency), or proteins added during fining (Allen, 1998).

Both grape skin and seed proanthocyanidins have epicatechins as the major compounds in the extended chains and catechins as the major terminal units. In addition grape skins contain two prodelfinidins that do not occur in grape seeds namely (+)-gallocatechin and (-)-epigallocatechin (Prieur *et al.*, 1994; Souquet *et al.*, 1996). Skin proanthocyanidins have a higher degree of polymerisation compared to seed proanthocyanidins (Prieur *et al.*, 1994; Souquet *et al.*, 1996). Despite the higher

concentration of tannins in grape seeds, skin tannins are expected to be extracted with less effort during winemaking because of their position (in vacuolar liquid, bound to vacuolar membrane and to the cell wall) and higher polarity (due to the prodelpinidins present) (Souquet *et al.*, 1996).

2.2.2 NON-FLAVONOIDS

2.2.2.1 Phenolic acids

Benzoic and cinnamic acids are phenolic acids present in grape skins and grape pulp (Figure 5a and 5b) (Ribéreau-Gayon, 1965). Although concentrations of most non-flavonoids are below their individual sensory threshold values, collectively some of these compounds may contribute to the sensory sensations of bitterness and harshness (Gawel, 1998; Nagel *et al.*, 1987; Vérette *et al.*, 1988). Cinnamic acids are predominantly esterified with tartaric acid to form caftaric and coutaric acid (Figure 5c) (Ribéreau-Gayon, 1965; Baranowski and Nagel, 1981; Lee and Jaworski, 1989). For *Vitis vinifera* grapes the respective concentrations of caftaric and coutaric acid are 124 mg/L and 17 mg/L in white cultivars (Ong and Nagel, 1978) and 167 mg/L and 22 mg/L in red cultivars (Table 2) (Singleton *et al.*, 1986). During vinification caftaric and coutaric acid hydrolyse to their free forms of caffeic and coumaric acid (Figure 5b) (Somers *et al.*, 1987). The caffeic and coumaric acid concentrations in wines are 125 mg/L and 30 mg/L respectively (Vérette *et al.*, 1988).

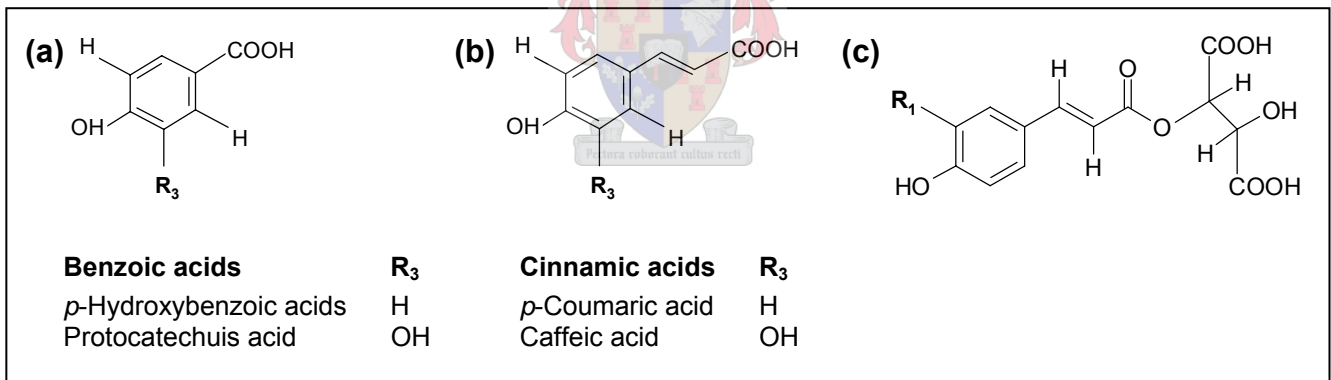


Figure 5. (a) Benzoic acids in grapes and wine. (b) Cinnamic acids in grapes and wine. (c) Derivatives of cinnamic acids and tartaric acid. R₁ = H, coutaric acid; R₁ = OH, caftaric acid (adapted from Ribéreau-Gayon *et al.*, 2000).

2.2.2.2 Stilbenes

Stilbenes are non-flavonoid compounds located in grape berry skins (Jeandet *et al.*, 1991; Lamuela-Raventós *et al.*, 1995). A particular stilbene, resveratrol or trihydroxy-3,5,4'-stilbene, has attracted widespread attention due to its healthful properties (Figure 6). Resveratrol compounds have anticarcinogenic and antitumor properties which reduce the risk of cardiovascular diseases and some types of cancer (Jang *et al.*, 1997; Falchetti *et al.*, 2001). Resveratrols are extracted during fermentation and reported concentrations in

wine range between 1 to 50 mg/L (Table 2) (Lamuela-Raventós *et al.*, 1995; Ribero de Lima *et al.*, 1999; Baptista *et al.*, 2001; Netzel *et al.*, 2003).

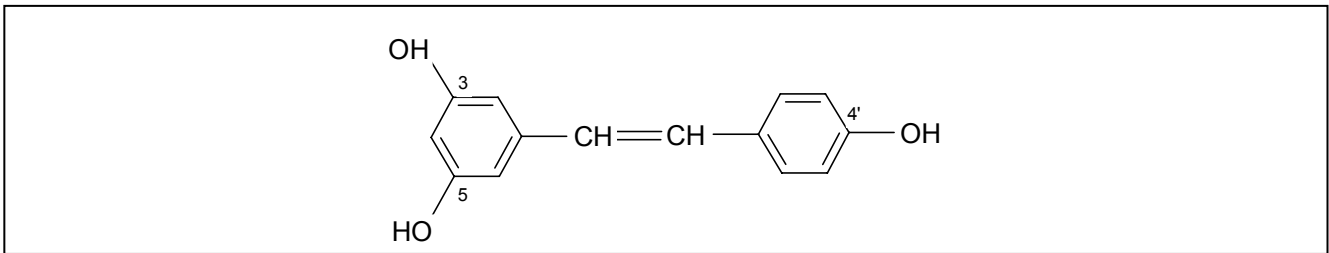


Figure 6. Trihydroxy-3,5,4'-stilbene or resveratrol (adapted from Ribéreau-Gayon *et al.*, 2000).

2.3 FACTORS INFLUENCING THE COLOUR OF ANTHOCYANINS

2.3.1 pH DEPENDENT ANTHOCYANIN COLOUR EQUILIBRIUM

Varying pH levels induce structural transformations of anthocyanins which affect the colour of anthocyanins. Anthocyanins exist in four different forms in solution: the red flavylium cation, the violet quinonoidal base, the colourless carbinol base and the pale yellow chalcone (Brouillard and Lang, 1990). Anthocyanins in the flavylium form are responsible for the orange or red colour of a particular medium (Brouillard, 1983). In relatively strong acidic aqueous solutions (pH lower than 3), anthocyanins display intense reddish colours and exist as flavylium cations (Figure 7). In the pH range 3.0-5.5, the flavylium cations and the neutral carbinol bases co-exist. Anthocyanin stability and colour intensity decreases towards pH neutrality (pH 5.5-6.5) and the quinonoidal bases largely predominate. At a pH of higher than 6.5, kinetic and thermodynamic competition occurs between the hydration reaction of the flavylium cation and the proton transfer reactions related to its acidic hydroxyl groups. The hydration reactions produce colourless carbinol pseudo-bases which equilibrate to the open yellow chalcones (Brouillard, 1982; Brouillard, 1988). The pK value for equilibrium between the red flavylium ion and its colourless pseudobase is 2.6, which favours the colourless form. This implies that at the average wine pH of 3.0, less than 50% of anthocyanins are in the red coloured forms. A decrease in red wine colour is often observed during malolactic fermentation, probably due to the increase in pH (Zoecklein *et al.*, 1995a).

2.3.2 COPIGMENTATION REACTIONS

Copigmentation reactions of anthocyanins were first reported by Robinson and Robinson (1931) and refer to the hydrophobic association of an anthocyanin chromophore with the planar electronically unsaturated part of a cofactor (Brouillard and Dangles, 1994). It is possible to distinguish between intramolecular copigmentation (the copigment is part of the anthocyanin molecule) and intermolecular copigmentation (the copigment is not covalently bound to the anthocyanin molecule) (Brouillard, 1983). Copigmentation causes (a) associated anthocyanins to display far greater colour than would be expected from the

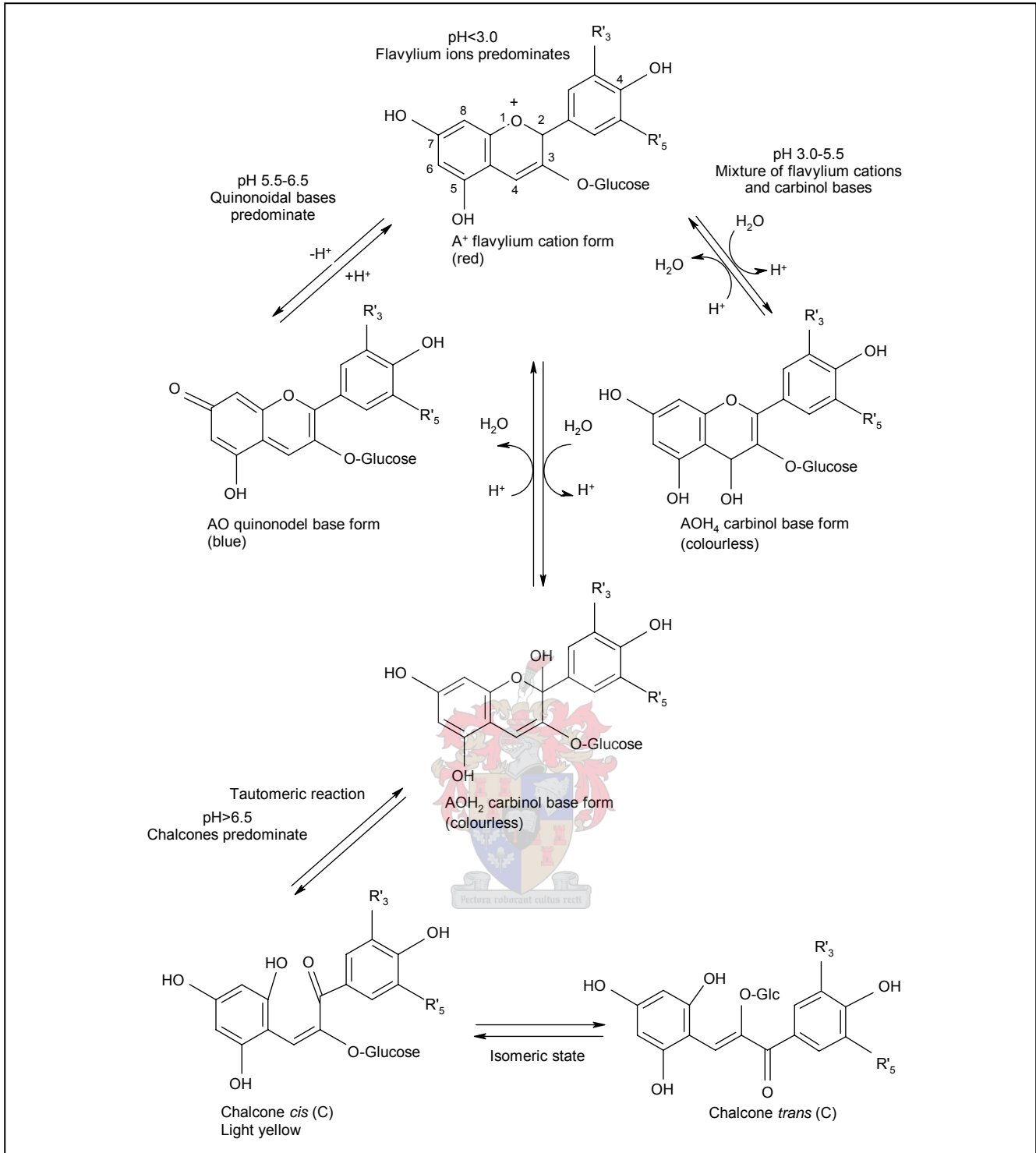


Figure 7. The anthocyanin equilibrium (adapted from Ribéreau-Gayon *et al.*, 2000).

anthocyanin concentrations (a hyperchromic shift) and (b) a change in the wavelength at which maximum absorbance is observed (a bathochromic shift), typically 5 to 20 nm higher, causing a transformation from the reddish to bluish hues (Boulton, 2001).

Most research of copigmentation has focused on monomeric compounds as cofactors because there is no evidence of polymeric phenols (tannins) being cofactors (Boulton, 2001). Potential cofactors include flavonoids, non-flavonoids, amino acids, and organic acids (Brouillard *et al.*, 1989; Boulton, 2001). The most stable copigment associations form

between the flavonols quercetin and quercetin-3-*o*-glucoside, and malvidin-3-*o*-glucoside (Baranac *et al.*, 1997). The observed red wine colour depends on a number of factors: anthocyanin and cofactor type, anthocyanin and cofactor concentration, molar ratio of anthocyanin to cofactor, pH, temperature, and the anions in solution (Brouillard and Dangles, 1994; Boulton, 2001; Darias-Martin *et al.*, 2001). There seems to be a minimum requirement of anthocyanins, approximately 18.5 mg/L, before significant copigmentation is traceable (Boulton, 2001). During wine ageing, copigmentation complexes disappear because of the conversion of monomeric anthocyanins into polymeric pigments. This transformation leads to an increase in colour stability against pH changes and bisulphite bleaching (Hermosín Gutiérrez *et al.*, 2005).

2.3.2.1 Intermolecular copigmentation

Red flavylium cations are susceptible to hydration reactions which change the coloured flavylium cations to colourless pseudobases. Intermolecular copigmentation of anthocyanins with other flavonoids and cofactors inhibits hydration reactions and protects the flavylium cations against water attack and subsequent colour loss (Brouillard, 1982). Intermolecular copigmentation also leads to enhancement of colour due to increased absorbance in the visible range of light and involves change from red to purple or blue colours (Asen *et al.*, 1972; Brouillard, 1982).

2.3.2.2 Intramolecular copigmentation

Intramolecular copigmentation is responsible for the colour stability of anthocyanins containing two or more aromatic acyl groups. Brouillard (1981) described intramolecular copigmentation as the formation of an intramolecular “sandwich” with aromatic acyl groups of two anthocyanin acyl groups on the outsides and the pyrylium ring of the flavylium in between, protecting it against nucleophilic addition of water at C2 and C4 positions. Figure 8 shows the hypothetical stacking mechanism protecting the pyrylium ring from water attack. Hydrophobic interactions between the two electron rich phenolic nuclei of the acyl groups and the electron deficient anthocyanidin (flavylium ion or quinonoidal anhydro-base) are responsible for this intramolecular π - π stacking. Intramolecular copigmentation protects the flavylium cation more efficiently against hydration reactions than intermolecular copigmentation (Brouillard *et al.*, 2003). Some important factors that affect intramolecular copigmentation are the structure of the acyl group, its position of attachment to the sugar, the structure of the sugar, and its location (Brouillard, 1988).

2.3.3 SELF-ASSOCIATION OF ANTHOCYANINS

Self-association occurs when the colour intensity of a solution increases more than linearly with an increase in anthocyanin concentration (Mazza and Miniati, 1993a) and is affected by the type and concentration of anthocyanins (Hoshino *et al.*, 1981; Hoshino *et al.*, 1982; Hoshino, 1991). Asen *et al.* (1972) was the first to suggest that this deviation from Beer-

Lambert's law was the result of anthocyanins in the flavylium ion form that underwent self-association (or vertical chiral stacking) in aqueous media (Figure 9). The principal driving force for this self-aggregation is the hydrophobic interactions between the various aromatic nuclei rather than hydrogen bonding. In contrast to copigmentation, self-association is characterized by a shift towards shorter wavelengths of maximum absorbance, that is, a hypsochromic shift (Hoshino *et al.*, 1981).

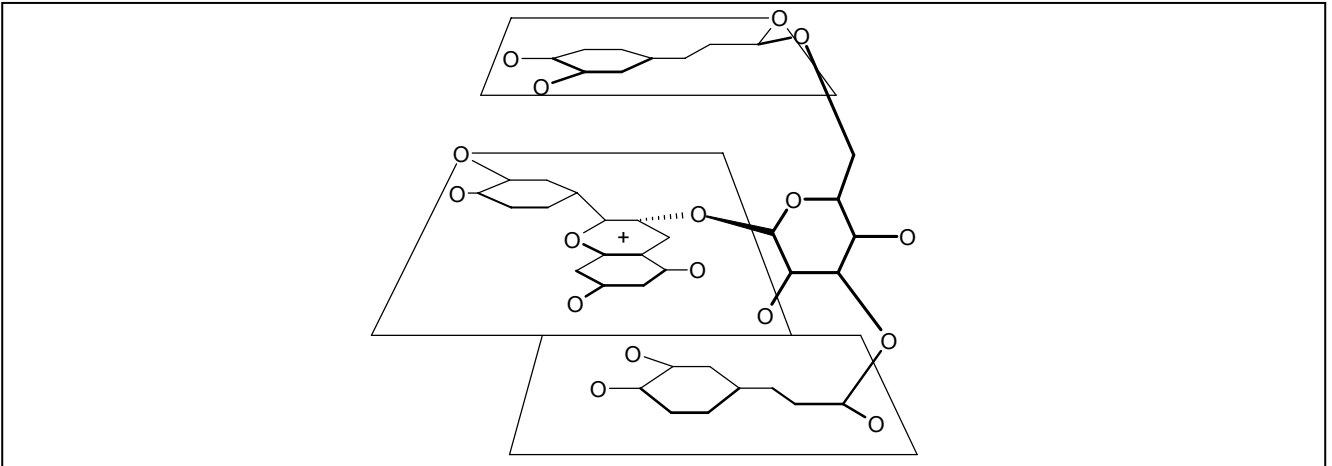


Figure 8. Model for the stacking of two acyl groups above and below the flavylium nucleus. The acyl residues were arbitrarily attached to the 3 and 6 positions of the 3-sugar and, for the sake of clarity, only the carbon skeleton and the oxygen atoms are represented (Brouillard, 1983).

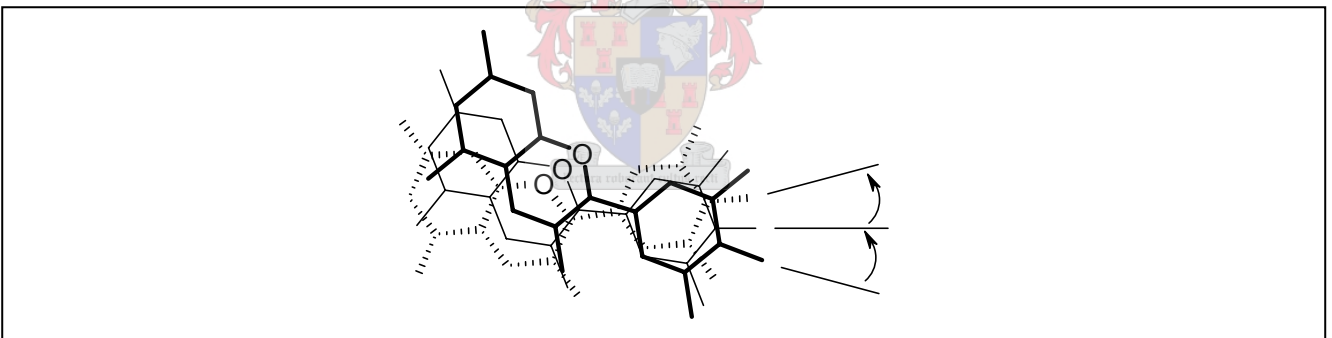


Figure 9. A model for the self-association (chiral stacking) of anthocyanin molecules (adapted from Haslam, 1998b).

2.3.4 ACETALDEHYDE MEDIATED POLYMERISATION REACTIONS

During red wine ageing, monomeric grape anthocyanins are progressively and irreversibly displaced by more stable polymeric pigments. In the presence of acetaldehyde, rapid reactions may occur between anthocyanins in the carbinol base form and flavanols resulting in stable complexes that are more coloured than the colourless anthocyanin forms (Figure 10) (Zoecklein *et al.*, 1995a). Acetaldehyde is a natural compound occurring in wines, produced either by yeast metabolism during fermentation or by ethanol oxidation (Atanasova *et al.*, 2002). The acetaldehyde mediated polymerisation reactions between anthocyanins and flavanols was first described by Timberlake and Bridle (1976a). In an

acid medium the acetaldehyde forms a carbocation that reacts with the active positions (C6 and C8) of a flavanol, leading to a carbocation intermediate, which in turn reacts with either another flavanol molecule or the hydrated form of an anthocyanin to form a violet coloured pigment (Timberlake and Bridle, 1976a; Fulcrand *et al.*, 1996). In wine acetaldehyde polymerisation reactions occurs as a result of the controlled oxidation during barrel ageing, when traces of acetaldehyde are produced by the oxidation of ethanol. The colour of the wine becomes more intense and changes tone, becoming darker after a few months of barrel ageing (Ribéreau-Gayon *et al.*, 2000).

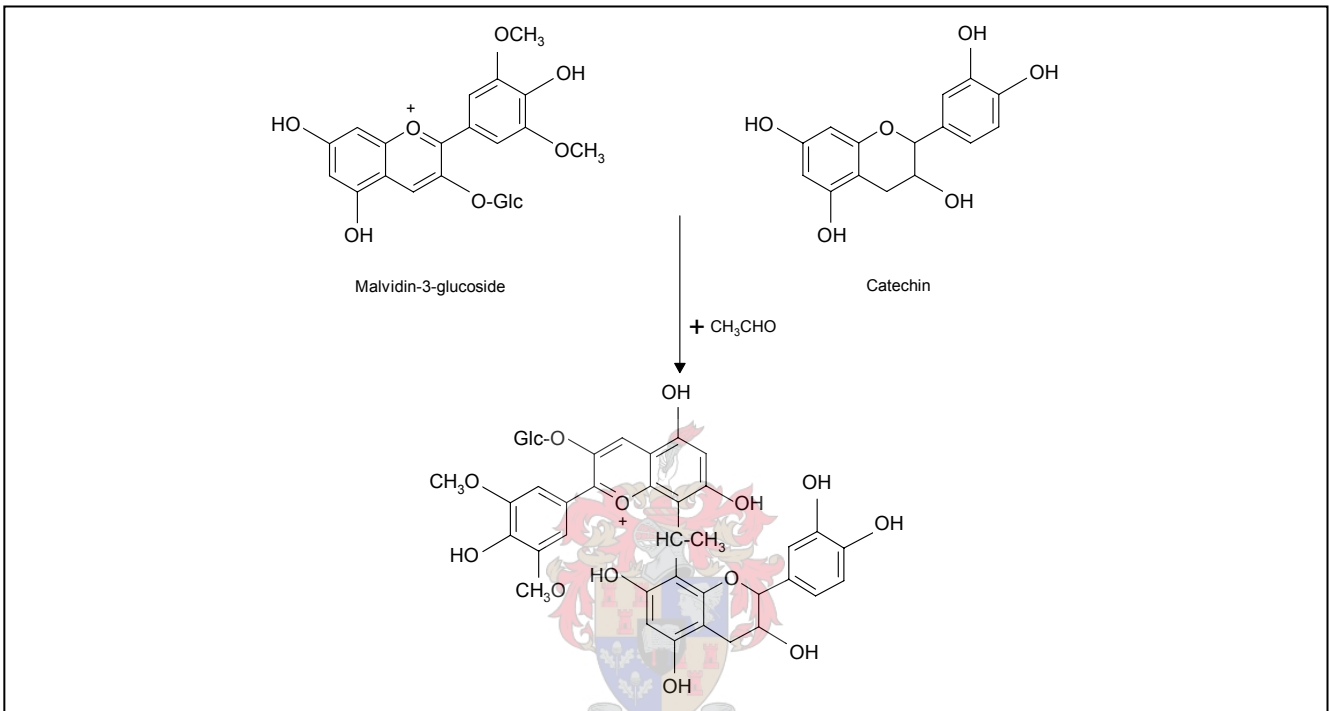


Figure 10. The acetaldehyde mediated polymerisation reaction between malvidin-3-glucoside and catechin (a flavanol) in an acid medium (adapted from Ribéreau-Gayon *et al.*, 2000).

2.3.5 OXIDATIVE DEGRADATION OF PHENOLIC COMPOUNDS

Enzymatic oxidation of phenolic compounds is primarily responsible for browning of grape must and wine. Besides the alteration of wine colour, browning also affects the sensory qualities of wine (reviewed in Mayer and Harel, 1979; Macheix *et al.*, 1991). In the presence of oxygen the enzyme group polyphenoloxidases catalyses enzymatic oxidation of phenolic compounds in grape must and wine (Macheix *et al.*, 1991) to form highly active quinones (Mayer and Harel, 1979). The quinones rapidly condense and may combine with amino sulphhydryl groups of proteins, as well as with anthocyanins, to form relatively insoluble brown polymers (Mayer and Harel, 1979). In grape juice the three most important parameters that determine browning intensity and the rate at which it appears are the phenolic concentration susceptible to oxidation, activity of polyphenoloxidases, and available oxygen. The two types of polyphenoloxidases responsible for oxidative browning are: *o*-diphenol oxygen oxidoreductase (*o*-DPO) and laccase (Macheix *et al.*, 1991). A natural “defence” mechanism exists against browning in must and wine involving caftaric

acid, the major phenol present in grape must prepared with limited oxidation and minimum extraction from skins and seeds. Caftaric acid is oxidised to its quinone which in turn reacts with the tripeptide glutathione to produce S-glutathionyl caftaric acid or the grape reaction product (GRP). The GRP is colourless and is not involved in further chemical reactions. Therefore, the caftaric acid quinones are no longer free to change to brown polymers and thereby browning is prevented (Margalit, 1997).

Oxydative degradations of anthocyanins are catalysed by oxygen and light. This reaction is primarily influenced by the type and concentration of alcohol. Controlled oxydative degradation of anthocyanins occurs during the barrel ageing of red wines. It is reported that malvidin is more resistant to oxidation than cyanidin (Ribéreau-Gayon *et al.*, 2000).

2.4 THE DEVELOPMENT OF ANTHOCYANINS AND TANNINS DURING RIPENING

Berry growth follows a double-sigmoid pattern corresponding to three growth periods with each growth period differing considerably in biochemical activity and subsequent grape composition (Figure 11) (Coombe, 1973; Coombe and Bishop, 1980; Brady, 1987). The most important developmental stages of *Vitis vinifera* grapevines are budburst, flowering, véraison (colour change and start of maturation) and harvest (grape maturity) (Jones and Davis, 2000). Véraison signals the end of the development period of grapes and the onset of the ripening process (Hrazdina *et al.*, 1984). This is marked by the initiation of anthocyanin formation in red varieties, the start of rapid sugar accumulation, a decrease in acidity and chlorophyll, and the onset of grape softening (Singleton and Esau, 1969c). Physiological maturity refers to the optimal levels of sugars, pH and titratable acidity for a specific wine style. Industrial maturity is the ripeness level which yield maximum weights of grapes and sugar concentrations. Technological maturity is associated with the optimum phenolic composition for achieving a specific wine style (Robredo *et al.*, 1991).

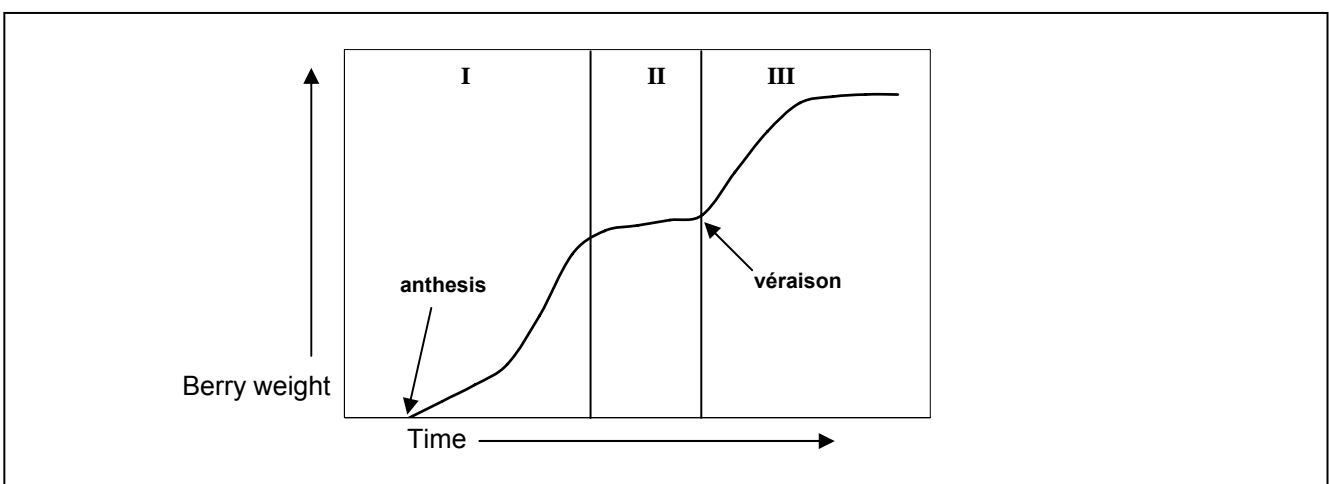


Figure 11. The double-sigmoid growth pattern of grape berries (Kennedy *et al.*, 2000a). **I.** First period of berry growth. **II.** Little change in berry size. **III.** Véraison followed by second period of berry growth.

2.4.1 ANTHOCYANINS

A number of studies have investigated the accumulation of anthocyanins and other phenolic compounds in grape berries during ripening (Hrazdina *et al.*, 1984; Fernández-López *et al.*, 1998; Yokotsuka *et al.*, 1999; Jordão *et al.*, 2001; Ryan and Revilla, 2003). Soon after the beginning of fruit formation, coinciding with, or just after véraison, there is a visible and measurable formation of anthocyanins (Pirie and Mullins, 1980; Hrazdina *et al.*, 1984; Yokotsuka *et al.*, 1999). Development of anthocyanins from véraison to full maturity follows a sigmoid curve that coincides to a considerable extent with the accumulation of soluble sugars (Hrazdina *et al.*, 1984; Pirie and Mullins, 1977; Pirie and Mullins, 1980). Anthocyanin formation during the early stages of berry development is limited to small areas, in contrast with mature berries where the subepidermal layer uniformly contains anthocyanins (Hrazdina *et al.*, 1984).

2.4.2 TANNINS

The maximum concentration of phenolic compounds in grapes does not normally coincide with the maximum accumulation of sugars (Maujean *et al.*, 1983). During grape maturation, phenolic compounds bind together or polymerise to form tannins (Zoecklein, 2001). Both grape seed (Romeyer *et al.*, 1986) and skin tannins (Fernández de Simon *et al.*, 1992; Kennedy *et al.*, 2001; Kennedy *et al.*, 2002) have a relatively low degree of polymerisation at véraison which increases during ripening, resulting in decreased astringency. As grapes mature sensory changes are observed from “hard” and bitter to astringent and finally to soft and supple during grape tasting. These sensory changes are the result of tannins polymerization together with products formed via the polymerization of phenolic compounds with other molecules (e.g. sugars and proteins). As seeds mature they change colour from green to dark brown (Zoecklein, 2001). During berry development there is an increase in the concentrations of anthocyanins associated with the tannin fraction (Kennedy *et al.*, 2001).

2.5 THE ANTHOCYANIN BIOSYNTHETIC PATHWAY

Grape anthocyanins are synthesized through the phenylpropanoid and flavonoid pathways (Figure 12) (Boss *et al.*, 1996c; Holton and Cornish, 1995). These pathways are regulated by enzyme activities (Hrazdina *et al.*, 1984) and gene expression (Boss *et al.*, 1996a). Both malonyl-CoA and 4-coumaroyl-CoA, the two types of direct flavonoid precursors, are derived from carbohydrates. Malonyl-CoA is synthesised from the glycolysis intermediate acetyl-CoA and carbon dioxide, the reaction being catalysed by acetyl-CoA carboxylase. The supply of 4-coumaroyl-CoA is more complex and involves the shikimate pathway, the main biosynthetic pathway to the aromatic amino acids phenylalanine and tyrosine in higher plants (Heller and Forkmann, 1988). Transformation of phenylalanine to cinnamic acid is catalysed by phenylalanine ammonia lyase (PAL) (Figure 12). Aromatic

hydroxylation of cinnamic acid by cinnamate 4-hydroxylase (C4H) leads to 4-coumarate which is further transformed to 4-coumaric acid which forms 4-coumaroyl-CoA by action of 4-coumarate:CoA ligase (4CL) (Heller and Forkmann, 1988).

Chalcone synthase (CHS) catalyses the formation of chalcone through the condensation of three molecules of malonyl-CoA and 4-coumaroyl-CoA (Figure 12) (Mazza and Miniati, 1993a). The formed C₁₅ chalcone is isomerised by a chalcone isomerase (CHI) into flavanone. Flavanone 3-hydroxylase (F3H) hydroxylates flavanones to form dihydroflavonols. Dihydroflavonol 4-reductase (DFR) catalyses the conversion of dihydroflavonols to leucoanthocyanidins. This is followed by the production of anthocyanidin from leucoanthocyanidins, which involves leucoanthocyanidin dioxygenase (LDOX). The final step involves the addition of a glucose residue to anthocyanidin catalyzed by UDP glucose-flavonoid 3-*o*-glucosyl transferase (UFGT) to form anthocyanin (Mazza and Miniati, 1993a; Mori *et al.*, 2005). Sections 2.5.1 and 2.5.2 highlight the importance of PAL and UFGT, two of the enzymes involved during anthocyanin biosynthesis.

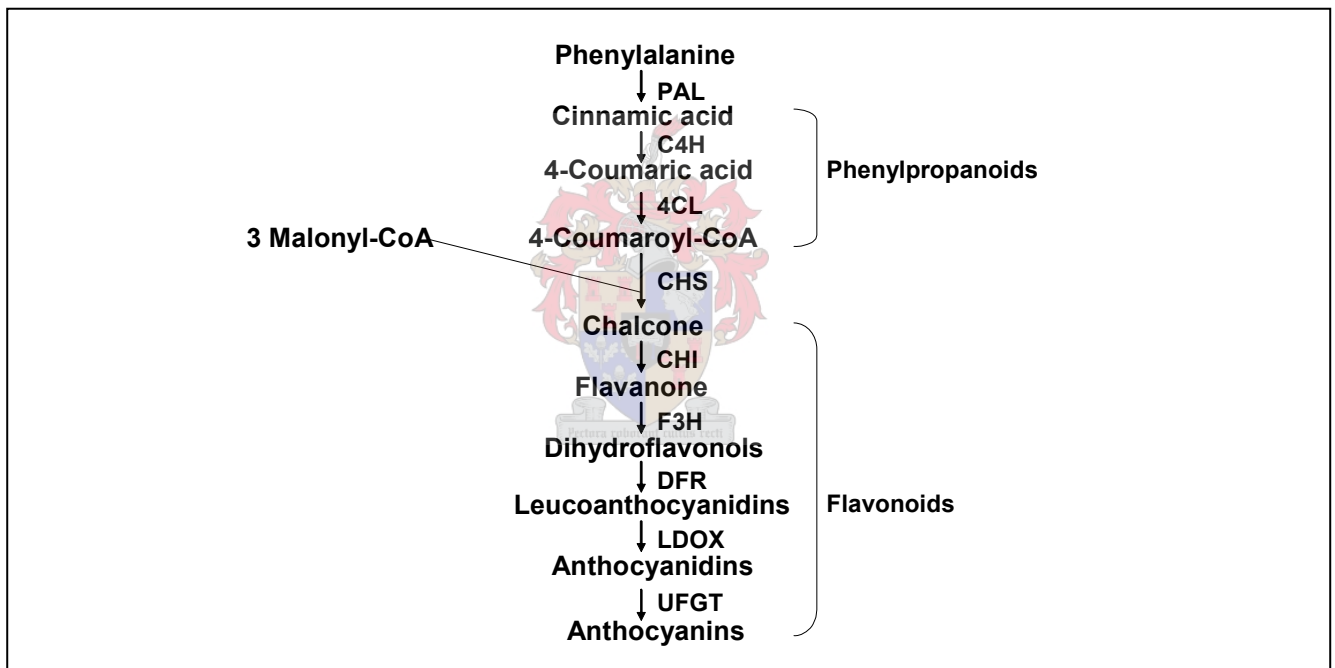


Figure 12. The major intermediates in the anthocyanin biosynthetic pathway (adapted from Jeong *et al.*, 2004). Abbreviations used: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase; UFGT, UDP glucose-flavonoid 3-*o*-glucosyl transferase.

2.5.1 PHENYLALANINE AMMONIA LYASE (PAL)

PAL is the key enzyme in the shikimate pathway that channels phenylalanine away from protein synthesis towards that of phenylpropanoid, flavonoids and anthocyanins (Hrazdina *et al.*, 1984; Roubelakis-Angelakis and Kliewer, 1985; Roubelakis-Angelakis and Kliewer,

1986). During berry development there is significant activity of PAL in skins and seeds, but this is followed by a swift decline in the enzyme activity towards véraison (Hrazdina *et al.*, 1984). There is no synthesis of anthocyanins during this period. In red grapes PAL increases again after véraison and there is a correlation between activity and colour intensity (Kataoka *et al.*, 1983; Hrazdina *et al.*, 1984). Apparently light is indispensable for PAL activity and therefore it has a major effect on anthocyanin biosynthesis and accumulation (Roubelakis-Angelakis and Kliewer, 1986). Even though PAL has been proposed to be the key enzyme in the phenylpropanoid pathway in many plants, it is likely that various other enzymes also regulate anthocyanin biosynthesis (Mori *et al.*, 2005).

2.5.2 UDP GLUCOSE-FLAVONOID 3-O-GLYCOSYL TRANSFERASE (UGFT)

The mRNA of all anthocyanin biosynthetic enzyme genes except that of UDP glucose-flavonoid 3-*o*-glucosyl transferase (UGFT) accumulate in the early berry development stage but decrease until véraison, thereafter the mRNA levels of all genes including that of UFGT increase in red cultivars (Boss *et al.*, 1996a; Kobayashi *et al.*, 2001). The mRNA of UFGT is only present in the grape skins of red cultivars which indicate that UFGT is critical for anthocyanin biosynthesis in grape skins (Boss *et al.*, 1996b; Boss *et al.*, 1996c; Kobayashi *et al.*, 2001).

2.6 VITICULTURAL FACTORS INFLUENCING THE PHENOLIC COMPOSITION OF GRAPES AND WINES

Many environmental factors and vineyard management practices influence grape phenolic composition and subsequent wine quality (reviewed in Du Plessis, 1984; Jackson and Lombard, 1993). Among the most important factors are solar radiation, temperature and irrigation. While these factors may not contribute to the rapid physiological or biochemical changes that occur during the development and maturation of grapes, their effects on shortening or lengthening these processes have to be recognized (Hrazdina *et al.*, 1984). This section gives a brief overview of the major viticultural factors that influence grape colouration.

2.6.1 IRRIGATION

Numerous studies have investigated the effects of vine water deficit on berry growth and ripening and as a factor determining the anthocyanin composition of grapes and wine quality (Matthews *et al.*, 1990; Ginestar *et al.*, 1998; Roby *et al.*, 2004). Vine water deficits generally lead to smaller berries (Bravdo, 1985; Matthews *et al.*, 1990; Kennedy *et al.*, 2002). Smaller berries have higher anthocyanin concentrations due to a concentrating effect of chemical compounds. Roby *et al.* (2004) found that grapes from low vine irrigation had a 45% increased anthocyanins concentration over grapes from highly irrigated vines. The corresponding loss in yield mass due to reduced berry growth was less than 20%.

Depending on the stage of berry development, water deficit has different effects. Pre-véraison water deficit results in the greatest reduction in berry weight compared with that of well-watered vines (Ginestar *et al.*, 1998). Post-véraison water deficit has an ignorable effect on berry weight (McCarthy, 1999).

Partial root zone drying (PRD) is a type of irrigation method where water is applied to one side of the vine for 10 to 15 days and then water is applied to the other side. If, during irrigation cycles, the “wet” side is sufficiently watered the “dry” roots are maintained in a healthy condition by water supplied to them from the wet roots. Dry *et al.* (2000) found no significant reduction in yields or berry size using PRD even though the total amount of irrigation was halved. The application of PRD increased berry anthocyanins and total phenolics (Dry *et al.*, 2000). Partial root zone drying can induce stress to vines if not applied with caution.

2.6.2 LIGHT EXPOSURE

Smart *et al.* (1985) adopted the term “microclimate” to define the environmental conditions within the immediate vicinity of the leaves and fruit of a grapevine. Depending on the grapevine canopy, leaves and bunches can develop in conditions varying from heavily shaded (shaded canopies) through to fully exposed (open canopies) (Haselgrove *et al.*, 2000). Both pre-véraison and post-véraison growth periods can be prolonged under heavily shaded conditions (Rojas-Lara and Morrison, 1989). This means that berry ripening, anthocyanin accumulation and berry growth in general are delayed. In most cases where grapes that developed in open canopies were compared to grapes that developed in shaded canopies, the exposed grapes had higher sugar concentrations, improved acid balance (lower juice pH and higher titratable acidity), and less unripe herbaceous fruit characters (Haselgrove *et al.*, 2000). Most research has focused on cluster microclimate, but the influence of shaded shoots and leaves have also been investigated (Crippen and Morrison, 1986). Unless otherwise stated this section of the review refers to the effects of the degree of bunch exposure to sunlight.

Researchers do not agree about the effect of sunlight on anthocyanin accumulation and concentrations. The opinion of some researchers has been that low light reduces anthocyanins and other flavonoids in grapes, while increased light increases flavonoid concentrations (Kliewer, 1970; Wicks and Kliewer, 1983; Morrison and Noble, 1990; Dokoozlian and Kliewer, 1996; Keller and Hrazdina; 1998). There have also been reports that different light treatments have either no effect on anthocyanin concentrations (Hunter *et al.*, 1995; Price *et al.* 1995) or even that high light causes decreased anthocyanin concentrations in grapes (Bergqvist *et al.*, 2001). Even when researchers agree that light stimulates the synthesis of anthocyanins, there are still different opinions about the precise time during berry development or ripening when sunlight is essential for maximum anthocyanin accumulation. Dokoozlian and Kliewer (1996) concluded that light exposure before véraison is critical for berry tissues to perceive environmental stimulation and

trigger anthocyanin biosynthesis. Keller and Hrazdina (1998) found that exposure of the grapevine canopy to better light conditions at véraison and Hunter *et al.* (1991) just after véraison resulted in the highest anthocyanin concentration in grape skins. Many investigations were limited to ripe fruit. Substantial proof exist that the accumulation of phenolic compounds in grape berries occurs prior to the onset of ripening (Kennedy *et al.*, 2000a; Kennedy *et al.*, 2000b; Downey *et al.*, 2003a). Both Haselgrove *et al.* (2000) and Downey *et al.* (2003b) have reported high concentrations of flavonols in the fruit at véraison as well as early in berry development and at flowering, emphasizing the importance of extending research of flavonoids to the earliest developmental stages.

Comprehensive studies of the influence of light on flavonoid synthesis in developing berries were conducted by Downey *et al.* (2004a and 2004b) who investigated flavonoid biosynthesis in Shiraz over three successive seasons. During two seasons bunch exposure had no effect on anthocyanin concentration. They suggested that there may be two systems regulating anthocyanin accumulation in grapes: an initial constitutive system that generates a base level of anthocyanins and an inducible system that is light-requiring. This study showed that grapes grown in the absence of light did accumulate anthocyanins, which indicates that light is not an absolute requirement for anthocyanin biosynthesis.

Downey *et al.* (2004b) also published data about the effects of different levels of bunch exposure to different levels of sun exposure on anthocyanin accumulation and wine colour of Cabernet Sauvignon. Anthocyanin concentrations were generally higher in exposed fruit and as exposure to light was increased, the anthocyanin concentrations also increased. Wine colour was also higher in wines made from more exposed fruit. It was concluded that light alone might not be the greatest influence on anthocyanin biosynthesis, but that the regional influence (most likely temperature) has a greater effect on anthocyanin concentration and composition than light. To achieve maximum colour development in warm regions, prolonged exposure of clusters to sunlight should be avoided because of the reducing effect that excessive fruit temperatures have on anthocyanin formation and subsequent concentrations (Haselgrove *et al.*, 2000; Bergqvist *et al.*, 2001; Spayd *et al.*, 2002). Complete fruit shading should also be avoided because some sunlight is needed for maximum anthocyanin synthesis and for balancing the composition of other fruit compounds (Spayd *et al.*, 2002).

2.6.3 BERRY TEMPERATURE

In addition to regulating photosynthesis and photo-morphogenesis, sunlight provides radiant energy that heats plant surfaces (Bergqvist *et al.*, 2001). The anthocyanin biochemical pathway is both light and temperature sensitive (Kliewer, 1970; Pirie and Mullins, 1980; Hrazdina *et al.*, 1984; Crippen and Morrison, 1986; Haselgrove *et al.*, 2000). The optimum temperatures for enzymes involved in the anthocyanin biosynthetic pathway are between 17° to 26°C (Pirie and Mullins, 1977). Anthocyanin biosynthesis and stability in grapes are favoured by warm daytime temperatures (15° to 25°C) and cool nights (10°

to 20°C) (Kliewer, 1970; Kliewer and Torres, 1972; Crippen and Morrison, 1986; Mori *et al.*, 2005). During experimental procedures the separation of the effects of temperature and sunlight on grape colour composition is extremely difficult.

2.6.4 BERRY VOLUME

Wine quality, colour density, total anthocyanins, and phenolics are negatively correlated with bunch and berry weight (Somers and Pocock, 1986). It is generally accepted that larger berries have greater solvent to solute ratios because of smaller skin:pulp ratios (Hardie *et al.*, 1996). Therefore berries with a smaller diameter are a desirable feature which increases the skin:pulp ratio and more potential extraction of anthocyanins from the grape skins.

2.6.5 SANITARY STATE

The health or sanitary state of grapes is known to affect the phenolic composition of red grapes and the subsequent colour and sensorial properties of red wines. The anthocyanin concentration of grapes infected with *Oidium tuckeri* or powdery mildew is lower than healthy grapes. Therefore wines made from healthy grapes have more colour than wines made from infected grapes because of the lower total anthocyanins and total phenolics concentrations of the infected berries (Amati *et al.*, 1996; Piermattei *et al.*, 1999). Any factor that leads to a reduction in red wine colour reduces the wine quality; therefore sound viticultural practices are essential to ensure a healthy harvest.

2.7 WINEMAKING PRACTICES INFLUENCING THE PHENOLIC COMPOSITION OF WINE

Numerous investigations and reviews evaluated the effects of winemaking practices and conditions on anthocyanins and other phenolic compounds (Dallas and Laureano, 1994; Bakker *et al.*, 1998; Kwiatkowski *et al.*, 2002; Sacchi *et al.*, 2005). Following grape harvest the first step of vinification involves the removal of stems, leaves, and other unwanted material (Figure 13). The next procedure entails crushing of the grapes to release the juice. The fresh must is treated with sulphur dioxide to inhibit both oxidative browning and the growth of unwanted microorganisms. In red wines, prolonged maceration enables the extraction of compounds from the pomace (skins and seeds). The extraction process occurs simultaneously with alcoholic fermentation. Fermentation may start spontaneously due to indigenous yeasts derived from grapes, or yeasts present on the surface of contaminated equipment. To prevent a sluggish or “stuck” fermentation and to obtain a wine with desirable bouquet and flavour, grape must is generally inoculated with a pure culture of a selected yeast strain (Jackson, 1994). An average red wine ferments 5 to 7 days. During fermentation most of the sugars are converted to alcohol by wine yeasts. In the presence of alcohol anthocyanins leach into the grape must after release from the

skins (Boulton *et al.*, 1996a). After partial or complete fermentation, the free-run grape must is allowed to flow away under gravity. Pressing of the pomace extracts most of the remaining grape must (press fraction) but the general quality is lower than that of the free-run grape must. After completion of alcoholic fermentation, the wine may be encouraged to undergo malolactic fermentation, a fermentation resulting in the degradation of malic acid to lactic acid. Malolactic fermentation is particularly beneficial in cool climate regions, where the reduction of acidity of wines improves their taste. After several weeks or months the wine is separated from the sediment - which settles out during spontaneous or induced clarification - through a process called racking. The sediment consists primarily of yeast and bacterial cells, grape cell debris, precipitated tannins, proteins, and potassium tartrate crystals. Prior to bottling, the wine may be fined to remove excess tannins and/or dissolved proteins and other materials that can lead to the development of haziness. Wines are chilled and filtered to further enhance clarification and stability. The final step in the production chain is bottling of the wine (Jackson, 1994).

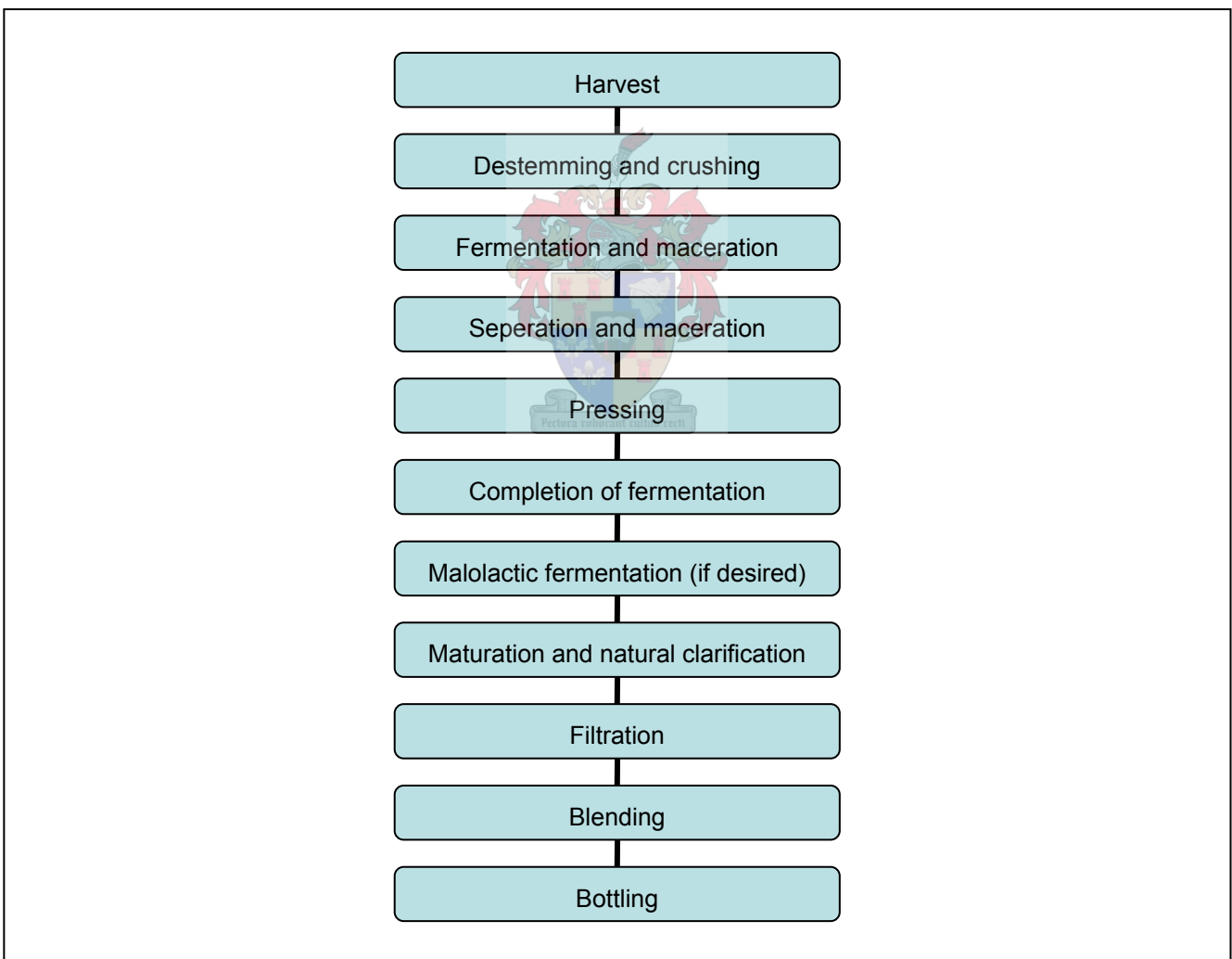


Figure 13. A flow diagram of red wine making (adapted from Jackson, 1994).

2.7.1 GRAPE PROCESSING

Vigorous crushing enhances the extraction and diffusion of anthocyanins and other phenolic compounds by increasing the skin surfaces in contact with the juice. However, excessive crushing of grape berries often leads to the extraction of astringent and bitter tannins that affect the final quality of the wine (Macheix *et al.*, 1991). Polymerisation of anthocyanins starts at grape crushing and reaches considerable proportions during fermentation on skins (Bakker *et al.*, 1986). Crushing also enhances enzymatic oxidation of phenolic compounds which may lead to browning of the must and the development of undesirable sensory characteristics of wines. The addition of sulphur dioxide (SO₂) as an antioxidant and antiseptic during crushing is widely accepted, but when added in excess may lead to stuck fermentations and bleached colour (Mazza and Miniati, 1993b). Gil-Muñoz *et al.* (1999) found that low temperatures of grapes at the moment of crushing increased the rate of polyphenol extraction during the first three to four days of alcoholic fermentation. However, as the fermentation progressed the temperature of the must increased, and the differences diminished. They concluded that night-time harvesting of grapes does not improve the quality of young red wines.

2.7.2 MACERATION TIME

The optimum length of skin fermentation time depends on cultivar, desired wine style and other factors (Auw *et al.*, 1996). Researchers report maximum colour extraction with skin contact times varying between 4 to 16 days (Auw *et al.*, 1996; Bakker *et al.*, 1998; Yokotsuka *et al.*, 2000; Gómez-Plaza *et al.*, 2002). Carbonic maceration produces fruity red wines ready for immediate consumption, but not suitable for ageing (Gómez-Míguez and Heredia, 2004). Intact whole grapes are surrounded with CO₂ which triggers the anaerobic metabolism of the grapes. The cell walls in the skin become permeable allowing the phenolic compounds to diffuse from the skin into the pulp. The phenolic diffusion is accelerated by the temperature (Gómez-Míguez and Heredia, 2004). Carbonic maceration produces wines with lower colour and overall quality when compared to wines made with traditional fermentation or thermovinification techniques (Rizzon *et al.*, 1999; Carnacini *et al.*, 1991; Pelligrini *et al.*, 2000; Gómez-Míguez and Heredia, 2004; Castillo-Sánchez *et al.*, 2005).

2.7.3 FERMENTATION TEMPERATURE

Cold soaking or cold maceration is a pre-fermentation technique that increases complexity, colour, and colour stability in red wines by enhancing phenol polymerisation (Zoecklein *et al.*, 1995a; Gómez-Plaza *et al.*, 2000). During cold maceration skins and seeds are permitted to soak for a period of 1 to 2 days at a temperature between 15° and 20°C prior to the initiation of fermentation. Colour extraction without the effects of ethanol on the grape cells is obtained. The must is usually pumped over once or twice daily to enhance the extraction (Boulton *et al.*, 1996b).

Another pre-fermentation technique is thermovinification that exploits heat to enhance colour extraction from skins. Thermovinification also destroys oxidases and ensures the oxidative stability of the wines. Grapes are crushed and generally destemmed. The mash is heated very rapidly (3 minutes) to approximately 70°C and kept at the temperature for 30 to 60 minutes. Thermally vinified wines are generally deeper in colour than wines fermented traditionally but it contains less anthocyanins and more polymeric compounds (Timberlake and Bridle, 1976b). It is important not to overextract and obtain wines with unacceptable bitter and astringent phenols (Boulton *et al.*, 1996b; Zoecklein *et al.*, 1995a).

Fermentation temperatures influence the type of winestyle. Winemakers generally use low fermentation temperatures (20°C to 25°C) to produce fresh, fruity, red aromatic wines (Zoecklein *et al.*, 1995a). Fermentation temperatures of approximately 30°C are optimal for the extraction and polymerisation of anthocyanins to obtain darker coloured wines (Gao *et al.*, 1997; Girard *et al.*, 2001).

2.7.4 PUMP-OVER OPERATION AND PRESSING

Enhanced colour and flavour extraction are obtained with the application of regular pump-over operations during red wine fermentation. These procedures involve the mixing of the skin cap formed with the juice once or twice a day by pushing the skins below the juice surface (punch-down) or by pumping the juice out and spraying it over the top of the skins (pump-over). Studies focussed on comparing different mixing methods have mostly found that the results depend on the grape variety. Generally in comparison with manual punch down treatments, mechanical punch down and pump over treatments significantly enhance the extraction and polymerisation of all phenolic compounds and produce wines with higher quality. Traditional pump over treatments tend to cause juice to infiltrate through gaps in the cap, leading to insufficient skin contact. To overcome this problem many wineries use sprinklers or splash plates (reviewed in Oberholster 2003; Sacchi *et al.*, 2005).

The timing of the separation of the pomace from the juice, as well as pressing, is a crucial step to ensure wines with an optimal phenolic balance. Anthocyanin extraction reaches a plateau after a few days but the extraction of phenols from skins and seeds can continue until separation takes place. An optimum balance between colour extraction and desirable phenol concentration must be obtained. Most winemakers base their timing of the separation and pressing on the sugar concentration or juice density (Boulton *et al.*, 1996b).

2.7.5 ENZYMES

The addition of enzymes during grape juice and wine processing is to increase juice yield and shorten maceration, clarification and filtration times (Muñoz *et al.*, 2004). Many trials have evaluated the potential of various enzymes to facilitate improved extraction of phenolic compounds, but contradictory results were reported. Some researchers reported

improved colour extraction with the use of commercial pectolytic and macerating enzyme preparations during vinification (Ough and Berg, 1974; Ough *et al.*, 1975; Shoseyov *et al.*, 1990; Zent and Inama, 1992). During another trial, after initial increased colour extraction, colour loss during storage occurred (Pardo *et al.*, 1999). Other researchers reported no significant colour differences between wines produced with or without the use of pectolytic enzymes (Haight and Gump, 1994). Enzyme manufacturers addressed these problems and nowadays manufacture more substrate specific enzymes. Improved wine colour, tannic structure, and quality of wines prepared with enzymes have since been reported (Guerrand and Gervais, 2002). However, the use of enzyme preparations still needs to be carefully considered since they can potentially alter wine composition, sensory properties and quality.

2.7.6 SULPHUR DIOXIDE

During vinification sulphur dioxide (SO_2) is primarily used for its antiseptic and antioxidative properties (Amerine *et al.*, 1980). The addition of SO_2 can result in temporary colour reduction. This is the result of a reversible reaction between the red flavylum ion and sulphite (HSO_3^-) which forms a colourless sulphite-anthocyanin complex. SO_2 decreases anthocyanin polymerization in wines (Dallas and Laureano, 1994; Bakker *et al.*, 1998). Once formed polymerized anthocyanins or tannin-anthocyanin polymers are resistant to decolourization by SO_2 because the physical site of sulphite binding is the same site at which polymerization reactions occur (Zoecklein *et al.*, 1995a). To obtain young red wines with good colour characteristics which will last during storage, addition of SO_2 at the moment of crushing is recommended (Gómez-Plaza *et al.*, 2002). SO_2 protects wines against excessive browning during maturation (Bakker *et al.*, 1998).

2.7.7 FINING TREATMENTS

Fining refers to the addition of adsorptive compounds to wine followed by the settling or precipitation of partially soluble compounds to remove or reduce the concentration of one or more undesirable compound (Zoecklein *et al.*, 1995b; Boulton *et al.*, 1996c). Reports exist that fining agents such as polyvinylpyrrolidone (PVPP), gelatin, or bentonite reduces anthocyanin and total phenolics concentrations and therefore alter the colour and sensory characteristics of wines (Sims *et al.*, 1995; Sarni-Manchado *et al.*, 1999; Gómez-Plaza *et al.*, 2000; Maury *et al.*, 2001). The sensory ratings of fined wines are very similar to those of unfined wines, but fined wines score better at taste than unfined wines (Castillo-Sánchez *et al.*, 2005).

In general PVPP binds and removes smaller molecular weight phenolic compounds while gelatin removes larger molecular weight phenolics (Sims *et al.*, 1995). Bentonite is used to reduce the protein concentration of wines but it also absorbs polyphenoloxidase, phenols, and other positively charged molecules (Main and Morris, 1991). Addition of bentonite and gelatin produces wines with lower colour, ionised anthocyanin and polymeric

compounds compared with PVPP-treated and untreated wines (Gil-Muñoz *et al.*, 1997; Gómez-Plaza *et al.*, 2000; Gómez-Plaza *et al.*, 2002). Presumably PVPP prevents the fixation and precipitation of anthocyanins with yeasts and solid parts that are removed following fermentation. The most suitable fining agent for young red wines which results in low reduction in colour seems to be PVPP (Gómez-Plaza *et al.*, 2002).

2.8 ANALYTICAL TECHNIQUES FOR THE QUANTIFICATION OF PHENOLIC COMPOUNDS (RELATED TO COLOUR) IN GRAPE JUICE, MUST AND WINE

Experimental procedures for the isolation and identification of individual red wine phenolic compounds are mostly complicated, time consuming and costly. The relatively low concentrations of these compounds in complex wine mediums and their structural diversity are both hindering factors (Håkansson *et al.*, 2003). The next sections present a brief overview of the theories behind different analytical methods for phenolics analyses. Detailed descriptions of the procedures are not discussed.

2.8.1 ABSORBANCE MEASUREMENTS

2.8.1.1 Spectrophotometric indices

Absorbance measurements are simple techniques to measure the colour of grape extracts and wine. Several forms of absorbance indexes exist. Sudraud (1958) described methods which use optical absorbancies at 420 and 520 nm for the calculation of colour density and hue of wine. The Office Internationale de la Vigne et du Vin (OIV) described a colour index where a wine's colour strength is the summation of the absorbancies measured at 420, 520 and 620 nm respectively (OIV, 1978). Along with the measures of Sudraud (1958), Somers and Evans (1977) also published other measures defining wine colour in natural conditions:

- Wine colour density (Absorbance units) = $\text{Absorbance}_{420\text{nm}}^* + \text{Absorbance}_{520\text{nm}}^{**}$
- Wine colour hue = $\text{Absorbance}_{420\text{nm}} / \text{Absorbance}_{520\text{nm}}$
- Total red pigments (Absorbance units) = $\text{Absorbance}_{520\text{nm}}$
- Total phenolics (Absorbance units) = $\text{Absorbance}_{280\text{nm}}^{***} - 4$

*Absorbance measurement at 420 nm provides an estimate of the concentration of yellow brown pigments present in wine.

**Absorbance measurement at 520 nm provides an estimate of the concentration of anthocyanins and other red coloured compounds present at natural wine pH conditions.

***Absorbance measurement at 280 nm provides an estimate of the phenolic concentration of wine.

Different pH and SO₂ levels influence the anthocyanin colour equilibrium (Dallas and Laureano, 1994; Bakker *et al.*, 1998). Comparison of absorbance measurements of different wines should be made under conditions of uniform pH and SO₂ to eliminate the

effects of these variables. Descriptions of these adjusted measurements are given by Iland *et al.* (2000b).

2.8.1.2 Folin-Ciocalteu value

The Folin-Ciocalteu value gives an estimation of the total phenolics present in wine. This method depends on an oxidation-reduction reaction and is based on the reactivity and reductive properties of the phenolic functional group. The number of –OH groups present in a sample are measured, assuming that light absorption increases when more –OH groups are present. This assessment uses oxidising agents, potassium permanganate and Folin-Ciocalteu reagent (a mixture of molybdenum and tungsten salts). The first reaction produces a yellow solution in the presence of indigo blue. The second reaction is characterized by a blue colouring, the absorption measured with a spectrophotometer at 760 nm (Ribéreau-Gayon *et al.*, 2000).

$$\text{Folin-Ciocalteu value or } I_{FC}^* = (\text{Absorbance}_{760\text{nm}} \times \text{dilution}) \times 20$$

* The value is between 10 and 100

2.8.1.3 OD280 value

The advantages of measuring the absorbance at 280 nm rather than doing the Folin-Ciocalteu test include time and repeatability. There are however certain molecules, such as cinnamic acids and chalcones, which have no absorption maximum at this wavelength. This does not pose a problem because these molecules are present in wine at very low concentrations and any error in the value will be very small and can be ignored. Red wine is diluted 1/100 and white wine 1/10 with distilled water. The absorbance is measured at 280 nm in a 10 mm optical path (Ribéreau-Gayon *et al.*, 2000).

$$\text{OD280 value or } I_{280}^* = \text{Absorbance}_{280\text{nm}} \times \text{dilution}$$

* The value is between 6 and 120

2.8.1.4 The Iland method for total anthocyanins and total phenolics in wine

A combined method for the determination of total anthocyanins and total phenolics in wine is described by Iland *et al.* (2000b). A wine sample is diluted 1:100 with 1 M HCl. Three hours after the dilution step spectral measures at 520 nm and 280 nm are recorded and if necessary readings converted to what it would be if measured in a 10 mm cell. The dilution factor is also taken into account during the calculations. Total red anthocyanins are expressed as absorbance units at 520 nm, whereas total phenolics are expressed as absorbance units at 280 nm.

2.8.1.5 The Iland method for total anthocyanins and total phenolics of grapes

Iland *et al.* (1996 and 2000a) also describe a method for the estimation of red anthocyanins and total phenolics of grapes. These parameters have been used by Australian and South African researchers in particular (Marais *et al.*, 2001; Damberg *et al.*

al., 2003; Cozzolino *et al.*, 2004a; Cynkar *et al.*, 2004; Marais and October, 2005). A 1 g fraction of homogenised grapes are extracted with 50% ethanol (pH 2) for one hour. The extract is centrifuged and diluted 1:10 with 1 M HCl. After three hours the absorbance values of the diluted HCl extract solution are recorded at 700 nm, 520 nm and 280 nm in a 10 mm cell. Red anthocyanins (expressed as mg anthocyanins per g berry) are calculated from the absorbance measurement at 520 nm. Total phenolics (expressed as absorbance units per g berry weight) are calculated from the measurement of absorbance at 280 nm. The measure of absorbance at 700 nm is a check for sample turbidity.

2.8.1.6 Glories method for measuring phenolic maturity of grapes

Glories described a method for measuring phenolic maturity in grapes (Glories and Augustin, 1993). The basic theory behind this method is the rapid extraction of anthocyanins from grape skins under first moderate acidic (pH 3.2 where the degree of extraction is comparable to that occurring during wine fermentation) and eventually extreme acidic conditions (pH 1 where all the anthocyanins are extracted and dissolved). In the extreme acidic medium the protein bonds of the proteophospholipid membrane are broken and the contents of the vacuoles released. The difference between the absorbance measures at 280 nm and 520 nm of the two different pH solutions reflects the tannin and anthocyanin extraction potential. This extractibility indicates the grape maturity level.

2.8.2 CHROMATOGRAPHIC METHODS

High performance liquid chromatography (HPLC) is a very sensitive chromatographic technique used in many research projects for the classification and quantification of phenolic compounds (Bakker *et al.*, 1986; Prieur *et al.*, 1994; Souquet *et al.*, 1996; Richey and Waterhouse, 1999). HPLC combined with a photo diode-array detector (DAD) allows for the simultaneous separation, identification and quantification of different anthocyanins in grapes and wines on the basis of their elution orders and spectral characteristics. The DAD permits the recording of the chromatographic analysis at several wavelengths and the visible-ultraviolet spectra of the different anthocyanins separated by HPLC at the same time (Hebrero *et al.*, 1988). Anthocyanins can be characterised according to their spectral properties (Table 3) (Hebrero *et al.*, 1988). Absorbance data collected at 313 nm and 365 nm are indicative of hydroxycinnamic acid derivatives and flavonols respectively. Although procedures can easily be automated the running time per sample is long, ranging from approximately 30 – 60 minutes or longer (Lamuela-Raventós and Waterhouse, 1994).

Reversed-phase HPLC (RP-HPLC) separates small phenolic compounds and individual anthocyanins can be quantified (Waterhouse *et al.*, 1999). RP-HPLC utilises a nonpolar stationary phase and a relatively polar mobile phase. More polar molecules elude before less polar molecules (Skoog *et al.*, 1998b). Normal-phase HPLC (NP-HPLC) is used to quantify the polymeric phenolics of wine and is adequate when only a total measure of monomeric anthocyanins is needed (Rigaud *et al.*, 1993). Proanthocyanidins

up to oligomers can be separated (Rigaud *et al.*, 1993). NP-HPLC employs a highly polar stationary phase and a relatively nonpolar solvent. Less polar molecules elute first while more polar molecules elute last (Skoog *et al.*, 1998b).

Table 3. Spectral characteristics of isolated anthocyanins from *Vitis vinifera* grapes in methanol, 0.01% HCl (adapted from Wulf and Nagel, 1978; Piergiovanni and Volonterio, 1980; Hebrero *et al.*, 1988).

Anthocyanin	λ_{\max} visible (nm)	λ_{\max} ultraviolet (nm)
Delphinidin-3-monoglucoside	541-542	278-279
Cyanidin-3-monoglucoside	530	282
Petunidin-3-monoglucoside	540	277-280
Peonidin-3-monoglucoside	528	280-281
Malvidin-3-monoglucoside	537-538	278-279

2.8.3 XYZ TRISTIMULUS VALUES

The Commission International de l'Eclairage (CIE) is an international organisation that develops and furthers methods and standards concerning light and colour (Datacolor, 2004a). In 1931 the CIE developed a colour space (CIE tristimulus system) based on data from colorimeters, instruments that can accurately measure the wavelengths of light (Computerworld, 2002). Since 1962 the official method of the Office Internationale de la Vigne et du Vin (OIV) has been based upon these concepts, which have been applied in attempts to standardise wine colour (Somers and Evans, 1977).

CIE tristimulus values X, Y, and Z, are coordinates of colour sensation, and form the foundation of the CIE colour space. The CIE XYZ tristimulus values is based on the three-component theory of colour vision, which states that the human eye/brain processes sensors (cones) for three primary colours: red, green and blue. All colours are seen as combinations of these primary colours (Datacolor, 2004a). These XYZ tristimulus values are calculated from transmittance values measured at wavelengths over the whole visible spectrum in specific conditions, using a spectrophotometer (Pérez-Magariño and González-Sanjosed, 2003). The XYZ tristimulus values are useful for defining colour, but the results are not easily visualised. This is because the XYZ colour space does not match perceived colour differences, nor does it describe brightness, the perceived amount of white in a colour (Computerworld, 2002).

2.8.4 CIELAB COLOUR SPACE

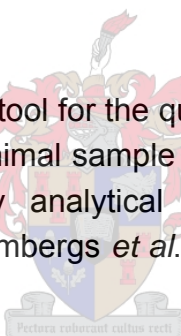
In 1976 the CIE adopted a new colour space, the CIELAB space. The CIELAB space is a mathematical transformation of the 1931 CIE tristimulus system. The 1976 CIELAB system organises colours so that numeric differences between colours agrees consistently well with visual perceptions (Datacolor, 2004b). The new colour space also includes brightness

measurements (Computerworld, 2002). The CIELAB space is a uniform three dimensional colour space defined by the colorimetric coordinates L^* , a^* and b^* (CIE, 1986). The vertical axis L^* is a measure of lightness, from totally opaque (0) to totally transparent (100). On the hue-circle, a^* is a measure of redness (or $-a^*$ of greenness) and b^* of yellowness (or $-b^*$ of blueness). The values of a^* and b^* vary between -60 and +60 (Carreño *et al.*, 1995; Gil-Muñoz *et al.*, 1997). Hue angle (H) and chroma (C) values are calculated from L^* , a^* and b^* coordinates as $H = \arctan b^*/a^*$ (degrees) and $C = [(a^*)^2 + (b^*)^2]^{0.5}$ (Carreño *et al.*, 1995).

The CIELAB colour system is widely used to evaluate food colours. Several researchers have used this system during research on grape and wine colour (Almela *et al.*, 1995; Carreño *et al.*, 1995; Carreño *et al.*, 1997; Negueruela, *et al.*, 1995; Ayala *et al.*, 1999). CIELAB measurements normally require advanced, costly spectrophotometers. Simplified methods to calculate tristimulus values for wines and brandies from absorbance measures at only a few wavelengths have been proposed (Ayala *et al.*, 1999; Pérez-Magariño and González-Sanjosé, 2003). Predicting CIELAB parameters from simple absorbance measures is more cost effective because inexpensive spectrophotometers can be used.

2.8.5 INFRARED SPECTROSCOPY

Infrared spectrometry is a resourceful tool for the qualitative and quantitative determination of molecular compounds. None to minimal sample preparation is required, which makes it a fast and environmentally friendly analytical technique to use in the laboratory environment (Skoog *et al.*, 1998a; Dambergs *et al.*, 2003; Palma and Barroso, 2002; Patz *et al.*, 2004).



2.8.5.1 Theory

The infrared region of the light spectrum ranges from $12\,800\text{ cm}^{-1}$ to 10 cm^{-1} wavenumbers or $0.78\text{ }\mu\text{m}$ to $1000\text{ }\mu\text{m}$ wavelengths. For the convenience of both application and instrumentation the infrared spectrum is divided into near-, mid-, and far-infrared radiation (Skoog *et al.*, 1998a) (Table 4). These margins are not clearly defined and differ between references. Infrared spectroscopy involves the observation of absorption in the mid-infrared region of the light spectrum (Williams and Norris, 2001).

Table 4. The infrared spectral regions (adapted from Skoog *et al.*, 1998a).

Region	Wavelength range (μm)	Wavenumber range (cm^{-1})	Frequency range (Hz)
Near	0.78 to 2.5	12 800 to 4000	3.8×10^{14} to 1.2×10^{14}
Middle	2.5 to 50	4000 to 200	1.2×10^{14} to 6.0×10^{12}
Far	50 to 1000	200 to 10	6.0×10^{12} to 3.0×10^{11}
Most used	2.5 to 15	4000 to 670	1.2×10^{14} to 2.0×10^{13}

Organic compounds have chemical bonds which vibrate or rotate when exposed to characteristic wavelengths of infrared radiation. These vibrations are the outcome of energy absorption at specific wavelengths in the IR region. The forms of vibration include *stretching*, *bending*, *twisting* and *rotating* (Skoog *et al.*, 1998a). For different chemical groups the absorbent wavelengths and natural frequency of vibration are unique and depend on the bond itself (C-C, C-O, O-H, etc) and on the molecular matrix. According to the Beer-Lambert law during absorption of infrared radiation the amplitude of the vibrational frequencies increases and is directly proportional to the concentration of the molecule in question (Skoog *et al.*, 1998a). The basic measurement obtained in infrared spectroscopy is an infrared spectrum. This is a plot of absorbance (or transmittance) vs. wavenumber calculated with the aid of complex mathematical procedures from the intensities of measured frequencies of vibration (Smith, 1999). The infrared spectrum of an organic matrix therefore contains valuable information regarding the structure and concentration of chemical functional groups within a sample.

2.8.5.2 Instrumentation

During the last decade more sophisticated and relatively inexpensive instruments for the mid-infrared region have been developed. The three types of commercially available instruments are: (1) dispersive grating spectrophotometers; (2) multiplex instruments; and (3) nondispersive photometers. More traditional infrared instruments have filters that restrict absorption measurements to a limited number of wavelengths which limit applications (Skoog *et al.*, 1998a). All infrared instruments need a source of uninterrupted infrared radiation and a sensitive infrared transducer. Sources of infrared radiation include Nernst Glowers, Globars, incandescent wires, mercury arcs, tungsten filament lamps and carbon dioxide lasers (Skoog *et al.*, 1998a). Infrared transducers include thermal transducers, pyroelectric transducers and photoconducting transducers. In the most recent infrared instruments Michelson interferometers are used to modulate all the typical frequencies of the infrared electromagnetic region (Skoog *et al.*, 1998a). Infrared spectrometers are based on the principle of interferometry. Interferometry exploits the wave nature of light and involves the splitting of a beam of light into two and then passing both through the sample being analysed before impressing them on an infrared detector creating a pattern called an interferogram (Skoog *et al.*, 1998a).

The Fourier Transform Infrared Spectrometer (FT-IR) is a type of multiplex instrument that collects every component of the full electromagnetic spectrum simultaneously at a detector (Skoog *et al.*, 1998a). This wave-like pattern is collected in less than one second and contains all the frequencies that make up the infrared spectrum (Pavia *et al.*, 2001). To extract information regarding the concentration of each component in the original spectrum produced on a multiplex instrument, the information must be decoded into its individual components. An infrared spectrum is established through decoding that predominantly utilises the classic mathematical tool known as Fourier transformation. The

total analysis time from spectrum collection to spectrum processing with Fourier transformation takes about 30 seconds (Foss Analytical, Denmark; Skoog *et al.*, 1998a).

2.8.5.3 Chemometrics

Infrared spectroscopy (IR) does not involve a direct measurement of chemical compounds. Absorbance measured by an IR-instrument must be transformed into a mathematical equation or a calibration model in order to predict the concentration of an analyte of interest. A calibration or training set of samples is analysed by standard laboratory methods to obtain reference values and the samples are also scanned by an infrared spectrometer. During the calibration process the information in the spectrum created by the IR instrument is correlated to the absolute values of the compounds measured with the standard laboratory method (Williams and Norris, 2001). Multivariate statistical procedures are used for developing calibration models from spectroscopic data. Principal component analyses (PCA) and partial least squares analyses (PLS) are specialized techniques often used during multivariate statistical procedures. Principal component analysis (PCA) is a data reduction or compression tool which allows the visualisation of interrelationships between different variables, the detection and interpretation of sample patterns, groupings, similarities or differences (Esbensen, 2002; Naes *et al.*, 2002). PLS is a bilinear regression modelling method where original x variables are projected onto a smaller number of factors or PLS components. Bilinear modelling methods are used for situations where collinearity exists among the original variables. The PLS algorithm is used to correlate IR spectra and analytical reference results obtained (Esbensen, 2002).

2.8.5.4 Applications of infrared spectroscopy for grape and wine analyses

In recent years applications of infrared spectroscopy in various chemical fields including the pharmaceutical, food and textile industries have escalated at a staggering rate. Applications in viticulture and oenology include analyses for parameters such as sugar, pH, alcohol, total acidity, glucose/fructose, malic acid, lactic acid, total phenols, acetic acid, tartaric acid, CO₂, glycerol, gluconic acid, sucrose, free amino nitrogen and ethyl carbamate (Gishen and Holdstock, 2000; Manley *et al.*, 2001; Kupina and Shrikhande, 2003; Patz *et al.*, 2004; Urbano-Cuadrado *et al.*, 2005). In general the technique has shown good prediction and repeatability for most of the parameters.

Near infrared spectroscopy (NIR) has been used for grape colour analyses (measured as total anthocyanins) (Gishen *et al.*, 2002; Dambergs *et al.*, 2003). Researchers also report positive results for the determination of phenolic concentrations in red wines with infrared spectroscopy (Cozzolino *et al.*, 2004b; Versari *et al.*, 2004; Grandjean *et al.*, 2005). An automated flow injection system with FT-IR spectroscopic detection has successfully been utilized to study the interaction of polyphenols (tannins) with proline-rich proteins (gelatine) (Edelmann and Lendl, 2002). Schneider *et al.* (2004) showed that FT-IR is a potential tool for the quantification of glycosidic precursors, the non-volatile, non-odorant compounds partly responsible for the varietal aroma of most wines from non-

aromatic grapes. Infrared spectroscopy together with chemometrics is also a potential technique for the discrimination of cultivars and origin of wines (Cozzolino *et al.*, 2003; Edelmann *et al.*, 2001; Roussel *et al.*, 2003) and distilled drinks (Palma and Barroso, 2002). There is a field of organic chemistry specifically interested in both the identification of organic compounds and structure clarification of unknown compounds by their IR spectra selected from IR spectral libraries or databases (Derendyaev *et al.*, 2004). For the successful application and implementation of this analytical tool knowledge about the theoretical principles of infrared spectroscopy is essential.

2.9 CONCLUSIONS

Researchers, viticulturists and winemakers are continually searching for accurate measures of grape and wine quality. It has been established that the anthocyanins and total phenolics concentrations in grapes are positively linked to final wine quality. The inclusion of these two parameters as part of grape quality control could provide important information regarding the quality and colour of grapes and wine. Analytical measures for the identification and quantification of anthocyanins and total phenolics concentrations in grapes and wines are mostly complex, time consuming and costly. Infrared spectroscopy is a simple, rapid, and accurate analytical technique fit for routine grapes and wine analysis. Improvements in infrared instrumentation such as the development of the WineScan FT 120 spectrometer have made this sophisticated technology accessible to the wine industry.

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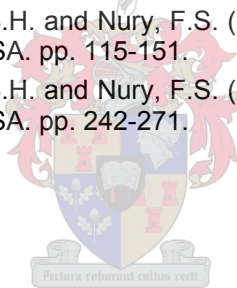
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RESEARCH RESULTS

The evaluation of **Fourier transform infrared spectroscopy (FT-IR)** for the quantification of **total anthocyanins and total phenolics of grapes**

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RESEARCH RESULTS

ABSTRACT

Fourier transform infrared spectroscopy (FT-IR) was evaluated as a rapid analytical method to measure the total anthocyanins and total phenolics concentration of red grapes. The large-scale investigation of 827 grape samples from 2004 and 2005 vintages was aimed at developing calibration models for the prediction of total anthocyanins and phenolics concentrations using the WineScan FT 120 instrument. The calibration set design aimed at evaluating the influence of vintage, sugar concentrations and *Vitis vinifera* cultivar (Cabernet Sauvignon, Merlot, Pinotage, Shiraz) on the prediction accuracy of calibration models. The calibration statistics obtained for the 2004 and 2005 (15.5-31.5°Brix) anthocyanin calibration set were $SEP = 0.17$ mg/g and $R^2 = 0.71$ and for the 2005 (15.5-31.5°Brix) calibration set $SEP = 0.15$ mg/g and $R^2 = 0.77$. Improved calibration statistics were obtained by using only samples with sugar concentrations equal to or higher than 23.5°Brix ($SEP = 0.13$ mg/g; R^2 validation set = 0.77). The calibration statistics obtained for the 2004 and 2005 total phenolics 2004 and 2005 (15.5-31.5°Brix) calibration set were $SEP = 0.12$ OD280/g and $R^2 = 0.68$ and for the 2005 (15.5-31.5°Brix) calibration set $SEP = 0.12$ OD280/g and $R^2 = 0.71$. More satisfactory prediction accuracy was obtained for a calibration set including 2005 vintage grapes with sugar concentrations lower than 23.5°Brix ($SEP = 0.13$ OD280/g; R^2 validation set = 0.74). For the cultivar calibration sets the best calibration statistics were obtained for 2005 Merlot grapes for the prediction of both anthocyanins ($SEP = 0.12$ mg/g; R^2 validation set = 0.84) and total phenolics ($SEP = 0.10$ OD280/g; R^2 validation set = 0.76). Principal component analysis (PCA) of grape homogenate spectra revealed clustering according to vintage (2004 and 2005), sugar concentration and pH. Inter-vintage (2004 and 2005) and intra- vintage (2005) cluster patterns were observed. The main variation between 2004 and 2005 grape samples could be ascribed to water content. Clustering within the 2005 vintage could not be interpreted on the basis of cultivar differences and must be investigated in future studies. PCA also lead to the identification of both outlier samples (samples with atypical spectra) and samples with unacceptable high errors of prediction.

3.1 INTRODUCTION

Grape quality assessment is a complex issue. The objective evaluation of grape quality and identification of characteristics that closely correlate with the perceived quality of the grapes and eventually the wine are focus areas for major wine producing countries worldwide (Gishen *et al.*, 2002; Francis *et al.*, 2004). Broad indicators of grape quality assessed in vineyards include balance between crop load and canopy capacity, evenness of ripening, vigour/no growing tips after véraison, exposure/sunburn, disease, and observable berry characteristics (size, seeds, colour, taste). Specific indicators of grape quality include chemical analyses of total soluble solids (mostly sugars, measured as

°Brix), pH and titratable acidity (Francis *et al.*, 2004). Phenolic compounds are associated with sensorial characteristics such as colour, flavour, astringency, bitterness, and hardness of red wines (Somers, 1971; Noble, 1994; Gawel, 1998). Wine colour intensity is considered an important indicator of wine quality and is highly correlated with the anthocyanin content of grapes and young wines (Somers and Evans, 1974; Jackson *et al.*, 1978; Iland, 1987; Marais *et al.*, 2001; Marais and October, 2005). Anthocyanins are the phenolic compounds responsible for the red colour of grapes and wines (Singleton and Esau, 1969). The determination of the total phenolic and anthocyanin concentration of grapes could allow better evaluation of vineyard management practices and support decisions regarding optimum harvest dates, grape quality classification, the identification of grape suitability for particular wine styles, streaming of grape juice inside the cellar and, more exact grape payment schemes (Damberg *et al.*, 2003).

Analytical techniques for the quantification of anthocyanins and total phenolics of grapes and wine include spectrophotometric methods (Somers and Evans, 1977; Iland *et al.*, 2000), Glories' method (Glories and Augustine, 1993), the Folin Ciocalteu method (Singleton and Rossi, 1965), and high performance liquid chromatography (HPLC) (Bakker *et al.*, 1986; Rigaud *et al.*, 1993). These reference methods can be time consuming, laborious, costly and generate large quantities of chemical waste. There is clearly a need for simple, rapid and cost effective analytical techniques at commercial wineries for the determination of anthocyanins and total phenolics in grapes and wine.

Infrared spectroscopy is an indirect analytical technique that requires minimum sample preparation and measures multiple compounds simultaneously within a few seconds (Skoog *et al.*, 1998). The technology is based on the measurement of frequencies of molecular vibrations upon the absorption of radiation in the infrared (IR) region of the electromagnetic spectrum. The near infrared region is defined by some sources as ranging from 12 800 to 4000 cm^{-1} and mid infrared region as ranging from 4000 to 200 cm^{-1} (Skoog *et al.*, 1998). Many of the vibrations can be assigned to specific bonds or groupings, such as the C-C, C-H, C=O, O-H and N-H groups (Williams and Norris, 2001a). In order to predict the concentration of a compound of interest a calibration model must first be developed. A calibration or training set of samples is analysed by standard laboratory methods to obtain reference values and the same samples are scanned by an infrared spectrometer. Spectral data are correlated with reference data using multivariate data analysis techniques and the constructed calibration equation is used to predict analytical results from the infrared spectra of new samples (Esbensen *et al.*, 2002).

Purpose built infrared spectrometers and the development of ready to use commercially available calibrations enabled the development of many applications for grape and wine analysis. These applications include the quantification of ethanol, reducing sugars, pH, titratable acidity, tartaric acid, malic acid, lactic acid, volatile acidity, acetic acid, gluconic acid, glucose, fructose, glycerol, sorbic acid and Folin C index (Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003; Patz *et al.*, 2004). Near infrared spectroscopy (NIRS) calibration models have been developed for the prediction of anthocyanins in grape homogenates (Damberg *et al.*, 2003) as well as for the prediction

of phenolic compounds in red wines (Cozzolino *et al.*, 2004). During a small-scale investigation using 20 samples Fourier transform infrared spectroscopy (FT-IR) calibration models have been developed for the quantification of anthocyanins in red wines (Versari *et al.*, 2004).

The aim of this study was to develop FT-IR calibration models for the quantification of total anthocyanins and total phenolics in grapes using a WineScan FT 120 instrument. In South Africa the Foss WineScan FT 120 spectrometer (Foss Analytical, Denmark) is widely used in routine wine analytical laboratories and it would be beneficial for the South African wine industry if the instrument could be calibrated for the quantification of anthocyanins and total phenolics concentrations of grapes. The ease of calibration model transfer between instruments for future widescale implementation in the South African wine industry was an important consideration for using of this technology and in particular the instrument, for this study. This project was the first large scale investigation of the distribution of anthocyanins and total phenolics in South African grapes and forms part of a larger program on establishing an objective system for grape quality assessment and control.

3.2 MATERIALS AND METHODS

3.2.1 GRAPE SAMPLING, STORAGE AND SAMPLE PREPARATION

Whole bunch samples of *Vitis vinifera* grape cultivars Cabernet Sauvignon, Merlot, Pinotage and Shiraz were collected at Distell (Distell Group (Pty) Ltd) during the 2004 (n = 120) and 2005 (n = 727) harvest seasons. The number of samples analysed during the 2004 vintage are lower than that analysed during 2005 due to the time required to optimise the laboratory methods during the 2004 vintage (see sections 3.3.1.1 to 3.3.1.4 and Chapter 4). All grape samples originated from the Western Cape region in South Africa. Cultivar sample numbers are not evenly distributed. No Pinotage grapes were analysed during the 2004 harvest season.

In 2004 grapes (mean \pm SD °Brix = 24.3 \pm 1.5) were sampled from loads delivered at the respective weighbridges of the Adam Tas and Bergkelder production facilities of Distell, Stellenbosch. In 2005 grapes were sampled in the vineyards as well as from loads delivered at the respective weighbridges. Vineyards samples were collected from the early stages of grape maturity until harvest (15-31°Brix). Each sample consisted of approximately 5 to 10 bunches of grapes. Depending on available storage space whole bunch samples were stored overnight at 0 or 4°C. On the day of analysis grape berries were picked from the various bunches within a sample and the weight of 100 berries from each sample was recorded. A colloid mill designed for homogenising soil samples was used to homogenise grape berries into a uniformly smooth textured paste within approximately 5 seconds.

3.2.2 LABORATORY ANALYSES

3.2.2.1 Total anthocyanins and total phenolics analyses

Total anthocyanins and total phenolic concentrations of grape homogenates were determined in duplicate according to the method described by Iland *et al.* (2000). After thorough mixing approximately 2 g of homogenate was transferred into a pre-tared test tube and the mass recorded. Aqueous ethanol (20 mL, 50% v/v) was added to the homogenate and the contents mixed on a rotating wheel for 1 hour at room temperature (22°C). After one hour the contents of the test tube was transferred to a centrifuge tube and centrifuged at 10 000 rpm for 10 minutes. A 1 mL volume of extract (supernatant) was acidified with 1 M HCl. Absorbance at 520 nm (A_{520}) and 280 nm (A_{280}) was measured after 3 hours, using a UV/visible spectrophotometer (Helios, Thermo Spectronic, England). The spectrophotometer was zeroed with distilled water prior to analysis. The total anthocyanin (expressed as mg of malvidin-3-glucoside per g berry) and total phenolic concentrations (expressed as absorbance units per g berry) were calculated as described by Iland *et al.* (2000). The calculation of total anthocyanins concentration from A_{520} utilizes a generic extinction coefficient for malvidin-3-glucoside (Somers and Evans, 1977; Iland *et al.*, 2000). The concentrations of total anthocyanins and total phenolics for a specific grape sample were calculated as the mean of duplicate grape homogenates analysed (see section 3.2.3.1, equation 1).

3.2.2.2 The evaluation of the effects of the use of filterpapers with different cross-sections on Fourier transform infrared (FT-IR) spectral measurements

The possible influence of the use of filterpapers with different cross-sections on grape homogenate analyses results obtained with the Foss WineScan FT 120 instrument were evaluated. In the Distell routine laboratory there are many filter-units which have cross-sections of 50 mm. The filter units prescribed by Foss have cross-sections of 185 and 240 mm but the Distell routine laboratory has only one of these filter units each. This means that a lot of time will be wasted cleaning these bigger filter units in between filtering samples. It is more practical to use the 50 mm cross-section filter-units because no cleaning of filter-units is necessary because there are enough filter-units in the laboratory. During the evaluation six grape homogenate samples were each split into three volumes and then filtered with the 50, 185 and 240 mm filter-units respectively. The sugar (°Brix), pH and titratable acidity (TA) for each sample were determined with the Foss WineScan FT 120 instrument (Foss Analytical, Denmark). Analyses results for each of the three treatments for the six samples were statistically analysed.

3.2.2.3 Fourier transform infrared (FT-IR) spectral measurements

Directly after homogenisation grape samples were centrifuged and filtered using filter paper circles graded at 20-25 μm with a diameter of 50 mm (Schleicher & Schuell, reference number 10312706). A WineScan FT 120 spectrometer (Foss Analytical, Denmark) fitted with a Michelson interferometer was used to generate Fourier transform

infrared (FT-IR) spectra. The number of scans generated per sample and the processing of the spectra are predetermined by the manufacturer and can not be changed by the user. Samples are pumped through a CaF₂-lined cuvette (37 μm path length) which is located in the heater unit of the instrument. The temperature of the samples is brought to 40°C before analysis. Samples are scanned from 5011–929 cm⁻¹ and transmittance recorded at the detector at 4 cm⁻¹ intervals, thereby collecting 1056 data points per spectrum. An interferogram is generated from 20 scans before being processed by Fourier transformation and corrected for background absorbance to generate a single beam transmittance spectrum. The ratio between the single beam transmittance spectrum of Zero liquid (Product number S-6060, Foss WineScan FT 120 Type 77110 and 77310 Operator's Manual, Foss Analytical, Denmark, 2001) and that of the sample, at each recorded data point, is used to generate the final transmittance spectrum for each sample. Two transmittance spectra are generated for each sample in order to monitor the absolute repeatability of the spectral measurements. Finally the transmittance spectra are converted into linearised absorbance spectra through a series of mathematical procedures (Foss WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001).

The WineScan instrument was cleaned prior to and between every sample using the S-470 Cleaning Agent Solution (Foss WineScan FT 120 Type 77110 and 77310 Operator's Manual, Foss Analytical, Denmark, 2001). Zero Liquid was scanned prior to filtered grape homogenates as well as at regular intervals during sample scanning to correct for background absorbance.

3.2.2.4 Determination of °Brix, pH and titratable acidity

The commercial calibration models provided with the WineScan were used to predict the °Brix, pH and total acid (in this study referred to as titratable acidity) of filtered grape homogenates scanned (Foss Analytical, Denmark, 2001). Table 1 shows the component ranges of samples used to establish the commercial calibrations.

3.2.3 STATISTICAL ANALYSES

The notation used for calibration statistics is described by Naes *et al.* (2002).

3.2.3.1 Statistical equations used for the processing of analytical data

Mean values and standard deviations for °Brix, pH and titratable acidity for grape homogenate sample sets were calculated according to equations 1 and 2, respectively. The standard error of laboratory (*SEL*) of the reference method was calculated according to equation 3.

Table 1. Component ranges of samples used to develop commercial calibrations supplied with the WineScan (adapted from Foss application notes, Grapescan Calibrations: Must – Brix; Must – pH; Must – Total acid).

Component	Commercial calibration		
	Must – Brix* ¹	Must – pH* ²	Must - Total acid* ³
	Range* ⁴	Range* ⁴	Range* ⁴
°Brix	8.34 - 23.10	8.41 - 23.70	13.32 - 23.70
pH	2.62 - 3.70	2.64 - 4.07	2.62 - 4.19
Titrateable acidity ^e	2.40 - 17.60	1.60 - 13.58	1.60 - 12.99
Tartaric acid (g/L)	3.09 - 11.00	2.10 - 12.02	2.00 - 11.70
Malic acid (g/L)	0.19 - 4.17	0.50 - 12.40	0.50 - 10.80

- *¹ Application note GrapeScan Calibrations, Must-Brix
 *² Application note GrapeScan Calibrations, Must-pH
 *³ Application note GrapeScan Calibrations, Must-Total acid
 *⁴ As determined by the reference method
 *⁵ Expressed as g/L tartaric acid

Mean

The mean is the average value of a variable in a specific sample set. The mean (\bar{x}) is calculated as the sum of the variable values (x_i), divided by the number of samples (n) (Snedecor and Cochran, 1980).

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

.....1

Standard deviation (SD)

The standard deviation (SD) is a measure of how widely values are spread around the mean. This corresponds to the range covered by the measured n samples in the sample set (Snedecor and Cochran, 1980).

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

.....2

Standard error of laboratory (SEL)

The standard error of laboratory (SEL) gives an indication of the accuracy of the reference method (Snedecor and Cochran, 1980).

$$SEL = \sqrt{\frac{\sum_{i=1}^n (x_{1i} - x_{2i})^2}{2n}}$$

.....3

Where x_{1i} and x_{2i} are the results of duplicate determinations and n is the number of samples.

3.2.3.2 One-way analysis of variance (ANOVA) and post-hoc analysis

One-way analysis of variance (ANOVA) and post-hoc analyses (Bonferroni tests) were performed to statistically interpret differences in result means (Statistica version 7.0, Tulsa, OK, U.S.A.). When multiple comparisons are done (as in this study) the reported probability levels can overestimate the statistical significance of mean differences and the Bonferroni test was used to make the necessary adjustments. Results were reported at the 5% significance level.

3.2.3.3 Principal component analysis (PCA)

Principal component analysis (PCA) is a data reduction method that allows visualisation of the information in a data set in a space defined by orthogonal principal components (PC's) which are therefore uncorrelated to each other (Naes *et al.*, 2002). PCA score plots allow the visualisation of interrelationships between different variables, the detection and interpretation of sample patterns, groupings, similarities or differences. Loadings plots are useful to understand how much each variable contributes to the meaningful variation in the data, and to interpret variable relationships (Esbensen, 2002; Naes *et al.*, 2002). For PCA FT-IR spectra were exported from the WineScan instrument to Microsoft Office Excel software (Microsoft Office Excel 2003) and subsequently to The Unscrambler Software (version 9.2, Camo AS, Norway). Duplicate FT-IR spectra were averaged. In this way a 2-dimensional data table was constructed where columns are defined by variables and rows defined by samples. The variables were scaled by multiplying each original absorbance value by the factor $1/SD$ (SD , standard deviation). The data set was mean centered which refers to the process where the origin of the PCA model is chosen as the average point in the data swarm. This process involves subtracting the mean of each variable from the original measurements (Esbensen, 2002; Naes *et al.*, 2002).

3.2.3.4 Identification and classification of spectral outlier samples

Visual inspection of duplicate scans of the same samples revealed five samples with uncharacteristic spectral properties. The duplicate scans for these five samples were visibly different and these five samples were removed from the data set. Additional outliers were identified as samples that had a large influence on the PCA models and six samples were removed from the data set according to this principle.

3.2.4 CALIBRATION PROCEDURES

3.2.4.1 Calibration set design

Two strategies were used to design calibration sets for quantification of total anthocyanins and total phenolics, respectively. The first design involved the random division of filtered grape homogenate spectra into three sets: 50% of spectra for a calibration set and two

validation sets, each containing 25% of spectra (Figure 1). Due to the large number of samples in the data set, two validation sets were used (Esbensen, 2002; Naes *et al.*, 2002). Samples were equally distributed between the calibration and validation sets on the basis of vintage, cultivar and sugar concentration.

The second strategy involved the random division of filtered grape homogenate spectra from the 2005 vintage into sets based on *Vitis vinifera* cultivar: 67% of spectra for a calibration set and 33% of spectra for a single validation set (Figure 2). For all four separate cultivar calibration models developed there was equal distribution between the calibration and validation sets regarding the sugar concentrations of samples.

3.2.4.2 Partial Least Squares Regression (PLS)

Partial least squares regression (PLS) was used to develop calibration models using the Advanced Performance Software Module version 2.1.0 of the WineScan instrument (Foss WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001). PLS is a bilinear regression modelling method where the original \mathbf{X} variables are projected onto a smaller number of factors or PLS components. Bilinear modelling methods are used for situations where co-linearity exists among the original variables. PLS components are calculated according to the same statistical procedures as PC's but PLS uses the \mathbf{Y} -data structure or \mathbf{Y} -variance, directly as a guide in decomposing the \mathbf{X} -matrix (Esbensen, 2002). In terms of this study PLS can be described as a projection of the total anthocyanins and phenolics concentrations data (y -variables) and the wavenumbers (x -variables) onto a number of PLS components that explain the most important information in both spectral and chemical data. The relationship between y and x variables can be described by the following equation

$$y = b + b_1x_1 + b_2x_2 + b_nx_n \quad \dots 4$$

where y is the dependent variable, $b_0 - b_n$ are the regression coefficients (b_0 is the intercept), and $x_0 - x_n$ represent the absorbance at the selected wavenumbers.

3.2.4.3 Wavenumber selection

Wavenumber selection was performed on calibration sets and used to establish calibration models. The Advanced Performance Software Module version 2.1.0 of the WineScan instrument was used for wavenumber selection (Foss WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001) and 15 filters (wavenumbers or groups of wavenumbers) per chemical compound are selected. These filters correspond to the wavenumbers at which the correlation between measured absorbance and the corresponding reference values for total anthocyanins and total phenolics respectively was highest. Only three regions are available for wavenumber selection ($2970-2434 \text{ cm}^{-1}$, $2272-1716 \text{ cm}^{-1}$ and $1542-965 \text{ cm}^{-1}$) to exclude noise from the spectral data.

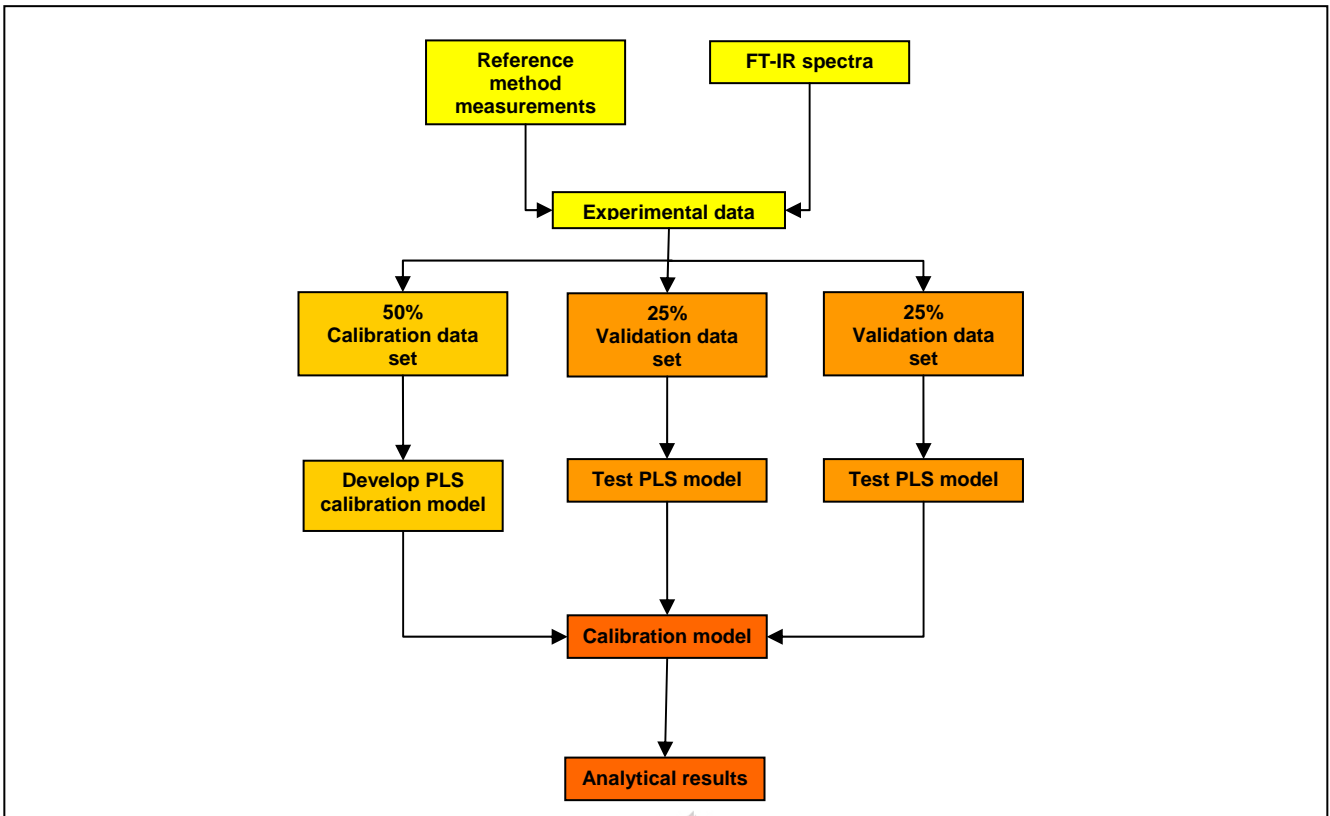


Figure 1. Diagram of the calibration set design involving all samples from the 2004 and 2005 vintages: 50% of sample numbers for calibration and two validation sets of 25% each.

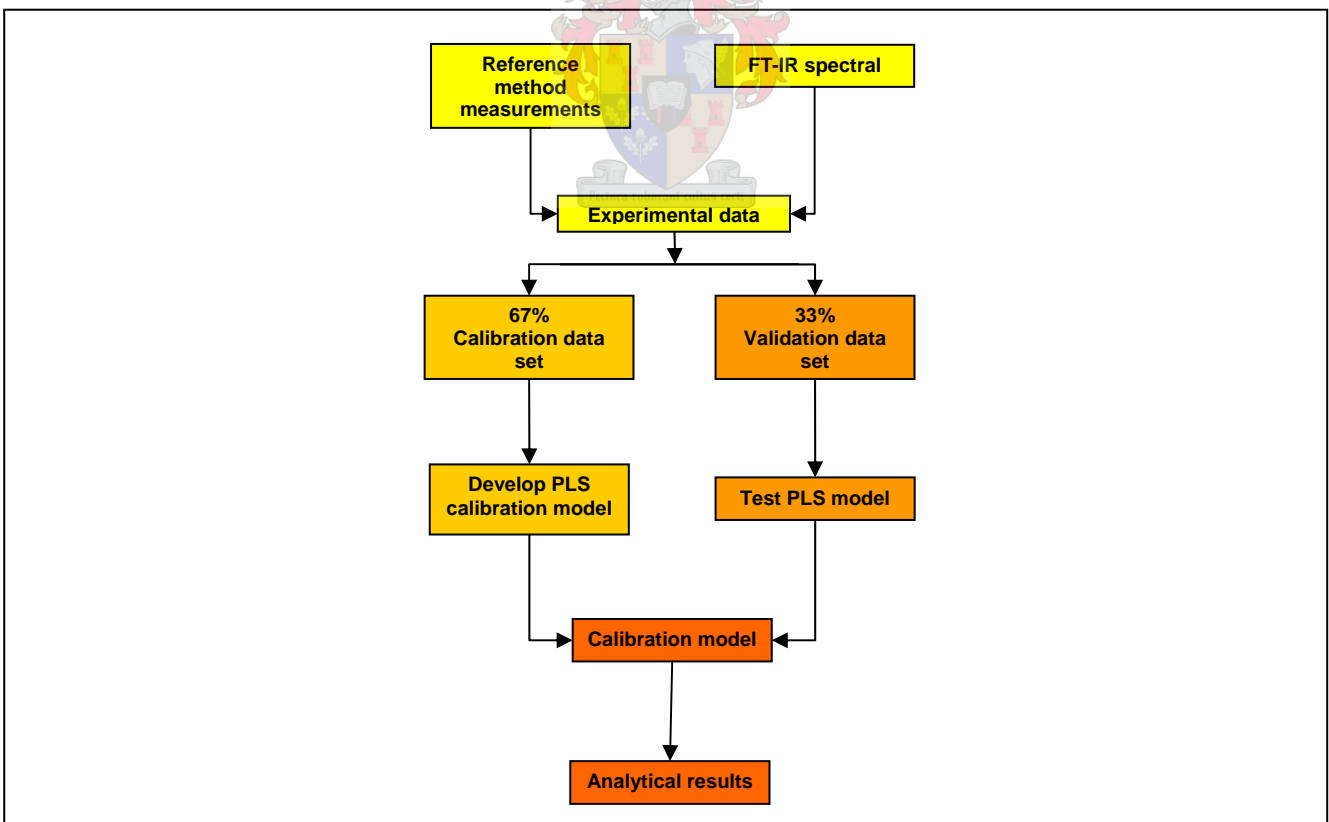


Figure 2. Diagram of calibration set design involving samples from the 2005 vintage: 67% of sample numbers for calibration and 33% for validation.

3.2.4.4 Assessment of the performance of calibration sets

Statistical indicators for the assessment of the performance of calibration models are listed below (Foss WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss Analytical, Denmark, 2001). The standard error of cross validation (*SECV*) (equation 5) gives an indication of the accuracy of the predictive ability of a calibration model using cross validation. The cross validation strategy involved keeping out successive groups of samples (10% of the total number of calibration samples) until all samples were kept out from the calibration set once, and using these subsets for calculating the calibration error.

The standard error of prediction (*SEP*) (equation 5) is an indication of the prediction accuracy of a calibration model when tested on independent validation sets. Bias (equation 6) is a measure of the prediction accuracy of a model and entails testing for a systematic difference between predicted and measured values. Bias was calculated as the mean value of residuals (differences between the reference values and the predicted values) (Esbensen, 2002). The RPD value (ratio of the standard deviation of the reference data of the validation set to the standard error of prediction) gives some indication of the efficiency of a calibration (equation 7). A RPD value of less than 3 indicates that a calibration model is fit for screening purposes, while a value of greater than 3 indicates that it is fit for quantification purposes (Williams and Norris, 2001b).

$$SECV \text{ or } SEP = \sqrt{\frac{\sum_{i=1}^n (y_i - \hat{y}_i - Bias)^2}{n-1}} \quad \dots\dots 5$$

$$Bias = \frac{1}{n} \sum_{i=1}^n (y_i - \hat{y}_i) \quad \dots\dots 6$$

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

$$RPD = \frac{SD}{SEP} \quad \dots\dots 7$$

3.3 RESULTS AND DISCUSSION

3.3.1 GRAPE SAMPLES AND REFERENCE LABORATORY ANALYSIS

The full details of an evaluation of the reference method analysis protocol (Iland *et al.*, 2000) are discussed in Chapter 4.

3.3.1.1 The evaluation of the effects of sample storage temperature on total anthocyanins and total phenolics concentrations

Due to the large number of samples received every day it was not possible to complete all analysis on the day of reception. No significant differences ($p > 0.05$) were found between

the total anthocyanins and total phenolics concentrations for samples analysed immediately or samples stored overnight at room-temperature (22°C), 0°C or 4°C (Table 2). Cynkar *et al.* (2004) reported no significant effects ($p \leq 0.05$) of overnight freezing (at -18°C) or storage at 4°C on the determination of total anthocyanins and total phenolics concentrations as well as total soluble solids using the reference method described by Iland *et al.* (2000). However, in the same study overnight freezing of the grapes did result in a significant ($p > 0.05$) raise in the pH values. Thus it is possible that sample storage at low temperatures (0°C) and homogenisation could have had significant effects on the pH and titratable acidity values obtained in this study. However, the effects of storage temperatures on pH and titratable acidity were not investigated in this study.

Table 2. The effects of sample storage temperatures on the total anthocyanins and total phenolics concentrations of grapes.

Storage treatment	n ^{*1}	Total anthocyanins (mg/g) ^{*3}	Total phenolics (OD280/g) ^{*4}
		Mean ^{*2} ± SD ^{*1}	Mean ^{*2} ± SD ^{*1}
Immediate, 22°C	5	1.62 ^a ± 0.65	1.57 ^a ± 0.40
Overnight, 22°C	5	1.67 ^a ± 0.66	1.65 ^a ± 0.38
Overnight, 4°C	5	1.55 ^a ± 0.84	1.61 ^a ± 0.50
Overnight, 0°C	5	1.57 ^a ± 0.67	1.64 ^a ± 0.43

Significance of least significant difference (LSD) test of treatment means at $p \leq 0.05$ and standard deviation. Means with different superscripts in the same column indicate statistically significant differences among treatments.

*¹ Abbreviations used: n, number of samples; SD, standard deviation

*² As determined by the reference method

*³ Expressed as mg anthocyanins per gram fresh berry

*⁴ Expressed as OD280 per gram fresh berry

3.3.1.2 Repeatability of the reference laboratory method

The determination of the repeatability of the reference laboratory method is fully described in section 4.2, Chapter 4. The exclusion of samples from original data sets based on poor reference repeatability is described in section 4.3, Chapter 4. Poorly repeated samples were removed from the original data sets and n = 89 samples of the 2004 vintage and n = 603 samples from the 2005 vintage were included in the final total anthocyanins data sets (total n = 792). For the final total phenolics concentrations data sets n = 95 samples of the 2004 vintage and n = 553 samples from the 2005 vintage were included (total n = 648).

3.3.1.3 Standard error of laboratory (SEL)

A detailed description of the calculation of the standard error of laboratory (SEL) values is provided in section 4.4, Chapter 4. The SEL values for samples included in the final anthocyanin data sets were 0.04 mg/g for samples from the 2004 vintage (n = 89) and

0.03 mg/g berry for samples from the 2005 vintage (n = 603). The SEL values for samples included in the total phenolics data sets were 0.05 OD280/g berry for samples from the 2004 vintage (n = 95) and 0.07 OD280/g berry for the samples from the 2005 vintage (n = 553).

3.3.1.4 The evaluation of the effects of the use of filterpapers with different cross-sections on FT-IR analysis results

The investigation results (Table 3) indicated no significant differences between filterpapers with cross-sections of 50, 185 or 240 mm. For this study filterpapers with cross-sections of 50 mm were used.

Table 3. The effects of the use of filterpapers with different cross-sections on FT-IR analysis results.

Filterpaper cross-section (mm)	n ^{*1}	Sugar (°Brix)	pH	TA ^{*3}
		Mean ^{*2} ± SD ^{*1}	Mean ^{*2} ± SD ^{*1}	Mean ^{*2} ± SD ^{*1}
50	6	20.3 ^a ± 1.1	3.67 ^a ± 0.23	6.9 ^a ± 0.5
185	6	20.5 ^a ± 1.0	3.69 ^a ± 0.17	3.9 ^a ± 0.2
240	6	20.3 ^a ± 1.2	3.67 ^a ± 0.23	6.9 ^a ± 0.4

*Significance of least significant difference (LSD) test of treatment means at p≤0.05 and standard deviation. Means with different superscripts in the same column indicate statistically significant differences among treatments.

^{*1} Abbreviations used: n, number of samples; SD, standard deviation; TA, titratable acidity

^{*2} As determined with the Foss WineScan FT 120 spectrometer

^{*3} Expressed as g/L tartaric acid

3.3.1.5 Descriptive statistics of all samples

Table 4 contains statistics regarding the sample numbers, sugar, pH, titratable acidity, total anthocyanins and total phenolics of grape homogenates from both the 2004 and 2005 vintages. The mean sugar concentration of 2005 samples is lower than that of the 2004 samples (see section 3.2.1). As expected (due to the inclusion of grapes of lower maturity in the 2005 sample set) the mean pH values of 2005 samples were lower than that of the 2004 samples. The lower mean pH value of 2005 vintage grape homogenates is also reflected in the higher TA concentration of the same set. The mean total anthocyanins concentration of the 2005 vintage grape homogenates was slightly higher than that of the 2004 vintage grape homogenates. Interestingly, the mean total phenolics concentrations for both vintages were similar. The wider range of maturities of grapes in the 2005 vintage sample sets is reflected in the slightly higher SD values for most of the chemical parameters when compared to that of the 2004 vintage sample sets.

3.3.2 ANALYSIS OF FOURIER TRANSFORM INFRARED (FT-IR) SPECTRA

Figure 3 displays the FT-IR spectrum of a filtered grape homogenate and reflects the combined absorbance of all IR-active components in the sample. The wavenumber regions of significant importance to this study include the wavenumbers 1542 to 965 cm^{-1} , sometimes referred to as the “fingerprint” area and various IR-bands, including those corresponding to the vibrations of the C-O, C-C, C-H, and C-N bonds occur in this region (Smith, 1999a). This area provides important information regarding organic compounds such as sugars, alcohols and organic acids present in a sample. Little variation in absorbance could be observed in the 5011-3627 cm^{-1} wavenumber region (Figure 3), but this area is known to contribute to significant noise in the spectra. The distinct absorbance peaks in the wavenumber regions 3626-2970 cm^{-1} and 1716-1543 cm^{-1} , are the result of the absorbance of water (Smith, 1999b). For calibration purposes the water and “noise” regions were excluded. Figure 4 shows the distinct variation between the spectra of filtered grape homogenates from the same vineyard block sampled approximately weekly during the 2005 harvest season.

3.3.3 PCA MODELING

In the explorative stages of PCA modeling all samples from the 2004 and 2005 vintages ($n = 827$) and all wavenumbers were used. The PCA score plot of PC1 versus PC2 showed two major clusters (Figure 5a) while the 2004 samples grouped together. Grape cultivars were not responsible for the clustering (Figure 5d). Samples from the 2004 vintage are located more towards the positive end of PC2. Both positive and negative loadings on PC1 explains the separation of the samples along PC1 (Figure 5c). Significant loadings are seen in the fingerprint (1542 to 965 cm^{-1}), water (3626-2970 cm^{-1} and 1716-1543 cm^{-1}) and “noise” (5011-3627 cm^{-1}) areas (Figure 5c).

In order to compare the effect of the water regions on the separation of samples, subsequent PCA was done using only the 3626-2970 cm^{-1} and 1716-1543 cm^{-1} water regions (Figure 6a) and, alternatively modeling by using all wavenumbers, except the two water regions (Figure 6b). The clear separation between the 2004 and 2005 samples based on the influence of water became very evident in this approach. It is interesting to speculate that this pattern could be related to the very low winter rainfall experienced in fresh berry2004 causing lower soil water reserves and subsequent lower water concentrations in the 2005 vintage grapes.

In order to evaluate the contribution of other important chemical compounds to the variation between grape homogenates, the dominating effect of water absorbance was eliminated and further analysis was done with water and “noise” variables deselected (Figure 7a and 7b). PC1 seemed to distinguish between samples based on the level of ripeness (measured as sugar concentration and pH value). Samples in groups 1, 2 and 3 had low ($y < 23.5^\circ\text{Brix}$), medium ($23.5^\circ\text{Brix} \leq y < 26.0^\circ\text{Brix}$) and high ($y \geq 26.0^\circ\text{Brix}$) sugar concentrations respectively (Figure 7a). The three groups based on arbitrary chosen sugar concentrations clearly separated along PC1. Samples in group 1 (low sugar concentrations) located towards the negative end of PC1 and were slightly more scattered

Table 4. Descriptive statistics of all samples.

Sample set	n ^{*1}	Sugar (°Brix)		pH		TA ^{*1} (g/L) ^{*4}		Total anthocyanins (mg/g) ^{*5}		Total phenolics (OD280/g) ^{*6}	
		Range ^{*2}	Mean ^{*2} ± SD ^{*1}	Range ^{*2}	Mean ^{*2} ± SD ^{*1}	Range ^{*2}	Mean ^{*2} ± SD ^{*1}	Range ^{*3}	Mean ^{*3} ± SD ^{*1}	Range ^{*3}	Mean ^{*3} ± SD ^{*1}
Cabernet Sauvignon 2004	37	20.4 - 26.3	24.4 ± 1.4	3.67 - 4.09	3.91 ± 0.11	3.2 - 5.1	4.1 ± 0.5	0.69 - 1.97	1.15 ± 0.23	0.76 - 1.64	1.31 ± 0.18
Cabernet Sauvignon 2005	239	17.6 - 29.3	23.5 ± 2.6	3.41 - 4.35	3.90 ± 0.18	3.0 - 8.9	4.5 ± 1.0	0.32 - 1.98	1.10 ± 0.34	0.57 - 1.78	1.22 ± 0.22
Merlot 2004	25	20.2 - 26.3	24.3 ± 1.7	3.63 - 4.04	3.84 ± 0.10	3.3 - 5.0	4.1 ± 0.4	0.69 - 1.40	1.07 ± 0.20	1.05 - 1.64	1.34 ± 0.15
Merlot 2005	155	17.7 - 29.4	24.0 ± 2.2	3.50 - 4.06	3.81 ± 0.13	3.0 - 7.4	4.2 ± 0.8	0.48 - 2.03	1.16 ± 0.28	0.61 - 1.96	1.37 ± 0.22
Pinotage 2005	64	18.2 - 28.9	24.5 ± 2.5	3.49 - 4.29	3.89 ± 0.16	4.0 - 6.6	5.1 ± 0.6	0.66 - 1.84	1.23 ± 0.25	1.01 - 2.08	1.53 ± 0.23
Shiraz 2004	37	21.4 - 27.4	24.3 ± 1.3	3.69 - 4.17	3.87 ± 0.13	2.6 - 10.0	4.3 ± 1.2	0.66 - 1.69	1.23 ± 0.23	0.95 - 1.74	1.37 ± 0.19
Shiraz 2005	145	15.5 - 31.5	24.1 ± 3.6	3.35 - 4.49	3.80 ± 0.21	2.6 - 10.0	4.3 ± 1.2	0.65 - 2.08	1.35 ± 0.32	0.78 - 1.93	1.41 ± 0.22
All cultivars 2004	89	20.2 - 27.4	24.3 ± 1.5	3.63 - 4.17	3.88 ± 0.12	2.8 - 5.1	3.9 ± 0.5	0.66 - 1.97	1.15 ± 0.23	0.76 - 1.74	1.34 ± 0.17
All cultivars 2005	603	15.5 - 31.5	23.9 ± 2.8	3.35 - 4.49	3.85 ± 0.18	2.6 - 10.0	4.5 ± 1.0	0.32 - 2.08	1.19 ± 0.33	0.58 - 2.08	1.34 ± 0.24

*1 Abbreviations used: n, number of samples; TA, titratable acidity; SD, standard deviation

*2 As determined with the Foss WineScan FT 120 spectrometer

*3 As determined with the reference method

*4 Expressed as g/L tartaric acid

*5 Expressed as mg anthocyanins per gram fresh berry

*6 Expressed as OD280 per gram fresh berry

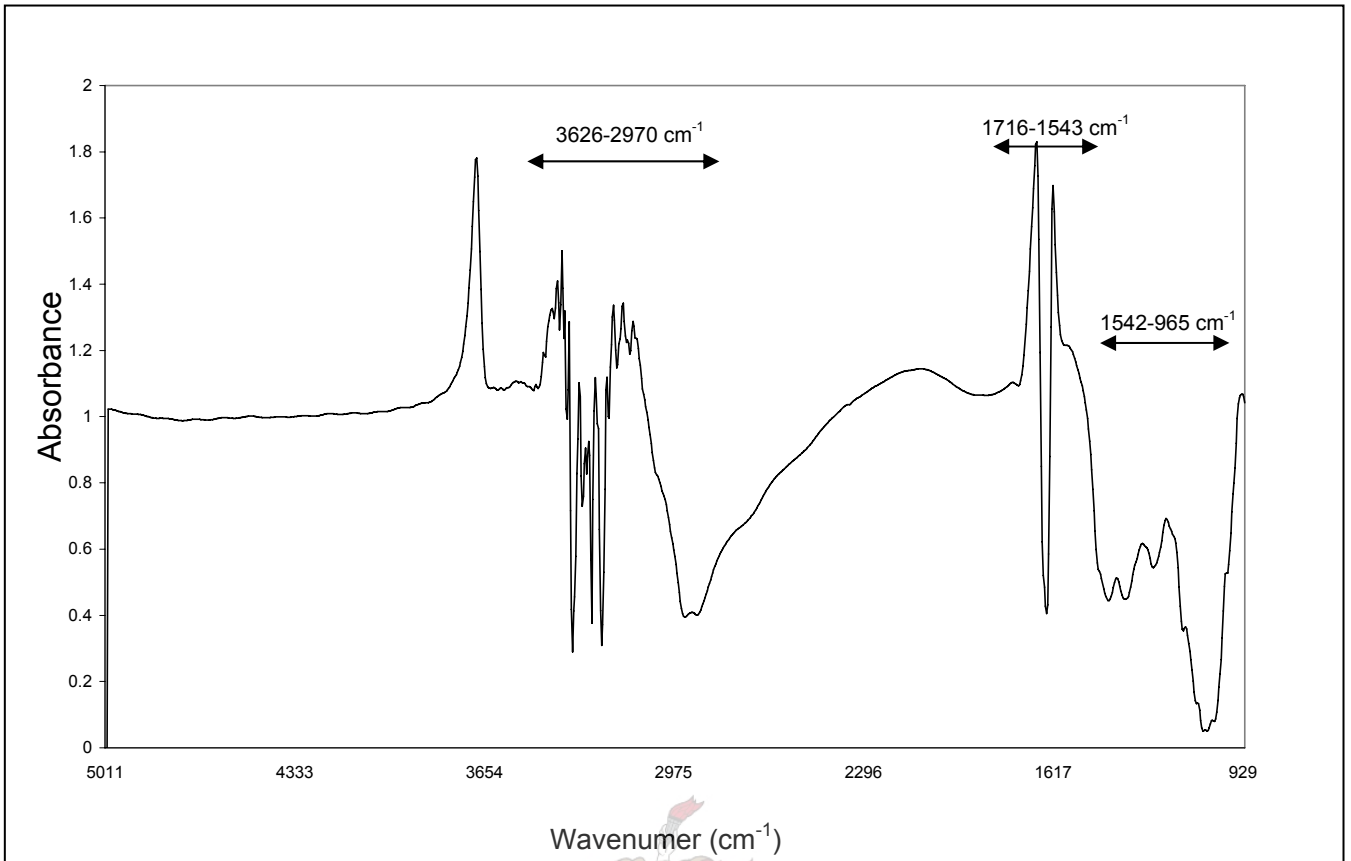


Figure 3. FT-IR spectrum of a filtered grape homogenate sample in the region 5011 – 929 cm⁻¹.

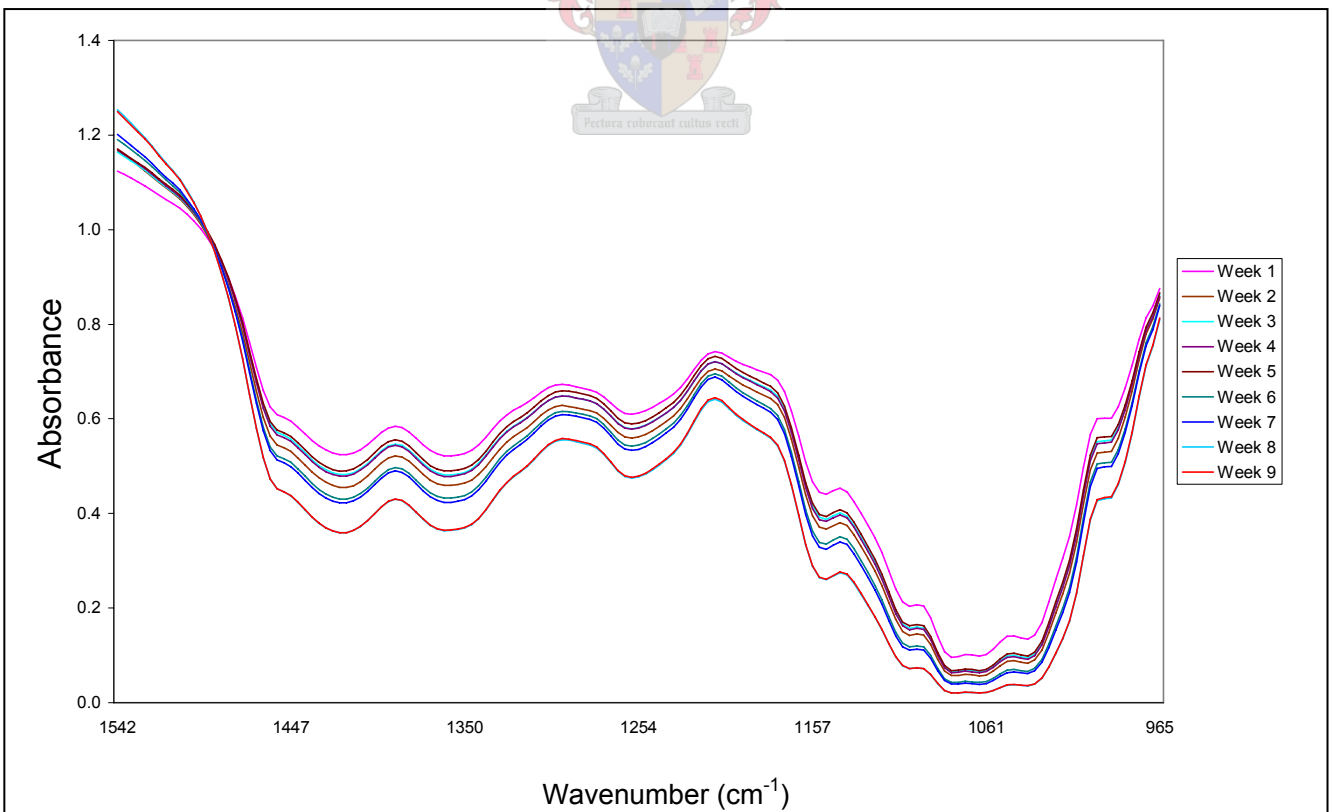


Figure 4. Spectral variation in the "fingerprint" area (1542-965 cm⁻¹) of FT-IR spectra of the same block sampled weekly during the 2005 vintage. Weekly sample dates: 1, 2 February 2005; 2, 9 February 2005; 3, 15 February 2005; 4, 22 February 2005; 5, 2 March 2005; 6, 9 March 2005; 7, 15 March 2005; 8, 23 March 2005; 9, 29 March 2005.

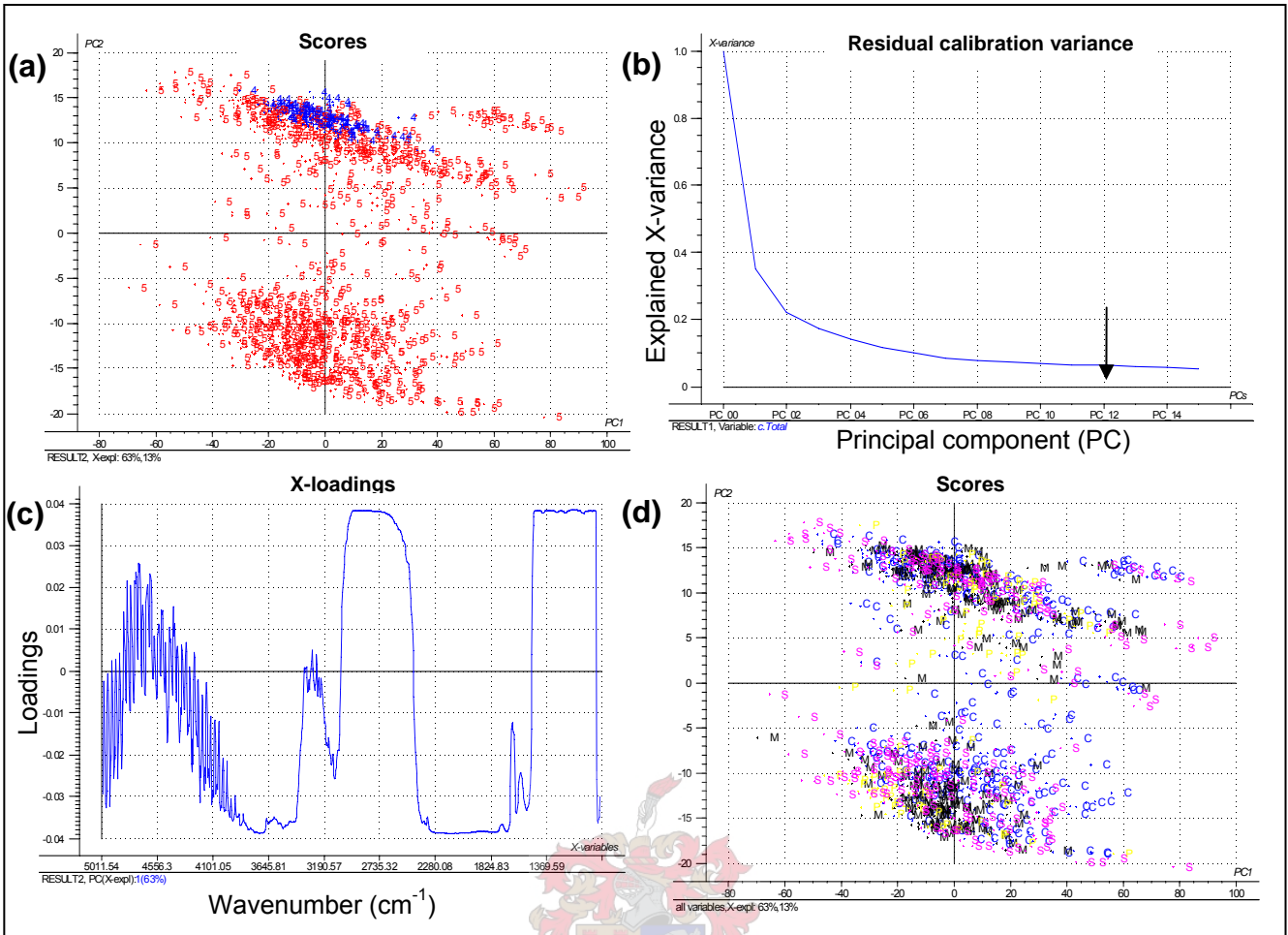


Figure 5. Modelling with all wavenumbers. PCA of grape homogenate samples ($n = 827$) of the 2004 and 2005 vintage. a) Score plot of PC1 (63% X-variance explained) and PC2 (13% X-variance explained), 4:2004 (blue markers), 4:2005 (red markers). b) Residual calibration variance plot showing that 12 PC's describe more than 95% of the X-variance. c) Loadings plot of PC1. d) Score plot of PC2 (12% X-variance explained) and PC3 (6% X-variance explained). C: Cabernet Sauvignon (blue markers), M: Merlot (black markers), P: Pinotage (yellow markers), S: Shiraz (pink markers).

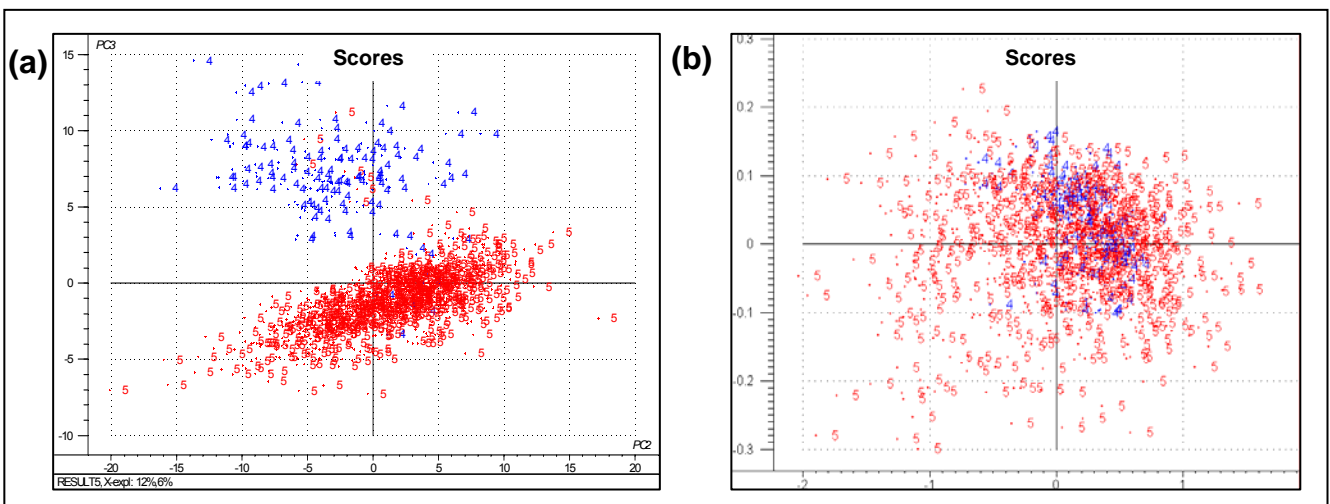


Figure 6. PCA score plot of PC1 versus PC2 of the FT-IR spectra of the total set of grape homogenates ($n = 827$) (a) including the two wavenumber regions $3626\text{--}2970\text{ cm}^{-1}$ and $1716\text{--}1543\text{ cm}^{-1}$, where water absorbs strongly, and (b) excluding the two wavenumber regions $3626\text{--}2970\text{ cm}^{-1}$ and $1716\text{--}1543\text{ cm}^{-1}$. 4:2004 (blue markers), 5:2005 (red markers).

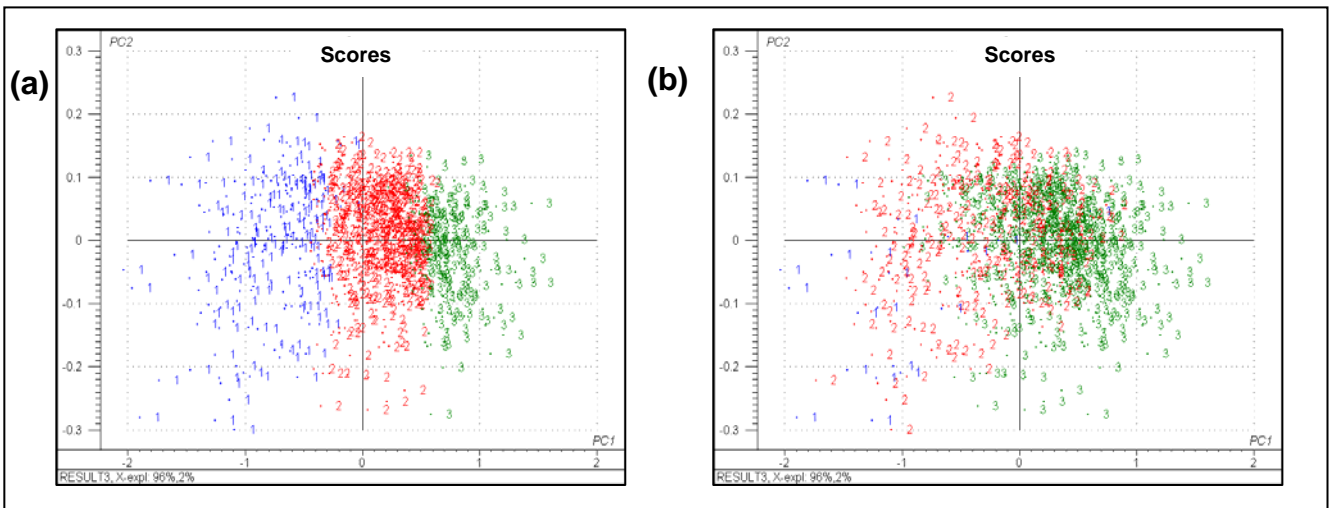


Figure 7. (a) PCA score plot of PC1 versus PC2 of the FT-IR spectra of grape homogenates from the 2005 vintage ($n = 603$) based on sugar concentrations. The $3626\text{-}2970\text{ cm}^{-1}$ and $1716\text{-}1543\text{ cm}^{-1}$ wavenumber regions, where water absorbs strongly, were excluded. 1: $x < 23.5^\circ\text{Brix}$ (blue markers), 2: $23.5^\circ\text{Brix} \leq x < 26.0^\circ\text{Brix}$ (red markers), 3: $x \geq 26.0^\circ\text{Brix}$ (green markers), and (b) PCA score plot of PC1 versus PC2 of the FT-IR spectra of grape homogenates from the 2005 vintage ($n = 603$) based on pH values. The $3626\text{-}2970\text{ cm}^{-1}$ and $1716\text{-}1543\text{ cm}^{-1}$ wavenumber regions where water absorbs strongly were excluded. 1: $x < 3.6$ (blue markers), 2: $3.6 \leq x \leq 3.9$ (red markers), 3: $x > 3.9$ (green markers).

than samples of the other two groups. The samples in groups 2 and 3 with medium and high sugar concentrations respectively, located towards the positive end of PC1.

Figure 7b illustrates the PCA results based on pH values. Samples in groups 1, 2 and 3 had pH values of $x < 3.6$, $3.6 \leq x \leq 3.9$ and $x > 3.9$ respectively. Although not as clear a separation as with the sugar concentrations, samples with lower pH values (Group 1) were located more towards the negative end of PC1 and samples with higher pH values (Group 3) more towards the positive end. These results suggested that the grape homogenates from diverse sugar concentrations would possibly have to be treated as separate groups in the design of sample sets for anthocyanin and total phenolics calibration. Other possible sources of variation between spectra of the grape homogenates that were investigated during PCA were type of *Vitis vinifera* cultivar, length of storage time before homogenisation as well as storage temperature. No variation could be attributed to any of these factors (data not shown).

Figures 8a and 8b show PCA score plots modelling the nine grape homogenates of which the “fingerprint” area spectra were graphically illustrated in section 3.3.2, Figure 4. These grape samples originated from the same vineyard block and were harvested approximately every week as the 2005 harvest season progressed. The PCA score plot where all wavenumbers were used (Figure 8a) showed that samples distributed according to grape maturity along PC1. PCA modelling excluding the $3626\text{-}2970\text{ cm}^{-1}$ and $1716\text{-}1543\text{ cm}^{-1}$ wavenumber regions was also done (Figure 8b). The PCA score plot of PC1 versus PC2 shows a distribution pattern of samples along PC1 related to grape maturity level. The sample harvested in week one (least mature sample) is located to the positive end of PC1. Grape samples harvested during weeks two to five are also located towards the positive side of PC1. On the negative side of PC1 are samples harvested during weeks six to nine (samples of highest maturity). These PCA results confirm that grape maturity is the major source of variation between the grape homogenates.

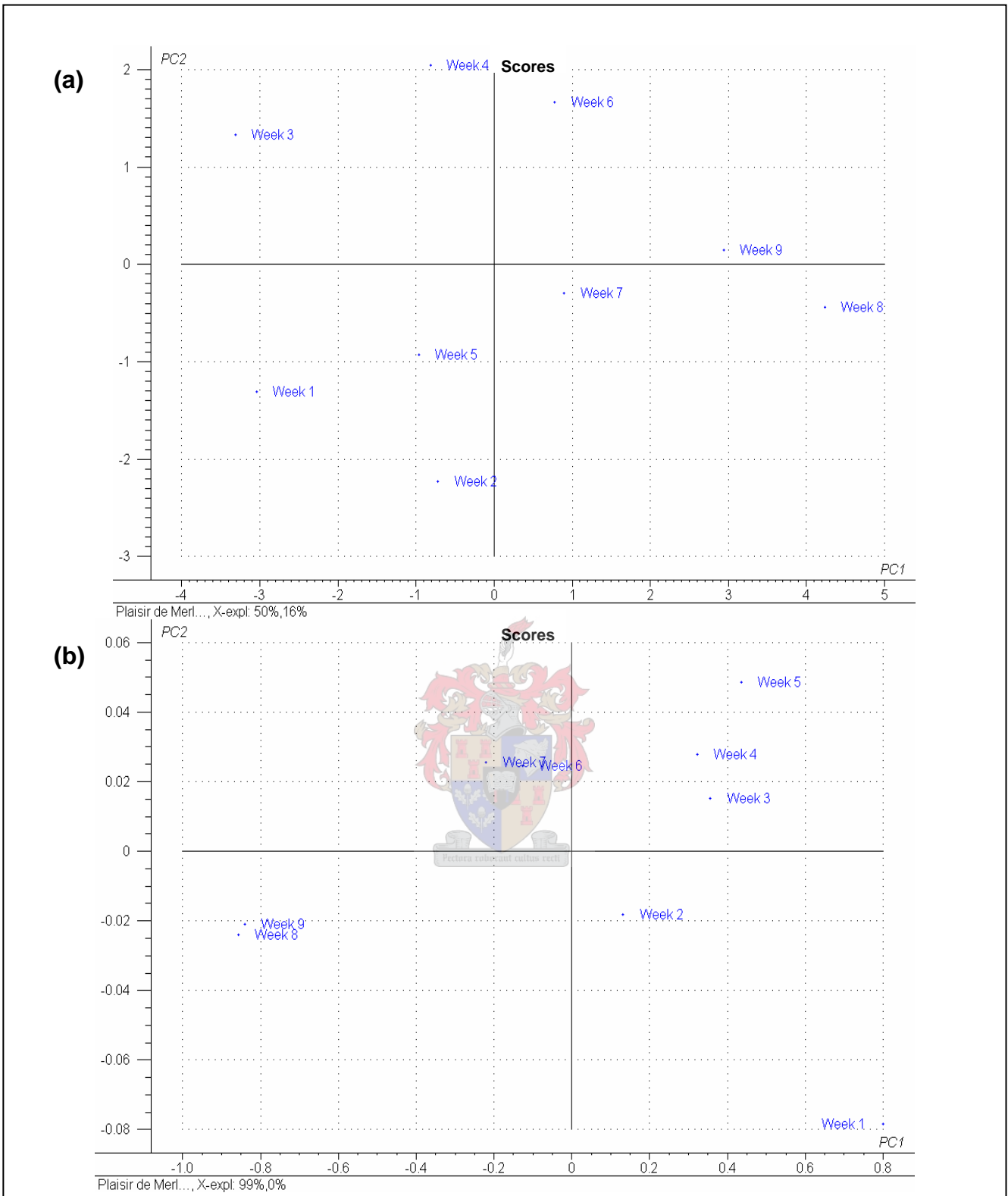


Figure 8. (a) PCA score plot of PC1 versus PC2 of the FT-IR spectra of grape homogenates from the same block sampled weekly during the 2005 vintage ($n = 9$). All wavenumbers were used, and (b) PCA score plot of PC1 versus PC2 of the FT-IR spectra of grape homogenates from the same block sampled weekly during the 2005 vintage ($n = 9$). The $3626\text{--}2970\text{ cm}^{-1}$ and $1716\text{--}1543\text{ cm}^{-1}$ wavenumber regions where water absorbs strongly were excluded.

3.3.4 THE EVALUATION OF THE EFFECTS OF CALIBRATION SET DESIGN FOR PREDICTION OF TOTAL ANTHOCYANINS IN GRAPE HOMOGENATES

Since the PCA score plots of filtered grape homogenate spectra revealed sample groupings according to vintage and sugar concentration, six calibration sets were designed to evaluate the effects of these factors on calibration models for total anthocyanins (Table 5). Three calibration sets involved samples from both the 2004 and 2005 vintages and respectively contained samples from all sugar levels, higher sugar levels ($x \geq 23.5^\circ\text{Brix}$) and lower sugar levels ($x < 23.5^\circ\text{Brix}$). The “cut-off” value of 23.5°Brix was arbitrarily chosen. The three remaining calibration sets only involved samples from the 2005 vintage and the before mentioned sugar concentrations were again used as differentiation criteria. No calibration sets were evaluated for samples from the 2004 vintage individually due to restricted sample numbers ($n = 89$). Also note that in the three calibration sets containing samples from both vintages a mere 10-15% of the sample numbers are from the 2004 vintage. Therefore, valid comparisons of vintage effects could not be made.

The aim of the different calibration sets was to evaluate the effect of calibration set design on calibration accuracy. Two individual independent validation sets were used to test the predictive accuracy of each calibration model. Table 6 contains calibration statistics for the six calibration sets. The three 2005 vintage calibration sets had slightly better predictive abilities than the three 2004&2005 vintage calibration sets. This might be explained by the fact that the three 2004&2005 calibration sets were not balanced with regard to sample numbers from the 2004 and 2005 vintages.

Prediction errors for total anthocyanins in grape homogenates using near-infrared spectroscopy (NIR) calibration models range from 0.09 to 0.16 mg/g. (Damberg *et al.*, 2003). The *SEP* values obtained in this study are in good agreement with these results. Of all six calibration sets the least accurate prediction results were obtained with the 2004&2005 All sugar, calibration model (*SECV* = 0.15 mg/g; *SEP Val1* = 0.17 mg/g; *SEP Val2* = 0.17 mg/g). In comparison with the five other calibration sets the best calibration statistics were obtained for the 2005 High sugar set (*SEP Val1* and *Val2* = 0.13 mg/g). Even though the *SECV* of the 2005 Low sugar set was lower than that of any of the other calibration sets (0.11 mg/g berry) the predictive ability of the model was not that accurate (*SEP Val1* = 0.15 mg/g; *SEP Val2* = 0.16 mg/g). Of the three 2004&2005 vintage calibration sets the 2004&2005 High sugar set had the best calibration statistics (*SECV* = 0.13 mg/g; *SEP Val1* = 0.15 mg/g; *SEP Val2* = 0.14 mg/g). Even though the *SECV* of the 2004&2005 Low sugar set was slightly lower (0.12 mg/g) than that of the 2004&2005 High sugar set, the *SEP* values of the two independent validation sets were slightly higher (*SEP Val1* = 0.15 mg/g; *SEP Val2* = 0.17 mg/g).

The SEL for the determination of total anthocyanins in the Distell laboratory were calculated as 0.03-0.04 mg/g berry and is in good agreement with the SEL of the AWRI laboratory (mentioned in section 3.3.1.5). The *SEP* values obtained for all six calibration sets were approximately three times higher than the SEL values for the sets. This is in good agreement with other published results (Damberg *et al.*, 2003). The RPD values of all six calibration models are well below 5. Table 7 illustrates the significance of some RPD values (Williams and Norris, 2001b). The RPD values of all six calibration models are well

below 5 indicating that all six calibration models are only fit for screening purposes and not for quantification purposes (Williams and Norris, 2001b).

The coefficient of determination (R^2) values for calibration sets ranged between 0.77 and 0.90. However these values provide an over optimistic view and must be interpreted along with the *SEP* and R^2 values for validation sets. For the validation sets of the six calibration models the R^2 values ranged from 0.68 to 0.81 which are considerable lower than the R^2 values of the calibration sets. Dambergers *et al.* (2003) reported R^2 values for NIR validation sets ranging between 0.86 and 0.96. The highest R^2 value was that of the 2005 Low sugar set ($R^2 = 0.81$). It is considered that the calibration model that gives the highest RPD value, together with the highest coefficient of determination, is the best (Williams and Norris, 2001b). According to this definition the 2005 Low sugar set is considered to be the best of all six calibration sets (RPD Val2 = 2.3). When considering the *SEP*, R^2 and RPD values of validations sets the 2005 High sugar set is considered to be the best of all six sets. Figure 9 shows the correlations between the total anthocyanins concentrations that were determined by the reference method and the estimated values predicted by the six calibration models.

Table 5. General statistics for total anthocyanins (mg anthocyanins/g berry) for calibration and validation sets as indicated.

Calibration set	Set	n ^{*1}	n ^{*1} 2004	n ^{*1} 2005	Range ^{*2}	Mean ^{*2} ± SD ^{*1}
2004&2005 All sugar	Cal ^{*1}	344	43	301	0.32 – 2.08	1.18±0.32
	Val ^{*1} 1	171	21	150	0.51 – 2.07	1.16±0.29
	Val ^{*1} 2	170	22	148	0.48 – 2.03	1.21±0.31
2004&2005 High sugar	Cal ^{*1}	211	31	180	0.60 – 2.08	1.24±0.28
	Val ^{*1} 1	104	15	104	0.73 – 2.03	1.26±0.28
	Val ^{*1} 2	105	14	91	0.69 – 2.07	1.26±0.26
2004&2005 Low sugar	Cal ^{*1}	130	13	117	0.32 – 1.80	1.07±0.34
	Val ^{*1} 1	63	6	57	0.51 – 1.70	1.03±0.30
	Val ^{*1} 2	64	6	58	0.51 – 2.03	1.11±0.37
2005 All sugar	Cal ^{*1}	282	-	282	0.32 – 2.08	1.19±0.33
	Val ^{*1} 1	142	-	142	0.53 – 2.07	1.18±0.30
	Val ^{*1} 2	144	-	144	0.48 – 2.03	1.26±0.31
2005 High sugar	Cal ^{*1}	179	-	179	0.60 – 2.08	1.25±0.28
	Val ^{*1} 1	88	-	88	0.73 – 2.03	1.26±0.27
	Val ^{*1} 2	89	-	89	0.69 – 2.07	1.29±0.27
2005 Low sugar	Cal ^{*1}	118	-	118	0.32 – 1.80	1.06±0.36
	Val ^{*1} 1	58	-	58	0.51 – 2.03	1.03±0.30
	Val ^{*1} 2	57	-	57	0.51 – 2.03	1.07±0.36

*1 Abbreviations used: n, number of samples; SD, standard deviation; Cal, calibration set; Val, validation set

*2 As determined by the reference method

Table 6. Calibration statistics for FT-IR measurement of anthocyanins (mg anthocyanins/g berry).

Calibration set	PLS* ¹ factors	SECV* ¹	R ² * ¹ Cal* ¹	SEL* ¹	SEP* ¹		R ² * ¹ Val* ¹		Bias		RPD* ¹	
					Val1* ¹	Val2* ¹	Val1* ¹	Val2* ¹	Val1* ¹	Val2* ¹	Val1* ¹	Val2* ¹
2004 & 2005 All sugar	12	0.15	0.77	0.03	0.17	0.17	0.68	0.71	0.011	-0.019	1.7	1.8
2004 & 2005 High sugar	11	0.13	0.77	0.03	0.15	0.14	0.69	0.70	-0.029	-0.042	1.9	1.9
2004 & 2005 Low sugar	12	0.12	0.87	0.04	0.15	0.17	0.75	0.77	0.039	-0.002	2.0	2.2
2005 All sugar	12	0.13	0.85	0.03	0.15	0.15	0.77	0.77	-0.001	-0.007	2.0	2.1
2005 High sugar	11	0.13	0.78	0.03	0.13	0.13	0.77	0.76	-0.023	-0.041	2.1	2.1
2005 Low sugar	11	0.11	0.90	0.03	0.15	0.16	0.76	0.81	0.031	0.0095	2.0	2.3

*¹ Abbreviations used: PLS, partial least squares; SECV, standard error of cross validation; R², coefficient of determination; SEL, standard error of laboratory; SEP, standard error of prediction; Cal, calibration; Val, validation; RPD, ratio of the standard deviation of the data to the standard error of prediction

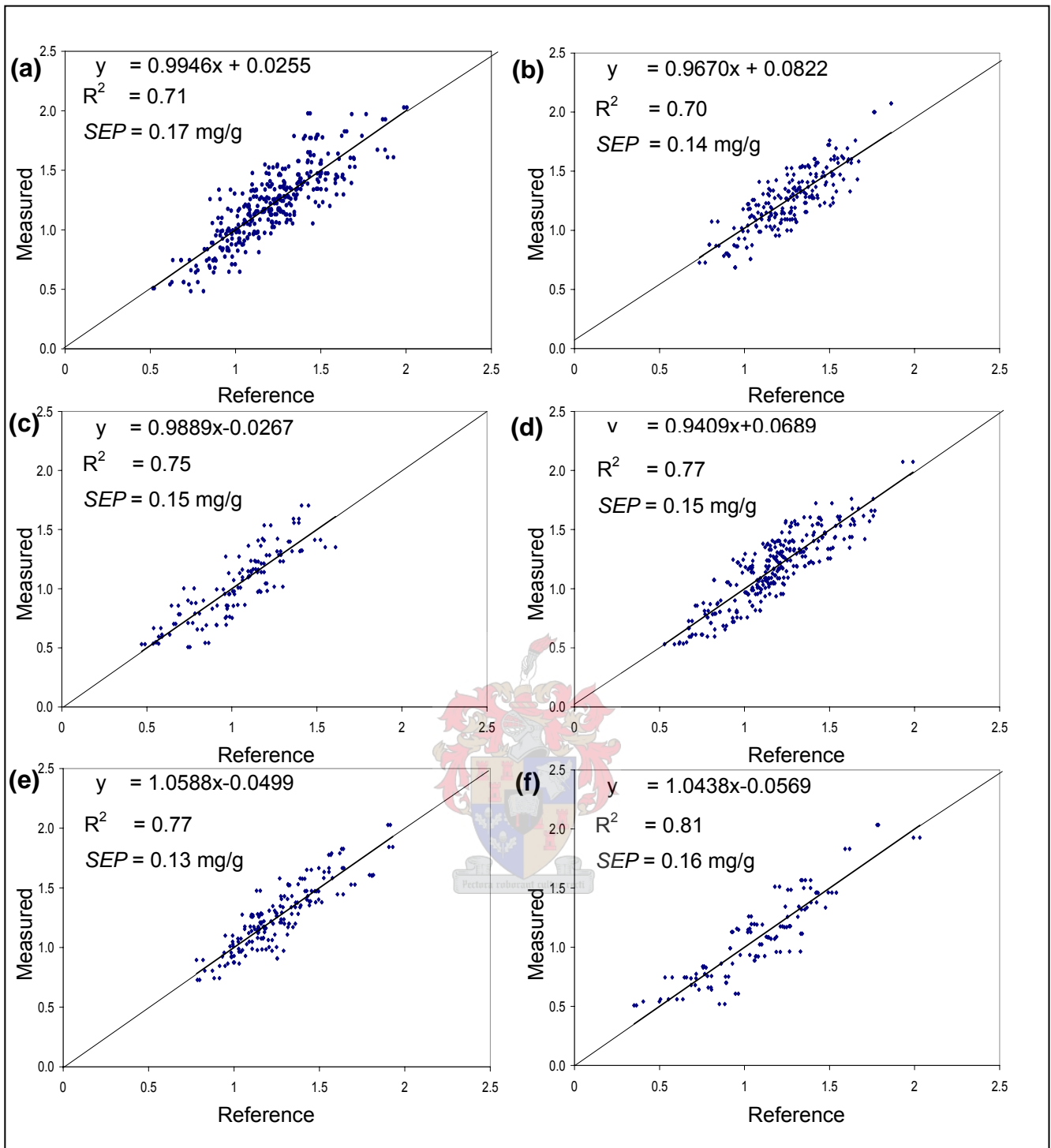


Figure 9. A comparison of the six calibration sets for total anthocyanins using a 25% subset of sample numbers as an independent validation set. Only regression statistics for the validation set with the best prediction and highest correlation statistics combined are shown for each of the six calibration sets. (a) 2004 & 2005 All sugar validation set2, and (b) 2004 & 2005 High sugar validation set2, and (c) 2004 & 2005 Low sugar validation set1, and (d) 2005 All sugar validation set1, and (e) 2005 High sugar validation set1, and (f) 2005 Low sugar validation set2.

Table 7. The significance of some RPD statistics (adapted from Williams and Norris, 2001b).

RPD* ¹ Value	Classification	Application
0.0-2.3	Very poor	Not recommended
2.4-3.0	Poor	Very rough screening
3.1-4.9	Fair	Screening
5.0-6.4	Good	Quality control
6.5-8.0	Very good	Process control
8.1+	Excellent	Any application

*¹ Abbreviation used: RPD, ratio of the standard deviation of the reference data of the validation set to the standard error of prediction

3.3.5 THE EVALUATION OF THE INFLUENCE OF CULTIVARS ON CALIBRATION SET DESIGN FOR TOTAL ANTHOCYANINS IN GRAPE HOMOGENATES

Grape homogenates from the 2005 vintage were divided into four sets according to *Vitis vinifera* cultivar. Separate calibration models were developed for the prediction of total anthocyanins for *Vitis vinifera* cultivars Cabernet Sauvignon, Merlot, Pinotage and Shiraz. Samples from all sugar concentrations were included in calibration sets because of the low sample numbers for each individual cultivar. Future efforts will aim to increase the sample numbers in each of the cultivar calibration models, especially that of Pinotage (n = 42). The prediction ability of all cultivar calibration sets were tested with independent validation sets. Table 8 shows the regression statistics for the calibration and validation sets of each cultivar.

The *SECV* values of the four cultivar calibration sets ranged from 0.10-0.12 mg/g and were generally lower than the *SECV* values for the six calibration sets including all cultivars ranging from 0.11-0.15 mg/g (section 3.3.4, Table 6). There was no significant gain in terms of the prediction accuracy for calibration models consisting of individual grape cultivars (*SEP* range, 0.13-0.18 mg/g) when compared to calibration models which included all cultivars (*SEP* range, 0.13-0.17 mg/g) (Table 8). The numbers of PLS factors for the cultivar calibration models were also slightly higher (12 and 13 factors) than that of the calibration models which included all cultivars (11 and 12 factors). It is important to select the optimum number of PLS factors when using PLS. Using too many components will cause “overfitting”. “Overfitting” means the developed calibration model will be very dependent on the calibration dataset and will give poor prediction results. Using too few components will cause “underfitting” and the calibration model will not be large enough to secure the variability in the data. To eliminate the “fitting” effect and achieve more accurate predictions, one solution is to use more samples to develop the calibration model (Naes *et al.*, 2002).

The prediction results obtained with the Merlot calibration model were satisfactory. Despite the low *SECV* value (*SECV* = 0.12 mg/g) the *SEP* value for Shiraz was

unsatisfactory ($SEP = 0.18$ mg/g). The contrast between statistics of the calibration and validation set for Shiraz are also reflected in the R^2 values (R^2 Cal = 0.84; R^2 Val = 0.72). The mean anthocyanin content of both the calibration and validation sets for Shiraz was the highest. The lower SEP and R^2 values for Shiraz are contradictory to earlier results where the best predictions were obtained for calibration sets with higher anthocyanin means (section 3.3.4, Table 6). Reported NIR calibration results for the total anthocyanins concentrations of Shiraz grape homogenates are: $n = 218$; $SECV = 0.10$ mg/g; $R^2 = 0.95$. NIR calibration results reported for Cabernet Sauvignon in the same study are: $n = 190$; $SECV = 0.10$ mg/g; $R^2 = 0.95$ (Dambergers *et al.*, 2003). The results for the calibration set of Cabernet Sauvignon was in good agreement with the results obtained in the before mentioned study ($n = 158$; $SECV = 0.12$ mg/g; $R^2 = 0.89$). Despite good calibration set results ($SECV = 0.10$ mg/g; $R^2 = 0.86$) testing with an independent validation set showed that the Pinotage calibration model was unsatisfactory ($SEP = 0.13$ mg/g; $R^2 = 0.62$). A possible explanation is that due to the low number of samples in the calibration set all possible sources of spectral variation are not included in the calibration set.

The standard error of laboratory values (SEL) for the cultivar calibration models ranged from 0.03 mg/g to 0.04 mg/g. The RPD values of the four calibration models range between 1.5 and 2.6. Therefore, the RPD values are well below the limit of 5 indicating that all four models are only fit for screening purposes (Williams and Norris, 2001b). The SD values of all four calibration sets ranged between 0.26 to 0.35 mg/g. The calibration model for Merlot is considered to be the best model because it has the lowest SEP value ($SEP = 0.12$ mg/g), highest RPD value (RPD = 2.6) together with the highest R^2 value ($R^2 = 0.84$).

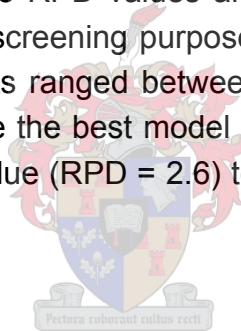


Table 8. General statistics for anthocyanins (mg anthocyanins/g berry) for individual cultivar sets as indicated.

Calibration Set	2005 Cabernet Sauvignon	2005 Merlot	2005 Pinotage	2005 Shiraz	Validation Set	2005 Cabernet Sauvignon	2005 Merlot	2005 Pinotage	2005 Shiraz
n ^{*1}	158	102	42	94	n ^{*1}	77	48	18	45
Range ^{*2}	0.32 – 1.93	0.49 – 1.69	0.66 – 1.84	0.65 – 2.07	Range ^{*2}	0.39 – 1.70	0.54 – 2.03	0.86 – 1.66	0.80 – 2.08
Mean ^{*2}	1.10	1.14	1.18	1.32	Mean ^{*2}	1.06	1.23	1.27	1.37
SD ^{*1}	0.35	0.27	0.26	0.30	SD ^{*1}	0.30	0.31	0.20	0.34
SECV ^{*1}	0.12	0.10	0.10	0.12	SEP ^{*1}	0.13	0.12	0.13	0.18
R ² ^{*1}	0.89	0.87	0.86	0.84	R ² ^{*1}	0.82	0.84	0.62	0.72
PLS ^{*1} factors	13	13	12	12	Bias	-0.019	-0.027	0.007	-0.016
SEL ^{*1}	0.03	0.04	0.04	0.03	RPD ^{*1}	2.3	2.6	1.5	1.9

^{*1} Abbreviations used: n, number of samples; SD, standard deviation; SECV, standard error of cross validation; SEP, standard error of prediction; R², coefficient of determination; PLS, partial least squares; SEL, standard error of laboratory; RPD, ratio of the standard deviation of the reference data to the standard error of prediction (SEP)

^{*2} As determined by the reference method

3.3.6 THE EVALUATION OF THE EFFECTS OF CALIBRATION SET DESIGN FOR PREDICTION OF TOTAL PHENOLICS IN GRAPE HOMOGENATES

Similar to the calibration sets discussed in section 3.3.2 grape homogenates were divided into six sets to assess the effects of vintage and sugar concentration on the performance of calibration models for the prediction of total phenolics in grapes (Table 9). Grape homogenates from the 2004 vintage were by far outnumbered by 2005 samples. Grape homogenates from both vintages were divided into three sets according to the sugar concentrations: all sugar levels, higher sugar levels ($y \geq 23.5^\circ\text{Brix}$) and lower sugar levels ($y < 23.5^\circ\text{Brix}$). Three calibration sets were designed that only contained samples from the 2005 vintage. The described sugar concentrations were again used as differentiation criteria. No calibration sets were evaluated for samples from the 2004 vintage individually.

Table 9. General statistics for total phenolics (OD280/g berry) for calibration and validation sets as indicated.

Calibration set	Set	n ^{*1}	n ^{*1} 2004	n ^{*1} 2005	Range ^{*2}	Mean ^{*2} ± SD ^{*1}
2004 & 2005 All sugar	Cal ^{*1}	325	48	277	0.61 – 1.96	1.34±0.23
	Val ^{*11}	159	23	135	0.71 – 1.84	1.33±0.21
	Val ^{*12}	156	23	133	0.58 – 2.01	1.34±0.25
2004 and 2005 High sugar	Cal ^{*1}	193	34	159	0.90 – 2.01	1.38±0.20
	Val ^{*11}	94	17	77	0.93 – 1.89	1.38±0.20
	Val ^{*12}	16	76	92	0.83 – 1.93	1.38±0.22
2004 and 2005 Low sugar	Cal ^{*1}	129	14	115	0.61 – 2.08	1.27±0.26
	Val ^{*11}	63	7	56	0.71 – 1.87	1.31±0.24
	Val ^{*12}	3	56	59	0.58 – 1.90	1.27±0.27
2005 All sugar	Cal ^{*1}	276	-	276	0.61 – 1.96	1.34±0.25
	Val ^{*11}	135	-	135	0.71 – 1.88	1.34±0.22
	Val ^{*12}	133	-	133	0.58 – 2.08	1.34±0.27
2005 High sugar	Cal ^{*1}	159	-	159	0.90 – 2.01	1.39±0.20
	Val ^{*11}	77	-	77	0.93 – 1.89	1.40±0.20
	Val ^{*12}	75	-	75	0.83 – 1.89	1.38±0.22
2005 Low sugar	Cal ^{*1}	115	-	115	0.61 – 2.08	1.25±0.27
	Val ^{*11}	55	-	55	0.71 – 1.87	1.30±0.25
	Val ^{*12}	55	-	55	0.58 – 1.90	1.26±0.27

*¹ Abbreviations used: n, number of samples; SD, standard deviation; Cal, calibration; Val, validation

*² As determined by the reference method

Table 10 contains calibration statistics for the six calibration sets. It was difficult to evaluate calibration statistics because diverse prediction results were obtained for the two independent validation sets of five of the six calibration sets. The calibration statistics of the 2005 vintage calibration sets are slightly better than that of the three 2004&2005 vintage calibration sets. Similar results were obtained for the anthocyanin calibration models using the same calibration set design. The standard error laboratory values (SEL's) for the determination of total phenolics of grape homogenates ranged between 0.06 and 0.07 OD280/g. Overall the coefficients of determination (R^2) for the calibration sets ranged from 0.66 to 0.82. Similarly the R^2 values for the validation sets ranged between 0.58 and 0.78. The 2005 Low sugar calibration model were judged to be the best of all six calibration models because of the low *SECV* value (*SECV* = 0.11 OD280/g), high R^2 values for validation sets (R^2 Val1 = 0.74 and R^2 = Val2 = 0.78) and consistency regarding the prediction of two independent validation sets (*SEP* Val1 and Val2 = 0.13 OD280/g). The 2005 Low sugar calibration model also had the highest *RPD* value (*RPD* = 2.1) of all six calibration sets but it was still much lower than a *RPD* value of 5. Any *RPD* value higher than 5 indicates that a calibration model is suitable for quantitative purposes. Note that the number of PLS factors for this model was lower than that used for the other two 2005 vintage calibration models. No improvement of calibration statistics was obtained for the two 2005 All sugars and High sugar calibration sets with the use of less PLS factors (data not shown). There was no significant difference ($p > 0.05$) between the *SEP* values obtained for the validation sets of the 2004&2005 vintage calibration sets. Based on the lower number of PLS factors used for the 2004&2005 Low sugar calibration model it appears to be the best model of the three 2004&2005 vintage calibration sets. Correlation between the total phenolics concentrations that were determined by the reference method and the estimated values predicted by the six calibration models are graphically represented in Figure 10.

Table 10. Calibration statistics for FT-IR measurement of total phenolics (OD280/g).

Calibration set	PLS* ¹ factors	SECV* ¹	R ² * ¹ Cal ^a	SEL* ¹	SEP* ¹		R ² * ¹ Val* ¹		Bias		RPD* ¹	
					Val1* ¹	Val2* ¹	Val1* ¹	Val2* ¹	Val1* ¹	Val2* ¹	Val1* ¹	Val2* ¹
2004 & 2005 All sugar	12	0.14	0.66	0.07	0.12	0.14	0.67	0.68	0.001	0.008	1.8	1.8
2004 & 2005 High sugar	12	0.11	0.69	0.07	0.12	0.15	0.63	0.55	0.003	0.029	1.7	1.5
2004 & 2005 Low sugar	11	0.13	0.76	0.06	0.12	0.15	0.74	0.70	-0.027	0.016	2.0	1.8
2005 All sugar	13	0.14	0.70	0.07	0.12	0.14	0.70	0.71	-0.007	0.011	1.8	1.9
2005 High sugar	13	0.11	0.70	0.07	0.12	0.15	0.61	0.58	-0.005	0.041	1.7	1.5
2005 Low sugar	11	0.11	0.82	0.06	0.13	0.13	0.74	0.78	-0.041	-0.012	1.9	2.1

*¹ Abbreviations used: PLS, partial least squares; SECV, standard error of cross validation; R², coefficient of determination; SEL, standard error of laboratory; SEP, standard error of prediction; Cal, calibration; Val, validation; RPD, ratio of the standard deviation of the data to the standard error of prediction

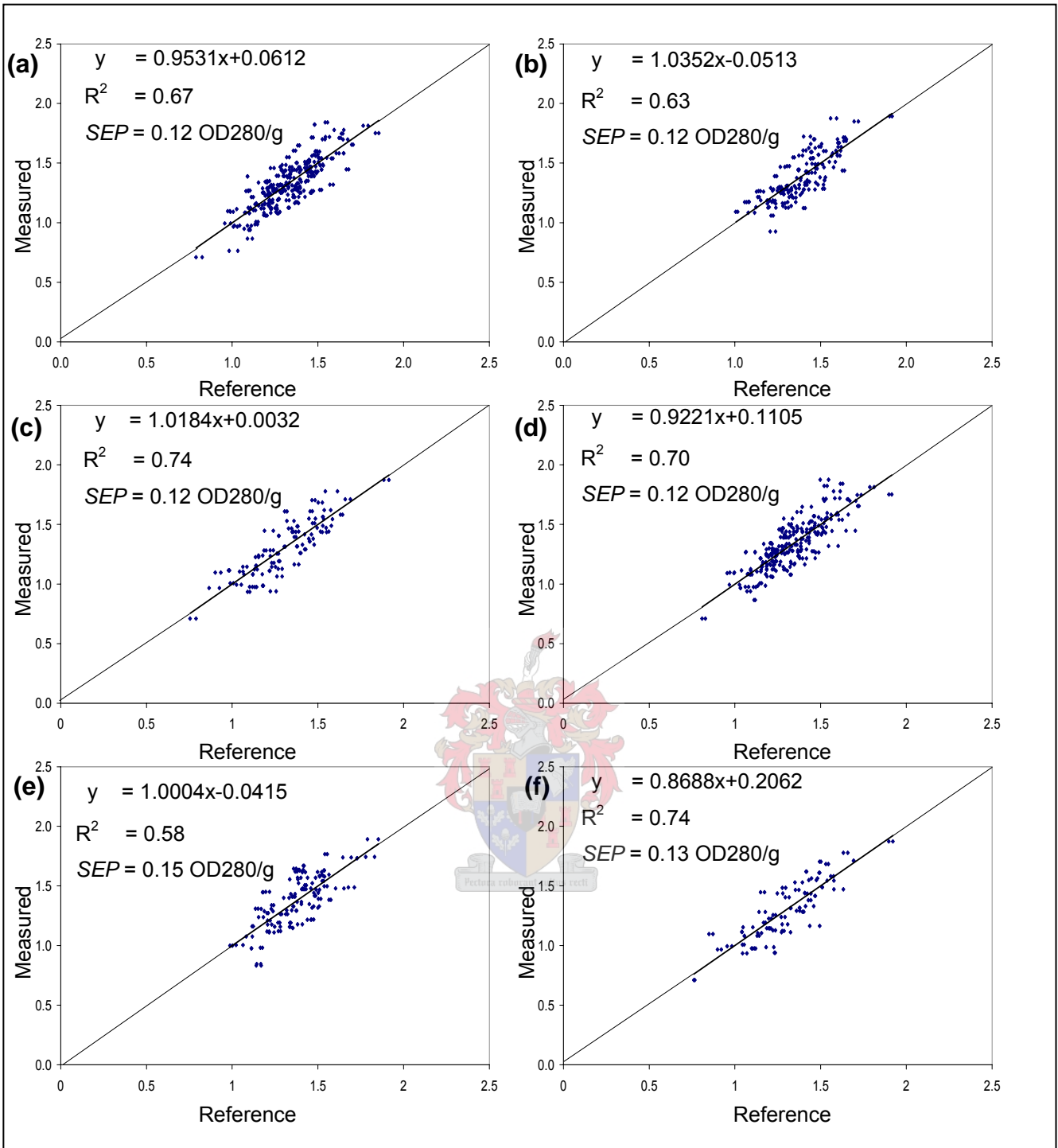


Figure 10. A comparison of the six calibration sets for total phenolics using a 25% subset of sample numbers as an independent validation set. Only regression statistics for the validation set with the best prediction and highest correlation statistics combined are shown for each of the six calibration sets. (a) 2004 & 2005 All sugar validation set1, and (b) 2004 & 2005 High sugar validation set1, and (c) 2004 & 2005 Low sugar validation set1, and (d) 2005 All sugar validation set1, and (e) 2005 High sugar validation set2, and (f) 2005 Low sugar validation set2.

3.3.7 THE EVALUATION OF THE INFLUENCE OF CULTIVARS ON CALIBRATION SET DESIGN FOR TOTAL PHENOLICS IN GRAPE HOMOGENATES

Four individual calibration models were established for the prediction of total phenolics of grape homogenates for the four *Vitis vinifera* cultivars from the 2005 vintage. The developed models included grape homogenates from all sugar concentrations. Table 11 contains the statistics for all four cultivar calibration models. The *SEP* values obtained by the cultivar calibration models were approximately two times higher than the *SEL*'s of the calibration sets. The prediction accuracy of the individual cultivar calibration models (*SEP* range, 0.10-0.14 OD280/g) was similar to that of the calibration models which included all four cultivars (*SEP* range, 0.12-0.15 OD280/g) (see section 3.3.4). The R^2 values of the validation sets for Cabernet Sauvignon, Pinotage and Shiraz were much lower than the R^2 values of the calibration sets. The highest calibration R^2 value was obtained for Pinotage ($R^2 = 0.91$) which was also the highest calibration R^2 value obtained in this study. However this calibration model delivered the lowest R^2 value of all the validation sets in this study ($R^2 = 0.54$). It seems as if the model is "overfitting" the calibration samples and therefore it gives poor prediction results. This may be because of the high number of PLS factors (15) used and the low number of samples in the calibration set ($n = 40$) (Naes *et al.*, 2002). Even lower prediction accuracy was obtained when the use of a lower number of PLS factors was investigated (results not shown). The Merlot calibration model were judged to be the best of all four cultivar calibration sets based on the low *SECV* (*SECV* = 0.11 OD280/g), low *SEP* (*SEP* = 0.10 OD280/g) and high R^2 values for both calibration ($R^2 = 0.77$) and validation ($R^2 = 0.76$). The Merlot calibration model also delivered the highest RPD value (RPD = 2).

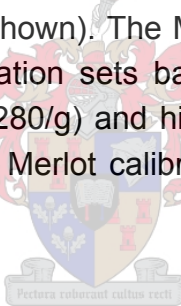


Table 11. General statistics for total phenolics (OD280/g) for individual cultivar sets as indicated.

Calibration Set	2005 Cabernet Sauvignon	2005 Merlot	2005 Pinotage	2005 Shiraz	Validation set	2005 Cabernet Sauvignon	2005 Merlot	2005 Pinotage	2005 Shiraz
n ^{*1}	141	88	40	94	n ^{*1}	69	42	19	46
Range ^{*2}	0.58 – 1.78	0.61 – 1.86	1.14 – 2.01	0.78 – 1.89	Range ^{*2}	0.76 – 1.73	0.81 – 1.74	1.18 – 1.90	0.92 – 1.93
Mean ^{*2}	1.22	1.37	1.53	1.40	Mean ^{*2}	1.22	1.37	1.55	1.40
SD ^{*1}	0.22	0.23	0.22	0.23	SD ^{*1}	0.22	0.20	0.20	0.22
SECV ^{*1}	0.09	0.11	0.07	0.11	SEP ^{*1}	0.13	0.10	0.14	0.14
R ² * ¹	0.82	0.77	0.91	0.78	R ² * ¹	0.65	0.76	0.54	0.61
PLS ^{*1} factors	11	11	15	14	Bias	-0.004	-0.036	-0.047	0.044
SEL ^{*1}	0.07	0.06	0.05	0.07	RPD ^{*1}	1.7	2	1.4	1.6

*¹ Abbreviations used: n, number of samples; SD, standard deviation; SECV, standard error of cross validation; SEP, standard error of prediction; R², coefficient of determination; PLS, partial least squares; SEL, standard error of laboratory

*² As determined by the reference method

3.4 CONCLUSIONS

This study was a large-scale investigation of the application of Fourier transform infrared spectroscopy (FT-IR) for the prediction of total anthocyanins and total phenolics concentrations of grapes. The commercial calibration models provided with the WineScan instrument have low prediction accuracies for the before mentioned parameters and new calibration models were developed. Principal component analysis (PCA) of FT-IR spectra enabled the early detection and interpretation of outlier samples. With the aid of PCA, grape sugar concentration and pH were identified as some of the major sources of variation between grape homogenates. The effects of calibration set design on calibration model performance were extensively investigated for both total anthocyanins and total phenolics. The influences of vintage, sugar concentration and *Vitis vinifera* cultivar on calibration set design and subsequent prediction accuracy were evaluated. Slightly better accuracy was obtained for restricted, defined data sets (grapes from a single vintage and cultivar). Overall the calibration models obtained for both anthocyanins and total phenolics were satisfactory and can be used for screening purposes. The calibration statistics for anthocyanin calibrations were slightly better than that of the calibrations for total phenolics. This was expected because the absorbance at 280 nm is not specific enough to robustly quantify grape phenolics (Herderich *et al.*, 2004). The use of an alternative reference method such as HPLC to quantify total phenolics will be considered in the future (Cozzolino *et al.*, 2004). It is clear that calibration models must be refined regarding robustness through continued research. More samples from all four *Vitis vinifera* cultivars from a wider calibration range are imperative to obtain calibration models with higher prediction accuracies and lower prediction errors.

The results in this study show that FT-IR spectroscopy shows promise as an analytical technique for the rapid prediction of total anthocyanins and total phenolics of grapes. This suggests that FT-IR can contribute to improved grape quality measurements. For the wine industry SEP's for calibration models for the determination of total anthocyanins and total phenolics concentrations of grapes of 0.10 mg anthocyanins/g berry and 0.10 OD₂₈₀/g berry will be acceptable. To successfully implement these measures as part of grape quality control data must be gained over several seasons. This will allow viticulturists and winemakers to develop sufficient experience with the interpretation of results of the two parameters. The goal for the South African wine industry is the industrial use of total anthocyanins and total phenolics of grapes as pre-harvest and at-weighbridge indicators of wine quality.

ABBREVIATIONS USED

ANOVA, analysis of variance; AWRI, Australian wine research institute; FT-IR, Cal, calibration; Fourier transform infrared; HPLC, high performance liquid chromatography; IR, infrared; LSD, least significant difference; n, number of samples; NIR, near-infrared; PC,

principal component; PCA, principal component analysis; PLS, partial least squares; R^2 , coefficient of determination; RPD, ratio of the standard deviation of the reference data to the standard error of prediction; SD, standard deviation; *SECV*, standard error of cross validation; SEL, standard error of laboratory; *SEP*, standard error of prediction; TA, titratable acidity; Val, validation.

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RESEARCH RESULTS

The evaluation of the reference analysis method protocol for the determination of total anthocyanins and total phenolics in grapes



REFERENCE ANALYSIS METHOD VALIDATION

4.1 INTRODUCTION

In a commercial environment it is beneficial to use simple analytical procedures with good repeatability. During the 2004 vintage the reference protocol for the determination of total anthocyanins and total phenolics (Iland *et al.*, 2000) was evaluated. The results obtained provided important information regarding the repeatability of the method as well as the standard error of laboratory as determined in the Distell laboratory.

4.2 EVALUATION OF REFERENCE ANALYSIS METHOD EXTRACTIONS

The reference analysis protocol (Iland *et al.*, 2000) is time consuming and a limited number of analyses can be performed by one analyst per day. Two extraction steps of grape homogenates are described: one hour in 50% v/v ethanol and a second supernatant extraction of three hours in 1 M HCl (Figure 1). The effects of six different extraction treatment times on total anthocyanins and total phenolics concentrations analyses results were investigated (Figure 1). Nine repetitions of nine different grape homogenates were performed. Two treatments with no HCl extraction steps had significantly lower total anthocyanins and total phenolics concentration values (Table 1). These results confirmed that the second supernatant extraction step involving HCl is crucial for maximum extraction of total anthocyanins and phenolics. There were no significant differences between four treatments involving a 60 minute extraction with ethanol and a second supernatant extraction step in HCl ranging from 0 to 180 minutes. These results suggest that the second extraction step in HCl could be cut back to 60 minutes resulting in a time saving of 120 minutes per sample. During this study it was decided to use the described procedure of Iland *et al.* (2000) of a 60 minute extraction step in ethanol and a 180 minute supernatant extraction step in HCl to enable exact comparisons between this investigation and other investigations using a minimum of a 180 minute HCl supernatant extraction step (Damberg *et al.*, 2003).

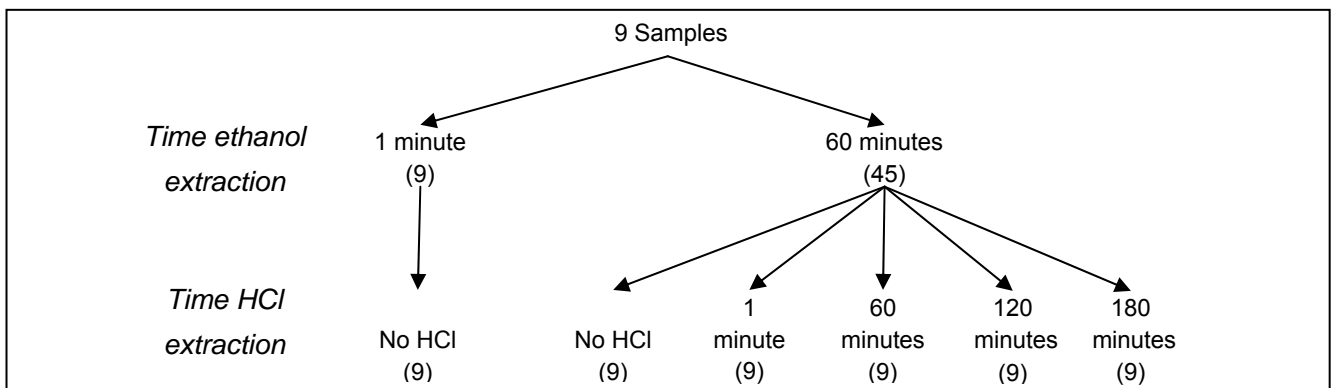


Figure 1. The experimental design to investigate the effects of different extraction steps on the total anthocyanin and total phenolic concentrations of grapes. Numbers in brackets represent numbers of replicates.

Table 1. The effects of different extraction times on the total anthocyanins and total phenolics concentrations of grapes.

Extraction time (minutes)		n* ¹	Total anthocyanins (mg/g)* ³	Total phenolics (OD280/g)* ⁴
50% v/v Ethanol	1 M HCl		Mean* ² ± SD* ¹	Mean* ² ± SD* ¹
1	No HCl	9	0.49 ^b ± 0.11	0.30 ^b ± 0.01
60	No HCl	9	0.52 ^b ± 0.08	0.30 ^b ± 0.01
60	1	9	0.95 ^a ± 0.19	1.32 ^a ± 0.24
60	60	9	0.94 ^a ± 0.22	1.28 ^a ± 0.22
60	120	9	0.95 ^a ± 0.23	1.29 ^a ± 0.24
60	180	9	0.95 ^a ± 0.23	1.28 ^a ± 0.23

Significance of least significant difference (LSD) test of treatment means at $p \leq 0.05$ and standard deviation. Means with different symbol superscripts in the same column indicate statistically significant differences among treatments.

*¹ Abbreviations used: n, number of samples; SD, standard deviation

*² As determined by the reference method

*³ Expressed as mg anthocyanins per gram fresh berry

*⁴ Expressed as OD280 per gram fresh berry

4.3 DETERMINATION OF THE REPEATABILITY OF REFERENCE ANALYSIS METHOD

Figures 2 and 3 show the box and whisker plots for eight repetitions of the reference protocol for total anthocyanins and total phenolics (Iland *et al.*, 2000) as determined on four grape homogenates of *Vitis vinifera* cultivars Cabernet Sauvignon, Merlot, Pinotage and Shiraz. The reference method showed good repeatability. Analyses of Merlot and Shiraz grape homogenates respectively delivered one repetition (outlier) with a total anthocyanins value which differed significantly from the 7 other repetitions (Figure 2). During laboratory analysis of the two identified outlier samples a small volume of the ethanol extracts were spilled which lead to subsequent inaccurate dilutions with HCl. According to the total phenolics results in Figure 3 the spillages had no obvious influence on the total phenolics results of the same Merlot and Shiraz grape homogenates. This investigation highlighted the importance of meticulous laboratory practices to ensure accurate analysis results.

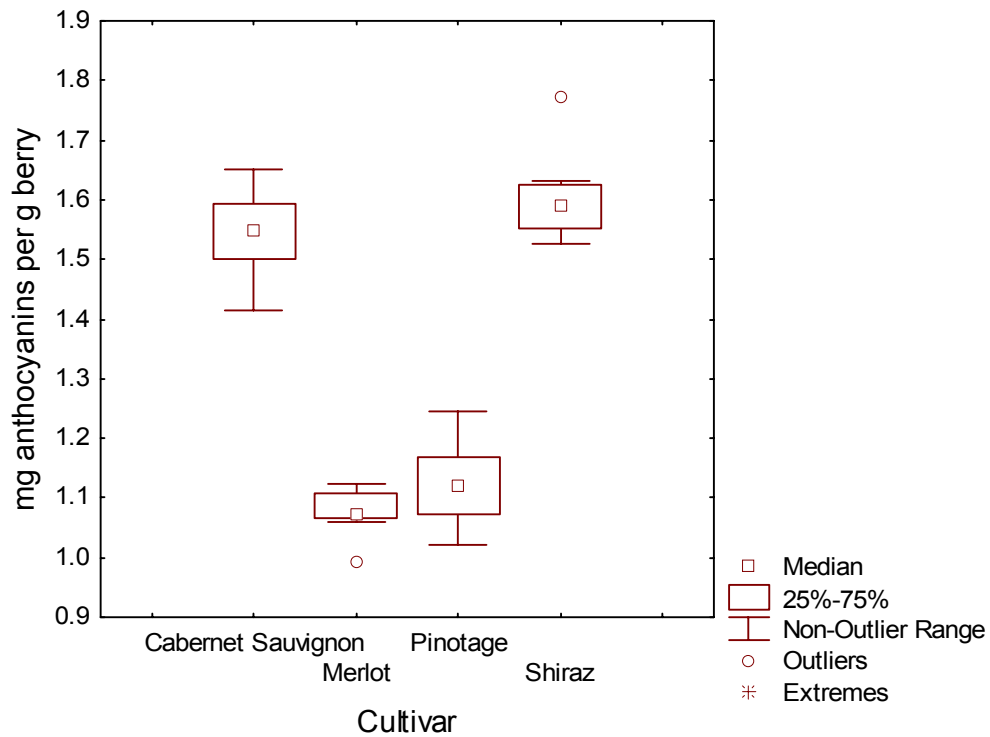


Figure 2. Box and whisker plot showing the repeatability of 8 total anthocyanins reference laboratory repetitions on grape homogenates of the *Vitis vinifera* cultivars Cabernet Sauvignon, Merlot, Pinotage and Shiraz.

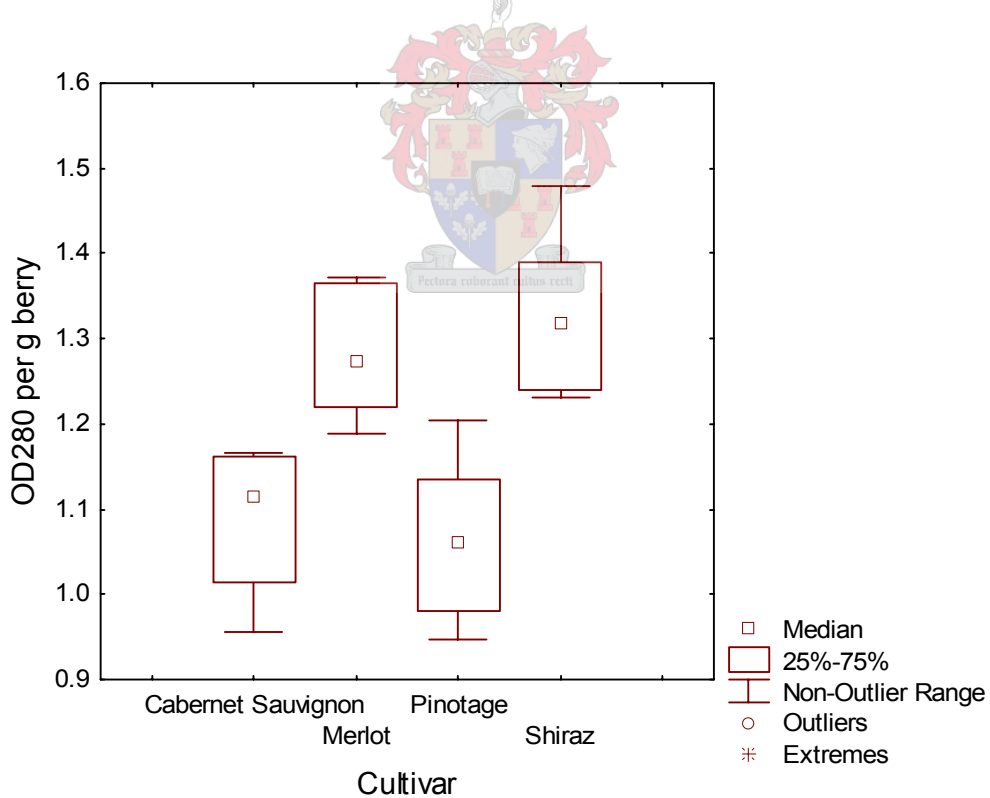


Figure 3. Box and whisker plot showing the repeatability of 8 total phenolics reference laboratory measurements on grape homogenates of the *Vitis vinifera* cultivars Cabernet Sauvignon, Merlot, Pinotage and Shiraz.

4.4 EXCLUSION OF SAMPLES WITH POOR REPEATABILITY FROM DATA SETS

Tables 2 and 3 contain respective 75th and 25th percentile values of eight reference repetitions for total anthocyanins and total phenolics of Cabernet Sauvignon, Merlot, Pinotage and Shiraz *Vitis vinifera* grape homogenates (see section 4.2). The differences between the 75th and 25th percentile values for each cultivar were calculated for both total anthocyanins and total phenolics (Tables 2 and 3). The difference between the 75th and 25th percentile values of Pinotage was the highest for both total anthocyanins (0.10 mg/g) and total phenolics (0.15 OD280/g) concentrations. It was decided to use these two values as criteria to decide which samples to exclude from sample sets based on poor repeatability. Therefore samples with duplicate determinations of grape homogenates with a total anthocyanins difference ($y_1 - y_2$) greater than 0.10 mg/g berry were excluded from anthocyanins data sets. For total phenolics data sets samples with a difference ($y_1 - y_2$) of greater than 0.15 OD280/g berry were excluded from total phenolics data sets. According to these criteria 31 of the 120 2004 vintage samples were excluded for total anthocyanins data sets (n included 2004 = 89) and 25 of the 120 2004 vintage samples were excluded from total phenolics data sets (n included 2004 = 95). 124 Samples of the 727 2005 vintage samples were excluded from anthocyanins data sets (n included 2005 = 603) and 153 samples for total phenolics data sets (n included 2005 = 553). Therefore, many samples were excluded from data sets to eliminate the effects of poorly repeated reference analysis on calibration models and subsequent prediction statistics.

Table 2. Statistics of 8 total anthocyanins reference repetitions on grape homogenates of *Vitis vinifera* cultivars, Cabernet Sauvignon, Merlot, Pinotage and Shiraz.

Cultivar	n* ¹	Mean* ^{2,3} ± SD* ¹	75 th Percentile* ³	25 th Percentile* ³	Difference between 75 th Percentile and 25 th Percentile* ³
Cabernet Sauvignon	8	1.54 ± 0.07	1.59	1.50	0.09
Merlot	8	1.08 ± 0.04	1.11	1.07	0.04
Pinotage	8	1.12 ± 0.07	1.17	1.07	0.10
Shiraz	8	1.60 ± 0.08	1.62	1.55	0.07

*¹ Abbreviations used: n, number of samples; SD, standard deviation

*² As determined by the reference method

*³ Expressed as mg anthocyanins per gram berry

Table 3. Statistics of 8 total phenolics reference repetitions on grape homogenates of *Vitis vinifera* cultivars, Cabernet Sauvignon, Merlot, Pinotage and Shiraz.

Cultivar	n* ¹	Mean* ^{2,3} ± SD* ¹	75 th Percentile* ³	25 th Percentile* ³	Difference between 75 th Percentile and 25 th Percentile* ³
Cabernet Sauvignon	8	1.09 ± 0.08	1.16	1.01	0.15
Merlot	8	1.29 ± 0.08	1.37	1.22	0.15
Pinotage	8	1.06 ± 0.09	1.13	0.98	0.15
Shiraz	8	1.33 ± 0.09	1.39	1.24	0.15

*¹ Abbreviations used: n, number of samples; SD, standard deviation

*² As determined by the reference method

*³ Expressed as OD280 per gram berry

4.5 DETERMINATION OF THE STANDARD ERROR OF LABORATORY (SEL)

The standard error of laboratory (SEL) values for the determination of total anthocyanins and total phenolics concentrations were calculated on the original vintage data sets and after samples were removed based on poor repeatability (final data sets) (Table 4). The SEL values for the original and final 2005 anthocyanin data sets were slightly lower than that of the two 2004 anthocyanin data sets. This was expected due to more repetitions and practice of the laboratory method resulting in increased reference method accuracy. The SEL for the determination of anthocyanins concentrations in the Australian Wine Research (AWRI) laboratory was reported as approximately 0.05 mg/g berry (Gishen *et al.*, 2002). All four SEL values for anthocyanin data sets were in good agreement with this value. Both the 2004 and 2005 final anthocyanin data sets had lower SEL values than the 2004 and 2005 original data sets. The lower SEL values of the final data sets indicate higher reference method accuracy that will be beneficial for calibration model development. Contrary to the total anthocyanins data sets the total phenolics SEL values for the original 2004 and 2005 data sets were exactly the same (0.09 OD280/g) and the SEL for the 2005 final total phenolics data set was even slightly higher than that of the 2004 final data set. A possible explanation for the contradictory SEL values for total anthocyanins and total phenolics is that the total phenolics reference might be more sensitive due to the absorption of components other than phenolic compounds at 280 nm.

Table 4. Standard error of laboratory values (SEL) for total anthocyanins and total phenolics data sets.

Vintage	n* ¹ Total anthocyanins data sets	SEL * ¹ Total anthocyanins data sets (mg/g)* ²	n* ¹ Total phenolics data sets	SEL * ¹ Total phenolics data sets (OD280/g)* ³
2004 Original data set	120	0.08	120	0.09
2004 Final data set	89	0.04	95	0.05
2005 Original data set	727	0.06	727	0.09
2005 Final data set	603	0.03	553	0.07

*¹ Abbreviations used: n, number of samples; SEL, standard error of laboratory

*² Expressed as mg anthocyanins per gram berry

*³ Expressed as OD280 per gram berry

4.6 CONCLUSIONS

The results obtained during the evaluation of reference analysis method protocol suggest that the second extraction step in HCL could be cut back from 180 to 60 minutes. This implies a big time saving during reference method analyses. However, these experiments must be repeated and thoroughly evaluated before such an adjustment to the reference analysis method can be made. An experiment showed that the reference analysis method has good repeatability. For calibration purposes a number of samples were removed from data sets based on poor repeatability of duplicate determinations to ensure that the optimal calibrations models could be obtained (Chapter 3). The standard error of laboratory values (SEL) for both total anthocyanins and total phenolics data sets were satisfactory low. For future analyses the aim is to keep the SEL for both total anthocyanins and total phenolics concentrations analyses at these low levels or to improve reference analysis method accuracy even further.

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RESEARCH RESULTS

The evaluation of the possibility to implement grape total anthocyanins and total phenolics concentrations analyses at a commercial winery in South Africa – a preliminary study

RESEARCH RESULTS

ABSTRACT

In the quest for more objective measures of grape quality, the total anthocyanins and total phenolics concentrations analyses of grapes were evaluated as two additional parameters to include as part of grape quality control at a commercial winery in South Africa for the cultivars Cabernet Sauvignon, Merlot, Pinotage and Shiraz. During this investigation 603 grape samples from the 2005 vintage were analysed for these two components. Principal component analysis (PCA) emphasized the importance of measuring many chemical components for the purpose of capturing enough information to discriminate between samples. It was established that as the sugar concentrations of grapes increase both the total anthocyanins and total phenolics concentrations increase. For the 2005 vintage the mean total anthocyanins concentration for Shiraz grapes was the highest (1.44 mg/g) followed by Pinotage (1.28 mg/g), Cabernet Sauvignon (1.22 mg/g) and Merlot (1.18 mg/g). Pinotage grapes had the highest mean total phenolics concentration (1.53 OD280/g) followed by Shiraz (1.47 OD280/g), Merlot (1.38 OD280/g) and Cabernet Sauvignon (1.26 mg/g). For all investigated *Vitis vinifera* cultivars a general trend was observed that earlier harvested grapes had significantly higher ($p \leq 0.05$) total anthocyanins and total phenolics concentrations than grapes harvested later in the season. Cabernet Sauvignon and Merlot grapes contained approximately 33% less anthocyanins in the later harvested grapes. Results showed that grape samples from regularly irrigated vineyards had lower total anthocyanins and total phenolics concentrations compared to dryland vineyards. An investigation involving the viticultural block grading revealed that the total anthocyanins and total phenolics concentrations of A graded grapes (higher grape quality) was not significantly higher ($p > 0.05$) than that of C graded grapes (lower grape quality). Consequently the addition of the total anthocyanins and total phenolics concentrations analyses of grapes as part of viticultural block classification protocol is recommended. The results of this study showed that the addition of the two parameters total anthocyanins and total phenolics concentrations as part of grape quality control will supply additional data according to which grapes can be more accurately classified.

5.1 INTRODUCTION

Increased grape production in certain parts of the globe causes large volumes of wines to flood wine markets. Wine consumers are spoilt for choice and offered high-quality wines at low prices. In these challenging conditions for wine producers one solution to remain economically viable is to improve wine quality and sell wines at higher price points. Presumably consumers will pay more money for wines of higher quality that offers more pleasure. To aid in the quest to improve the quality of wines it is important to identify grape components and winemaking practices that contribute most to final wine quality and to develop objective measures of these.

More information about the chemical composition of grapes is to the advantage of both grape producers and winemakers. The standard quality assessment protocol of grapes at most wine cellars in South Africa involves the pre-assessment of viticultural practices and the determination of chemical parameters such as total soluble solids (TSS), pH value and titratable acidity (TA). These measurements, without doubt, are important indicators of grape quality but there is a need for additional objective analyses that correlate with grape quality. Phenolic compounds such as tannins are associated with sensory sensations such as astringency and mouth-feel of wine (Herderich and Smith, 2005) and it is widely speculated that wine tannins are correlated to red wine quality (Herderich *et al.*, 2004). Wine colour intensity is an important indicator of wine quality. The anthocyanin concentration of grapes is highly correlated with wine colour intensity (Somers and Evans, 1974; Jackson *et al.*, 1978; Iland, 1987; Francis *et al.*, 1999; Marais *et al.*, 2001; Marais and October, 2005). In a study investigating the relationship between grape colour and wine quality in South Africa, Marais and October (2005) found that Shiraz showed the most potential to predict wine quality based on grape colour to a 66-71% level of accuracy. On the other hand Pinotage and Cabernet Sauvignon did not show such a positive correlation. These correlations were valid for grape anthocyanin concentrations of up to 1.7 mg anthocyanins/g berry weight. Therefore the determination of the total phenolics and anthocyanin concentration of grapes provide additional objective measures of grape quality.

The measurement of the total phenolic and anthocyanin concentration of grapes could allow better evaluation of different vineyard management practices and provide additional information to make more informed decisions regarding optimum harvest dates of grapes. The addition of total phenolics and anthocyanin concentration of grapes as quality classification parameters could lead to more accurate classification of grapes, the refinement of payment schemes, better streaming of grape must inside the cellar to fit particular wine styles and achieve maximum phenolic composition and wine colour within different wine quality categories instead of having one average blend (Damberg *et al.*, 2003).

This study was a preliminary evaluation of the implementation of the two chemical parameters total anthocyanins and total phenolics concentrations as part of routine quality monitoring of grapes at Distell (Distell Group (Pty) Ltd), a commercial winery in South Africa. The objectives of this study were to draw statistically significant conclusions about (1) the distribution of total anthocyanins and total phenolics concentrations in grapes from different maturity levels, cultivars, irrigation treatments and origin delivered to Distell; and (2) establish whether the total anthocyanins and phenolics concentrations of grapes correlates with conventional chemical parameters used for the classification of grape quality as well as grape grading according to current viticultural practices.

5.2 MATERIALS AND METHODS

5.2.1 GRAPE SAMPLES AND HOMOGENATE PREPARATION

During the 2005 vintage 603 whole bunch grape samples were collected from Distell (Distell Group (Pty) Ltd), South Africa. Grape bunches were either picked in the vineyards or sampled from loads delivered at the respective weighbridges of the Adam Tas and Bergkelder wine production facilities. Distell viticulturists did pre-harvest grape quality assessments. Vineyard blocks were classified taking into account factors such as the vineyard location, the standard of vineyard management practices and the sensory evaluation of grapes. Block history with regards to vineyard management practices and wine quality from previous vintages had also been taken into account during block grading. Vineyard blocks were classified as A, B or C with grapes from A and C blocks expected to be of the highest and lowest quality, respectively. Samples consisted of 5 to 10 bunches of grapes each and were of *Vitis vinifera* cultivars Cabernet Sauvignon, Merlot, Pinotage and Shiraz. All grape samples originated from the Western Cape region in South Africa (Figure 1) and were received on the day of picking. Due to limited storage space samples were stored overnight at 0 or 4°C. Grape berries were hand picked from bunches and kept in containers until analysed. For each sampled block a randomly picked sub sample of 100 berries was weighed and the mean berry weight recorded. Subsequently grape berries were homogenised for approximately 5 seconds in a colloid mill designed for homogenising soil samples and the resulting uniformly smooth pastes were analysed on the same day of homogenisation.

5.2.2 QUANTIFICATION OF TOTAL ANTHOCYANINS AND TOTAL PHENOLICS

The total anthocyanins and total phenolics concentrations of grape samples were determined in duplicate on grape homogenates according to the method described by Iland *et al.* (2000). Results were reported as mean values (section 4.2.4.1, equation 1). See Chapter 4 for full details of an evaluation of the reference protocol.

5.2.3 DETERMINATION OF SUGAR, pH, TITRATABLE ACIDITY, TARTARIC ACID, MALIC ACID AND POTASSIUM AND OTHER CHEMICAL COMPONENTS

Grape homogenates were centrifuged and supernatants filtered using filter paper circles graded at 20-25 µm with a diameter of 50 mm (Schleicher & Schuell, reference number 10312706). The sugar (°Brix), titratable acidity (TA), tartaric acid, malic acid, potassium concentrations and pH as well as other chemical components were determined with a WineScan FT 120 spectrometer (Foss Analytical, Denmark). Due to clogging of the cuvette the instrument was cleaned between every sample using the S-470 Cleaning Agent Solution and zeroed at regular intervals using Zero Liquid S-6060 (Foss WineScan FT 120 Type 77110 and 77310 Operator's Manual, Foss Analytical, Denmark, 2001). The commercial calibration models available with the WineScan were used to quantify the mentioned chemical components (Foss Analytical, Denmark). During

this study no validation of the commercial calibration models was made but the commercial calibration models were validated previously (personal communication Distell) and all FT-IR analyses were performed on the same instrument.

5.2.4 STATISTICAL ANALYSIS

5.2.4.1 Statistical equations

Standard statistical equations were used to calculate the means and standard deviations values for sugar, pH, titratable acidity (TA), tartaric acid, malic acid and potassium (equations 1 and 2) (Snedecor and Cochran, 1980). Correlations between chemical components were calculated according to equation 3 (Snedecor and Cochran, 1980).

$$\text{Mean or } \bar{x} = \frac{\sum_{i=1}^n x_i}{n} \quad \dots\dots 1$$

$$\text{Standard deviation or SD} = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n-1}} \quad \dots\dots 2$$

$$\text{Correlation or } r = \frac{\frac{1}{n} \sum_i (x_i - \bar{x})(y_i - \bar{y})}{S_x S_y} \quad \dots\dots 3$$

where S_x and S_y are the standard deviation of x and y respectively.

5.2.4.2 One-way analysis of variance (ANOVA) and post-hoc analysis

Result means data were subjected to one-way analysis of variance (ANOVA) and post-hoc analysis (Bonferroni tests). When multiple comparisons are done the reported probability levels overestimate the statistical significance of mean differences. The Bonferroni adjustment is used to make it more difficult for any one test to be significant. Results were reported at the 5% significance level. The Statistica version 7.0 software (Tulsa, OK. U.S.A.) was used for all statistical analyses.

5.2.4.3 Multivariate data analysis

Principal component analysis (PCA) of the chemical data obtained with the WineScan was done with The Unscrambler Software (version 9.2, Camo AS, Norway). For this purpose data were organised in one two-dimensional matrix table where the columns were defined by X-variables (in this study the various chemical components quantified by the WineScan), while the rows were defined by the grape homogenate samples. PCA models the interrelationships between the different variables and facilitates the detection and interpretation of sample patterns, similarities and differences (Esbensen, 2002, Naes *et al.*, 2002). Each principal component (PC)

is a linear function of the original variables and collectively they describe in descending order the main structured information in the data. Every PC can be interpreted individually, as they are calculated orthogonal to one another. X-variables were scaled by multiplying each of the original values by $1/SD$ (SD, standard deviation).

5.3 RESULTS AND DISCUSSION

5.3.1 ORIGIN OF GRAPE SAMPLES

Grape samples of the 2005 vintage used in this study originated from diverse terroir units in the Western Cape, South Africa which differ amongst others with regard to altitude above sea-level, soil types, rainfall, wind or sea breezes and temperatures (Figure 1). More than half of the number of samples ($\pm 62\%$) originated from Stellenbosch ($n = 373$). A large number of samples also originated from the nearby Paarl region ($n = 104$). The three areas Darling ($n = 28$), Malmesbury ($n = 46$) and Philadelphia ($n = 6$) are collectively referred to as the Swartland area ($n = 80$). Fewer samples (15 or less) were analysed for the following areas: Durbanville ($n = 14$), Walker Bay ($n = 10$), Overberg ($n = 9$), Somerset West ($n = 8$), Philadelphia ($n = 6$), Piketberg ($n = 2$) and Robertson ($n = 1$). Information regarding the origin of two samples was not available.

5.3.2 RELATIONSHIPS BETWEEN TOTAL ANTHOCYANINS CONCENTRATIONS, TOTAL PHENOLICS CONCENTRATIONS AND GRAPE CHEMICAL PARAMETERS

In order to model the relationships between the samples on the basis of chemical composition, the chemical components quantified by the commercial calibrations on the WineScan instrument were used. At the time of this study, only the commercial calibrations for Brix, pH, titratable acidity, tartaric acid, malic acid and potassium had been validated and were used for modelling (See Appendix 1). Relationships between grape homogenate samples showed no obvious clustering in PCA analysis (Figure 2). On PC1 positive X-loadings for Brix, pH, tartaric acid and potassium and negative X-loadings for titratable acidity and malic acid explain the most important variance between the samples. Positive X-loadings for mg anthocyanin/g berry and OD280/g berry explained the separation of samples towards the positive end of PC2.

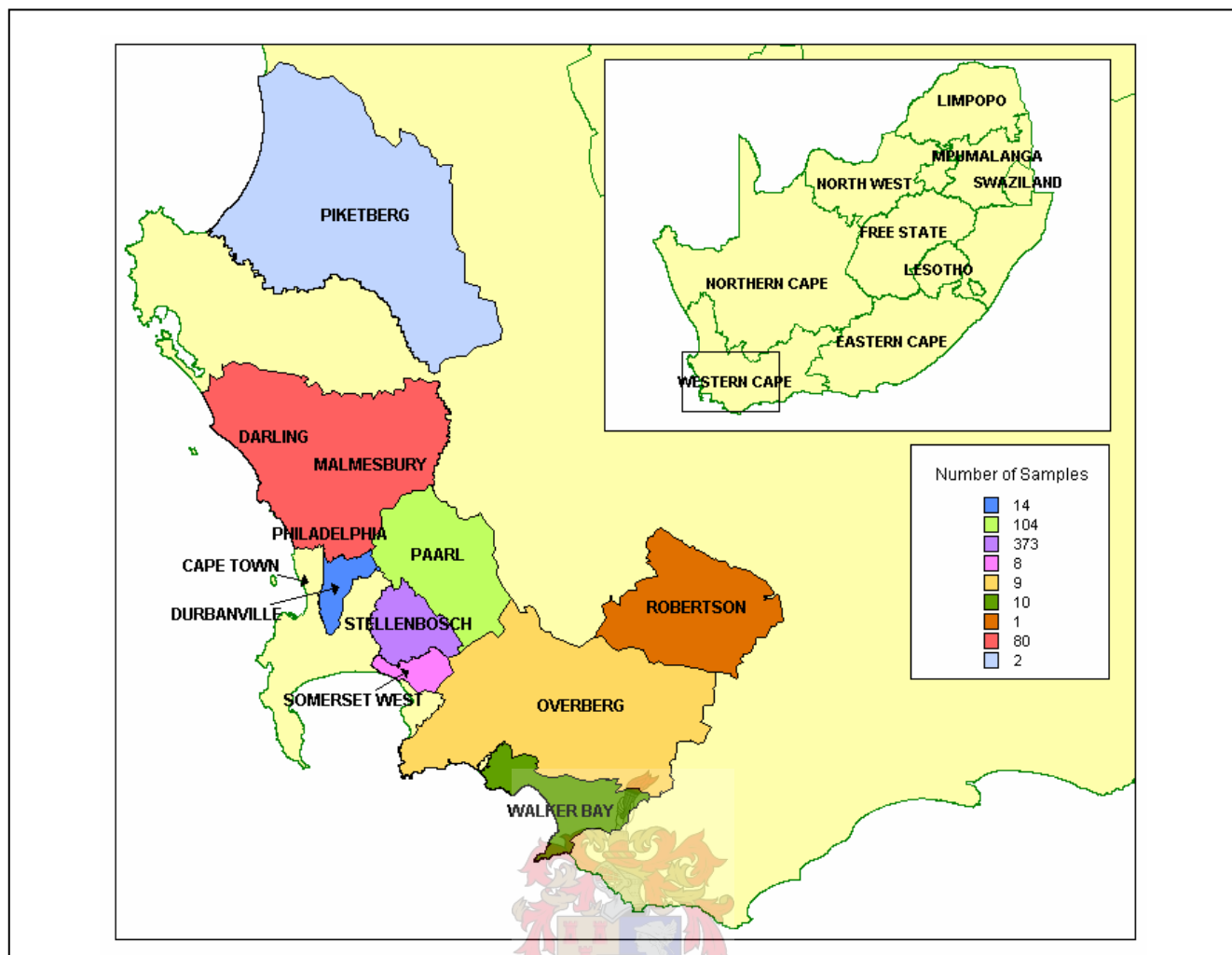


Figure 1. A map of the Western Cape region of South Africa illustrating the different areas where the samples used in this study originated from.

In subsequent analysis all the chemical components available on the WineScan were used (Figure 3). The components are included in the WineScan commercial calibration Must-Sanitary state (Foss application notes, GrapeScan Calibration: Must – Sanitary State). Only the commercial calibrations for °Brix, pH, titratable acidity, tartaric acid, malic acid and potassium have been validated. With all the additional X-variables included in modelling the Pinotage samples located primarily along the positive ends of PC1 and PC2 and clearly had higher loadings for some variables (Figure 3a). On PC1 positive X-loadings for Brix, density, glucose-fructose, pH, potassium, tartaric acid, ethyl acetate and colour intensity and negative X-loadings for titratable acidity, malic acid and lactic acid explained the most important variance between the samples. On PC2 positive X-loadings for isoamylacetate and gluconic acid contributed considerably to the separation of samples on the positive end of PC2 (Figure 3b). Gluconic acid is correlated with acetic acid bacteria or with the fungal grapevine pathogen *Botrytis cinerea*. On most of the grapes no infection was observed but these results indicate that trace levels of these pathogens did occur. Isoamylacetate is an ester aroma compound associated with the distinct aroma of Pinotage wines. This result was highlighted further by the 3-dimensional score plot (Figure 4) and showed that the Pinotage samples separated primarily on PC2, while the

Cabernet Sauvignon, Shiraz and Merlot samples showed similar patterns of separation along PC1.

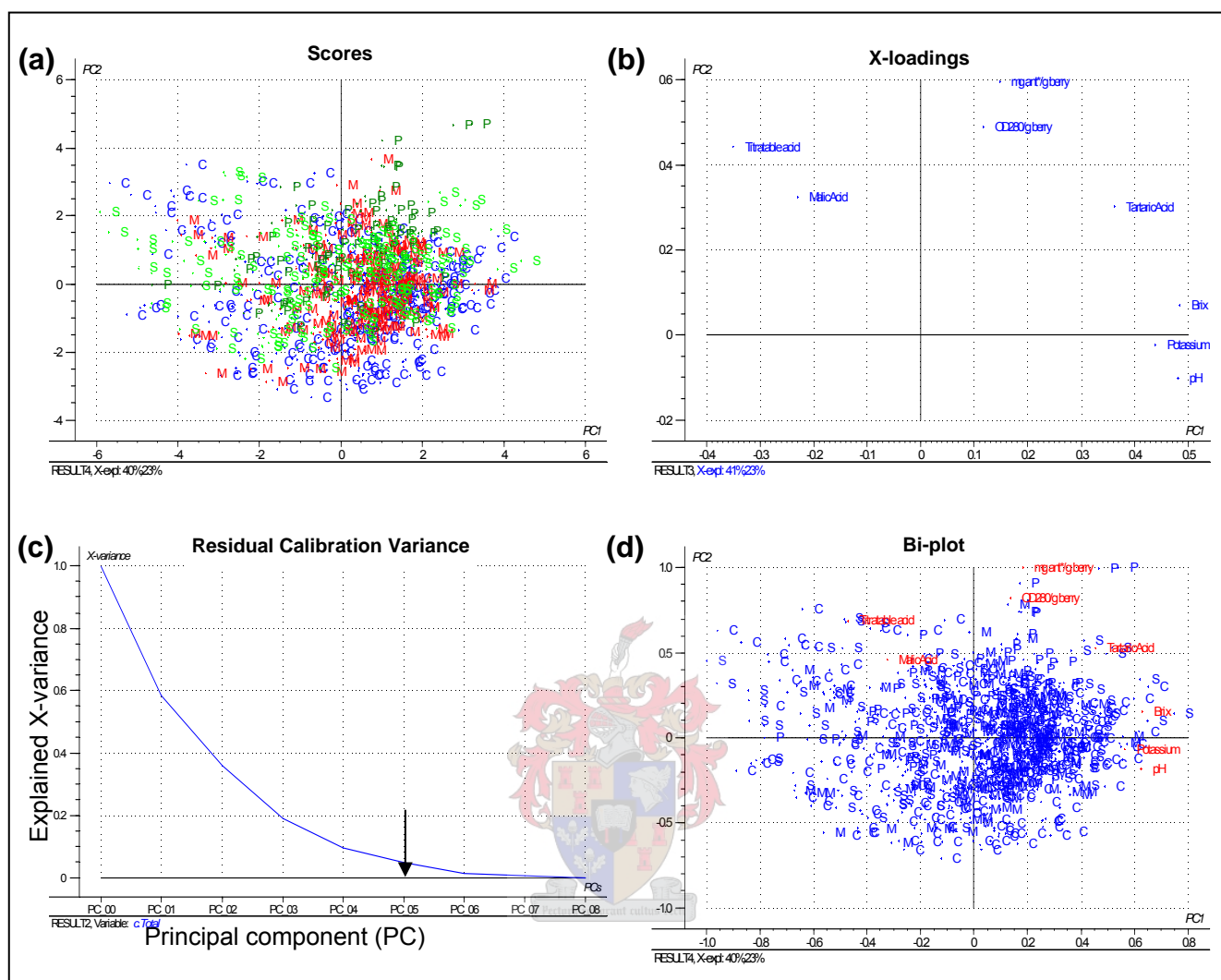


Figure 2. PCA of grape homogenate samples ($n = 603$) of the 2005 vintage. a) Score plot of PC1 (40% X-variance explained) and PC2 (23% X-variance explained). C: Cabernet Sauvignon (blue markers), M: Merlot (red markers), P: Pinotage (dark green markers), S: Shiraz (light green markers). b) Loadings plot of PC1 and PC2. c) Residual calibration variance plot showing that 5 PC's describe more than 95% of the X-variance. d) Scores and loadings from the PCA model showing relationships between samples and X-variables in the data set. X-variables (red markers), cultivars (blue markers).

On PC1 (Figure 5) positive X-loadings for ethylacetate, mesoinositol, butanediol, FolinC Index, color intensity, tartaric acid and negative X-loadings for lactic acid explained important variation in the samples. On PC2 positive X-loadings for titratable acidity, lactic acid and negative X-loadings for mannitol and volatile acidity explained variation along PC2. These results highlight the importance of measuring many chemical components for the purpose of capturing enough information to discriminate between samples. This result was of particular interest for the purposes of identifying which parameters to include in the assessment of grape

quality and could point to differential scales required by the various grape cultivars Cabernet Sauvignon, Merlot and Shiraz and the variance between the Pinotage samples.

Linear regressions were done to establish the extent of the relationships between chemical components of grapes (WineScan results) and the total anthocyanins and total phenolics concentrations respectively. Positive correlations were obtained between the sugar concentrations of grapes and the respective total anthocyanins ($r = 0.36$) and total phenolics ($r = 0.30$) concentrations. Slightly stronger correlations were established between the tartaric acid concentrations of grapes and the respective total anthocyanins ($r = 0.43$) and total phenolics ($r = 0.40$) concentrations. However, none of the before mentioned correlations were strong. Regression analysis revealed no correlations between the respective total anthocyanins and total phenolics concentrations and the pH values, malic acid and potassium concentrations of grapes.

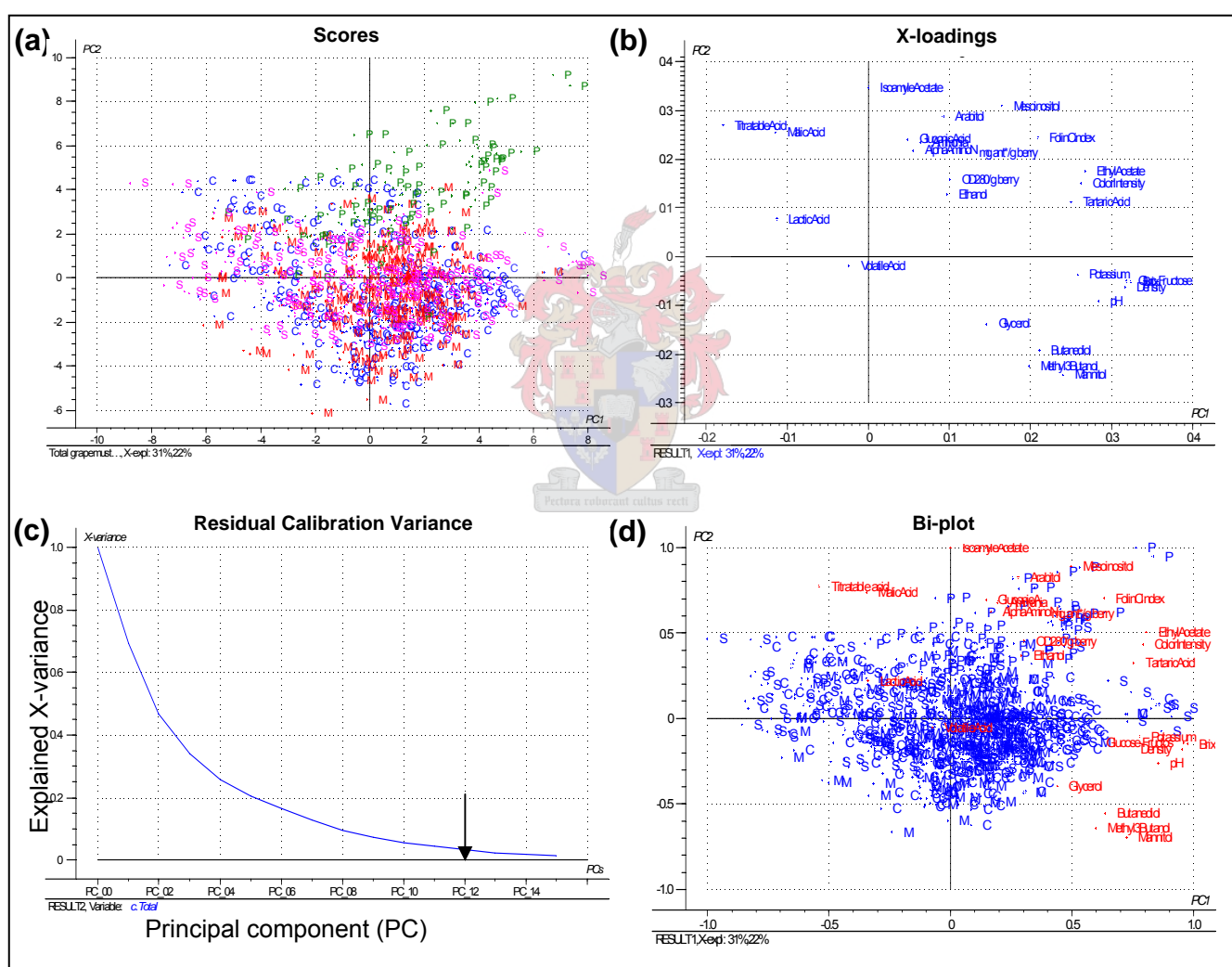


Figure 3. PCA of grape homogenate samples (n=603) of the 2005 vintage. a) Score plot of PC1 (40% X-variance explained) and PC2 (23% X-variance explained). C: Cabernet Sauvignon (blue markers), M: Merlot (red markers), P: Pinotage (green markers), S: Shiraz (pink markers). b) Loadings plot of PC1 and PC2. c) Residual calibration variance plot showing that 12 PC's described more than 95% of the X-variance. d) Scores and loadings from the PCA model showing relationships between samples and X-variables in the data set.

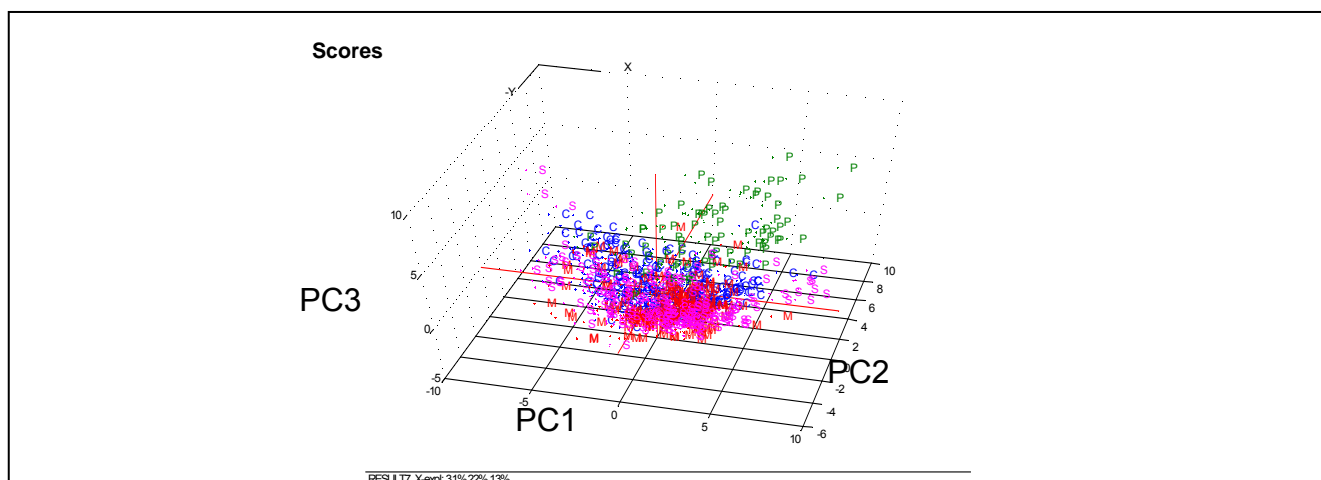


Figure 4. PCA of grape homogenates (n = 603, of the 2005 vintage). Three-dimensional score plot of PC1 (31% X-variance explained), PC2 (33% X-variance explained) and PC3 (13% X-variance explained). C: Cabernet Sauvignon (blue markers), M: Merlot (red markers), P: Pinotage (green markers), S: Shiraz (pink markers). Positive loadings for X-variables on PC2 explain most of the variation in the Pinotage samples.

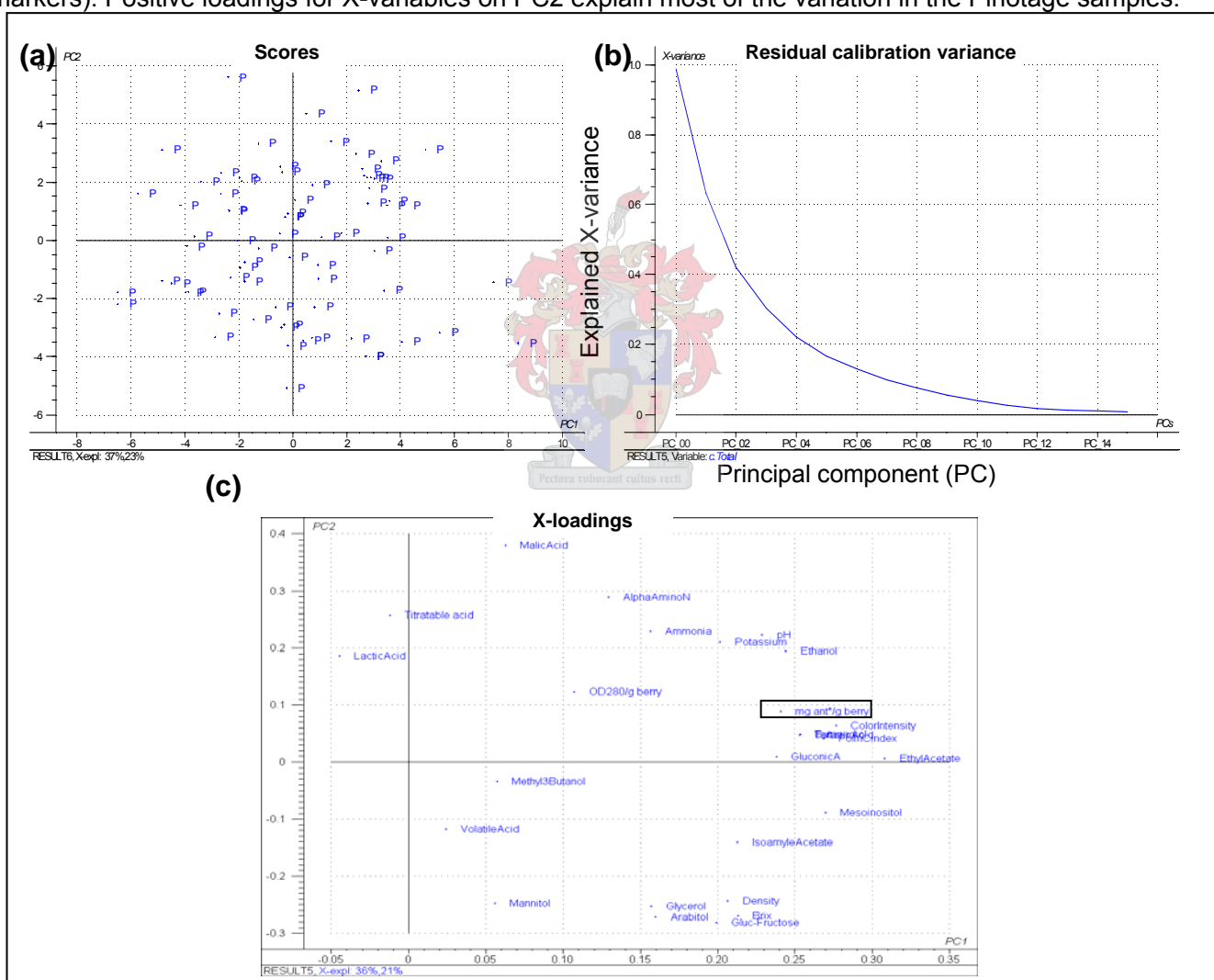


Figure 5. PCA of Pinotage homogenates (n = 64, vintage 2005). a) Score plot of PC 1 (37% X-variance explained) and PC2 (23% X-variance explained). b) Residual calibration variance plot showing that 12 PC's described more than 95% of the X-variance. c) Loadings plot of PC1 and PC2. Variables highlighted in the square were butanediol, FolinC index, Tartaric acid.

5.3.3 GRAPE ANALYSIS RESULTS

Sample statistics for sugar, pH, TA, tartaric acid, malic acid, potassium, berry weight, total anthocyanins and total phenolics concentrations are summarized in Table 2. Samples were grouped according to grape maturity (sugar or °Brix), grape cultivar, irrigation treatment and viticultural block grading. The results of post-hoc analysis (Bonferroni tests) of the result means, obtained from one-way ANOVA, are summarized using superscripts (Table 2). The high pH levels and potassium concentration as well as very low TA levels are expected due to the fact that the analyses were done on homogenised grapes. This would have released potassium from the grape skins which would cause the precipitation of potassium bitartrate and the subsequent increase in pH. Grape homogenisation might have influenced the results of grape chemical components, for example the mean titratable acidity values were lower and the mean pH values higher than expected. As a result of homogenisation potassium from grape skins were released and might have caused the precipitation of acids and caused the subsequent increased pH values of homogenates.

5.3.3.1 Distribution of grape chemical parameters in relation to sugar concentration

Data of all grape homogenates ($n = 603$) were divided into three sets using the sugar concentration (°Brix) of each sample as variation criterion (Table 2). The separation according to the arbitrary chosen °Brix intervals divided grape homogenates into three samples sets: low sugar ($y < 23.5^\circ\text{Brix}$), medium sugar ($23.5^\circ\text{Brix} \leq y < 26^\circ\text{Brix}$) and high sugar ($y \geq 26^\circ\text{Brix}$) (Table 2). Most grape homogenates were categorized as low sugar ($n = 240$) or medium sugar ($n = 241$). The ANOVA results (Table 2) revealed that the parameters sugar, pH, potassium and tartaric acid among all three grape maturity levels differed significantly ($p \leq 0.05$). Grape homogenates from low sugar grapes had the lowest and samples from high sugar grapes the highest respective potassium and tartaric acid concentrations and pH values. Therefore, with increasing grape maturity potassium concentrations increase which leads to an increase in pH values. The TA and malic acid concentration of grape homogenates from low sugar grapes were significantly higher ($p \leq 0.05$) than that of both sample sets with higher sugar concentrations. The berry weights of low and medium sugar grapes were significantly higher ($p \leq 0.05$) than the berry weights of high sugar samples. The lower berry weights of high sugar berry samples could be due to prolonged exposure of the berries to sunlight, subsequent moisture loss and berry shrinking probably indicating over ripe grapes. Total anthocyanins and phenolics concentrations differed significantly between the three sets of sugar concentrations ($p \leq 0.05$) (Figures 4a and 4b). Concentrations of both total anthocyanins and total phenolics increased with increasing sugar concentrations or grape maturity level. Non-overlap of error-bars indicates statistical significance at level 0.05.

Table 2. General statistics for data sets.

Sample set	n* ¹	Sugar (°Brix)	pH	Titrateable acidity (TA)* ⁴	Tartaric acid (g/L)	Malic acid (g/L)	Potassium (mg/L)	Berry weight (g)	Total anthocyanins (mg/g)* ⁵	Total phenolics (OD280/g)* ⁶
		Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean ± SD* ¹	Mean* ³ ± SD* ¹
All samples	603	23.9 ± 2.8	3.85 ± 0.18	4.5 ± 1.0	6.3 ± 0.7	3.3 ± 1.0	2439 ± 489	1.47 ± 0.22	1.19 ± 0.33	1.34 ± 0.24
<u>Sugar concentration</u>										
Low sugar	240	21.0 ^c ± 1.8	3.75 ^c ± 0.18	5.0 ^a ± 1.2	5.9 ^c ± 0.7	3.8 ^a ± 1.0	2218 ^c ± 480	1.51 ^a ± 0.23	1.07 ^c ± 0.36	1.27 ^c ± 0.27
Medium sugar	241	24.8 ^b ± 0.7	3.89 ^b ± 0.13	4.2 ^b ± 0.7	6.5 ^b ± 0.6	3.0 ^b ± 0.8	2498 ^b ± 373	1.49 ^a ± 0.21	1.21 ^b ± 0.29	1.36 ^b ± 0.21
High sugar	122	27.5 ^a ± 1.2	3.98 ^a ± 0.15	4.0 ^b ± 0.6	6.9 ^a ± 0.5	3.0 ^b ± 0.8	2756 ^a ± 502	1.36 ^b ± 0.18	1.37 ^a ± 0.23	1.46 ^a ± 0.18
<u>Cultivar</u>										
Cabernet Sauvignon	127	25.5 ^b ± 1.3	3.98 ^a ± 0.12	4.2 ^b ± 0.5	6.7 ^{ab} ± 0.4	2.9 ^c ± 0.5	2773 ^a ± 330	1.33 ^c ± 0.18	1.22 ^b ± 0.27	1.26 ^c ± 0.20
Merlot	108	25.2 ^b ± 1.1	3.85 ^c ± 0.11	4.0 ^c ± 0.6	6.6 ^{bc} ± 0.6	2.5 ^d ± 0.4	2329 ^c ± 292	1.56 ^a ± 0.20	1.18 ^b ± 0.26	1.38 ^b ± 0.19
Pinotage	44	25.9 ^{ab} ± 1.6	3.90 ^{bc} ± 0.15	5.0 ^a ± 0.5	7.0 ^a ± 0.6	4.4 ^a ± 0.9	2480 ^{bc} ± 450	1.51 ^{ab} ± 0.16	1.28 ^b ± 0.23	1.53 ^a ± 0.22
Shiraz	84	26.6 ^a ± 2.0	3.93 ^{ab} ± 0.16	3.8 ^c ± 0.6	6.4 ^c ± 0.6	3.2 ^b ± 0.6	2684 ^{ab} ± 547	1.42 ^{bc} ± 0.19	1.44 ^a ± 0.27	1.47 ^{ab} ± 0.19
<u>Irrigation</u>										
Irrigated	304	25.7 ^a ± 1.6	3.92 ^a ± 0.14	4.1 ^b ± 0.6	6.6 ^a ± 0.6	3.0 ^a ± 0.8	2575 ^a ± 448	1.45 ^a ± 0.20	1.24 ^a ± 0.28	1.39 ^a ± 0.21
Dryland	37	25.8 ^a ± 1.8	3.96 ^a ± 0.13	4.4 ^a ± 0.7	6.7 ^a ± 0.6	3.2 ^a ± 0.9	2684 ^a ± 405	1.36 ^a ± 0.21	1.37 ^a ± 0.27	1.44 ^a ± 0.18
Supplementary irrigation	19	25.6 ^a ± 1.3	3.87 ^a ± 0.14	4.4 ^{ab} ± 0.8	6.7 ^a ± 0.5	3.3 ^a ± 0.8	2530 ^a ± 318	1.48 ^a ± 0.21	1.40 ^a ± 0.21	1.38 ^a ± 1.83
<u>Block grading</u>										
A graded	118	25.6 ^a ± 1.5	3.88 ^b ± 0.13	4.4 ^a ± 0.7	6.7 ^a ± 0.6	3.2 ^a ± 0.9	2487 ^b ± 390	1.50 ^a ± 0.22	1.33 ^a ± 0.29	1.42 ^a ± 0.20
B graded	179	25.8 ^a ± 1.5	3.94 ^a ± 0.14	4.0 ^b ± 0.5	6.6 ^a ± 0.5	2.8 ^b ± 0.6	2635 ^a ± 409	1.43 ^{ab} ± 0.21	1.21 ^b ± 0.27	1.33 ^b ± 0.20
C graded	61	25.8 ^a ± 2.0	3.94 ^a ± 0.17	4.2 ^a ± 0.7	6.8 ^a ± 0.7	3.2 ^a ± 1.0	2625 ^{ab} ± 575	1.38 ^a ± 0.14	1.31 ^{ab} ± 0.28	1.51 ^a ± 0.19

Significance of least significant difference (LSD) test of treatment means at p≤0.05 and standard deviation. Means with the same superscript in the same column are not significantly different (p>0.05).

*¹ Abbreviation used: n, number of samples; SD, standard deviation

*² As determined by the WineScan

*³ As determined by the reference method

*⁴ Expressed as g/L tartaric acid

*⁵ Expressed as mg anthocyanins per gram berry weight

*⁶ Expressed as OD280 per gram berry weight

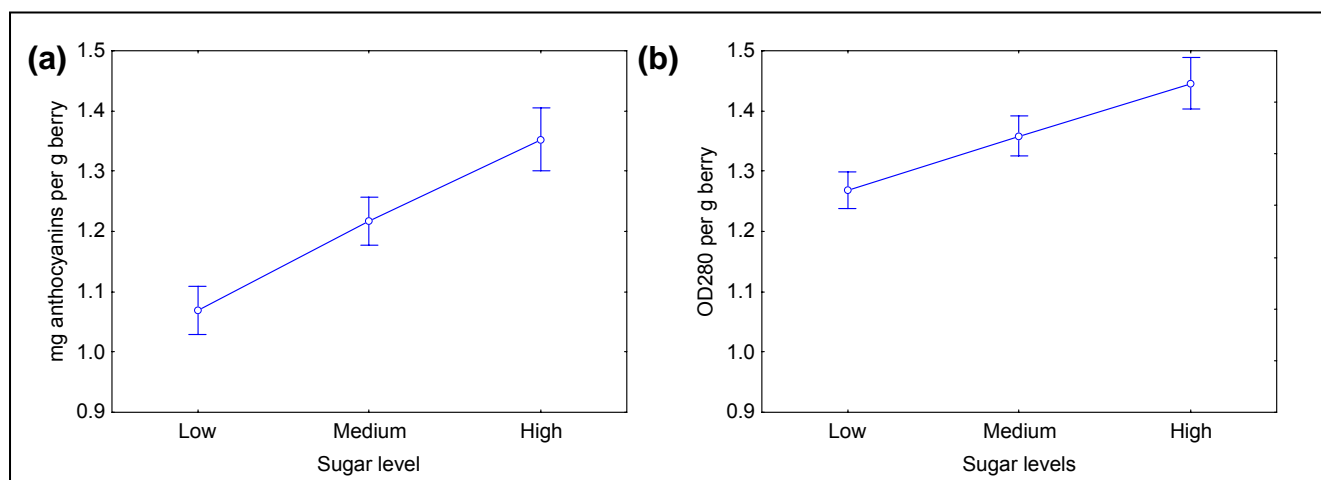


Figure 4. Significance of the relationship between the sugar concentration and (a) total anthocyanins, and (b) total phenolics concentrations.

5.3.3.2 Distribution of grape chemical parameters in *Vitis vinifera* cultivars

Data were compared of four sample sets consisting of homogenate samples of *Vitis vinifera* cultivars Cabernet Sauvignon (n = 127), Merlot (n = 108), Pinotage (n = 44) and Shiraz (n = 84) (Table 2). To compare grape samples at more or less the same physiological maturity level only samples with sugar concentrations $y \geq 23.5^\circ\text{Brix}$ were included in the sample sets. Due to the fact that samples were collected in a commercial environment there was an uneven distribution of the number of samples per cultivar. Note that more data from more vintages are needed before final conclusions can be made regarding the total anthocyanins and total phenolics concentrations of the four *Vitis vinifera* cultivars investigated during this study.

The sugar concentration of Shiraz was the highest and was significantly higher ($p \leq 0.05$) than that of Cabernet Sauvignon and Merlot. Merlot had the lowest sugar concentration (Table 2). No significant association between the sugar concentration of Pinotage and that of the other three cultivars could be established ($p > 0.05$). However, it was slightly lower than the sugar concentration of Shiraz but higher than that of Cabernet Sauvignon and Merlot.

The pH value of Merlot was the lowest and differed significantly ($p \leq 0.05$) from the pH values for Cabernet Sauvignon and Shiraz. The pH value of Cabernet Sauvignon was the highest and differed significantly ($p \leq 0.05$) from that of Merlot and Pinotage. Shiraz and Pinotage had intermediate pH values and did not differ significantly from each other ($p > 0.05$). The TA, tartaric acid and malic acid concentrations of Pinotage was significantly higher than that of all the other cultivars ($p \leq 0.05$). The cultivar with the lowest TA, tartaric acid and malic acid content was Merlot. Cabernet Sauvignon had the highest potassium concentrations, followed by Shiraz and Pinotage. The potassium concentration of Merlot was significantly lower ($p \leq 0.05$) than the potassium concentrations of Cabernet Sauvignon and Shiraz. The berry weight of Merlot was the heaviest and was significantly higher

($p \leq 0.05$) than that of Shiraz and Cabernet Sauvignon. Neither the berry weights of Merlot nor Shiraz was significantly different from that of Pinotage ($p > 0.05$).

Shiraz had a significantly higher total anthocyanin concentration ($p \leq 0.05$) than any other cultivar (Figure 5a). Pinotage had the highest total phenolics content and differed significantly ($p \leq 0.05$) from that of Merlot and Cabernet Sauvignon (Figure 5b). Cabernet Sauvignon had a significantly lower total phenolics concentration than that of any other cultivar ($p \leq 0.05$). The ANOVA results together with the PCA results discussed in section 5.3.2 indicate that the phenolic composition of Pinotage differs from the other cultivars investigated in this study. This was also found by Rossouw and Marais (2004).

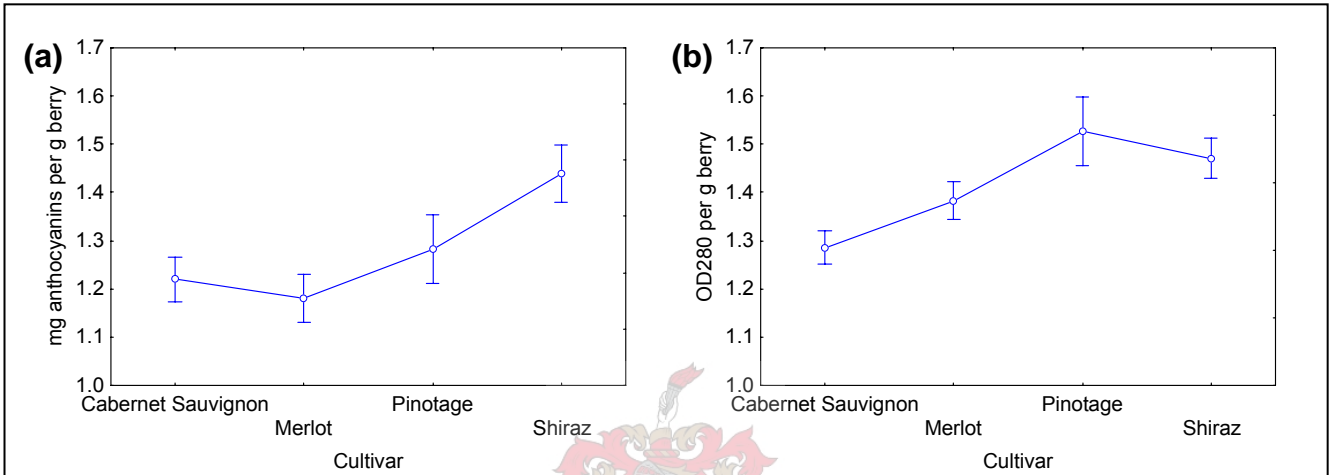


Figure 5. Significance of the relationship between the *Vitis vinifera* cultivar, and (a) total anthocyanins concentrations, and (b) total phenolics concentrations.

5.3.3.3 The effects of time of maturity and harvest on grape chemical parameters

Botting *et al.* (1996) reported that the greater the delay in maturity (measured as time to reach a sugar concentration of 22.5-23.5°Brix) the lower the concentration of berry anthocyanins. There are reports that the first grapes to reach maturity for a given variety within a particular climatic zone will produce the best wine (Coombe and Iland, 1987; Howell, 2001). In this study the effects of the time of maturity and harvest on total anthocyanins concentrations of grapes were investigated individually for each of the four *Vitis vinifera* cultivars (Table 3). Grape samples with a sugar concentration of $y \geq 23.5^\circ\text{Brix}$ were divided into three groups: early, intermediate and late according to days elapsed from the first day of harvest. The number of harvest days for Merlot was the highest ($n = 50$) and for Pinotage the lowest ($n = 35$). One way ANOVA results for all the data sets regarding the sugar and total anthocyanins concentrations are summarized in Table 3. For both Cabernet Sauvignon and Merlot there were significant differences between the early, intermediate and late harvested groups ($p \leq 0.05$) (Figures 6a and 6b). There was also a significant difference between the early and late harvested groups for Shiraz ($p \leq 0.05$) (Figure 6d). However, for Pinotage there was no significant differences between the mean total anthocyanins concentrations of the early, intermediate and late harvested groups

($p > 0.05$) (Figure 6c). Normally Pinotage is the first red grape cultivar harvested in South Africa and the earlier harvest dates of Pinotage (data not shown) as well as the shorter harvest day period ($n = 35$) may be reasons why the mean total anthocyanins concentrations of Pinotage did not decrease over time. Overall the mean total anthocyanins concentrations for all four *Vitis vinifera* cultivars decreased as the harvest season progressed. In the case of Cabernet Sauvignon and Shiraz most of the grapes were harvested in the intermediate and late periods of the season. The possible effects of chemical composition variation between early and late harvested grapes on wine quality should also be evaluated. The results indicate that viticultural practices should be further investigated to decrease the time of grapes to reach maturity.

Table 3. The effects of time of maturity and harvest on the total anthocyanins concentrations of *Vitis vinifera* cultivars.

Cultivar	Harvest season period	Days from the 1 st day of harvest	Total n ^{*1}	Block grading n ^{*1}			Sugar (°Brix)	Total anthocyanins (mg/g) ^{*4}
				A	B	C	Mean ^{*2} ± SD ^{*1}	Mean ^{*3} ± SD ^{*1}
Cabernet Sauvignon	Early	1-16	16	3	11	2	24.4 ^b ± 0.8	1.58 ^a ± 0.19
	Intermediate	17-32	49	13	28	8	25.7 ^a ± 1.1	1.27 ^b ± 0.21
	Late	33-48	62	10	43	9	25.7 ^a ± 1.5	1.08 ^c ± 0.22
Merlot	Early	1-16	29	12	14	3	25.1 ^a ± 1.0	1.43 ^a ± 0.22
	Intermediate	17-32	54	20	29	5	25.0 ^a ± 1.1	1.16 ^b ± 0.18
	Late	32-50	25	5	16	4	25.7 ^a ± 1.2	0.93 ^c ± 0.18
Pinotage	Early	1-11	12	4	-	8	24.9 ^b ± 1.2	1.35 ^a ± 0.37
	Intermediate	10-19	20	10	6	4	26.7 ^a ± 1.3	1.31 ^a ± 0.14
	Late	20-35	12	10	-	2	25.3 ^{ab} ± 1.5	1.17 ^a ± 0.12
Shiraz	Early	1-16	14	7	2	5	25.5 ^b ± 2.2	1.64 ^a ± 0.22
	Intermediate	17-32	40	20	14	6	25.9 ^b ± 1.5	1.44 ^{ab} ± 0.29
	Late	32-49	30	4	16	10	27.9 ^a ± 1.8	1.34 ^b ± 0.22

Significance of least significant difference (LSD) test of treatment means at $p \leq 0.05$ and standard deviation. Means with the same superscript in the same column are not significantly different ($p > 0.05$).

*1 Abbreviation used: n, number of samples; SD, standard deviation

*2 As determined by the WineScan

*3 As determined by the reference method

*4 Expressed as mg anthocyanins per gram berry weight

Subsequently the effects of the time of harvest on the total phenolics concentrations of grapes were investigated. Grape homogenate data were divided according to the same criteria as that used during the total anthocyanins investigation (Table 4). There were significant differences between the mean total phenolics concentrations of the early and late harvested groups for both Cabernet Sauvignon and Merlot ($p \leq 0.05$) (Figures 7a and 7b). However there were no significant differences between the early, intermediate or late harvested groups for both Pinotage and Shiraz ($p > 0.05$) (Figures 7c and 7d). There was a decrease in the mean total phenolics concentrations of grapes as the harvest season progressed but it does not seem to be as marked as the decrease in the mean total anthocyanins concentrations. It seems as if delayed maturity has a more pronounced effect on decreasing anthocyanin than phenolic concentration of grapes.

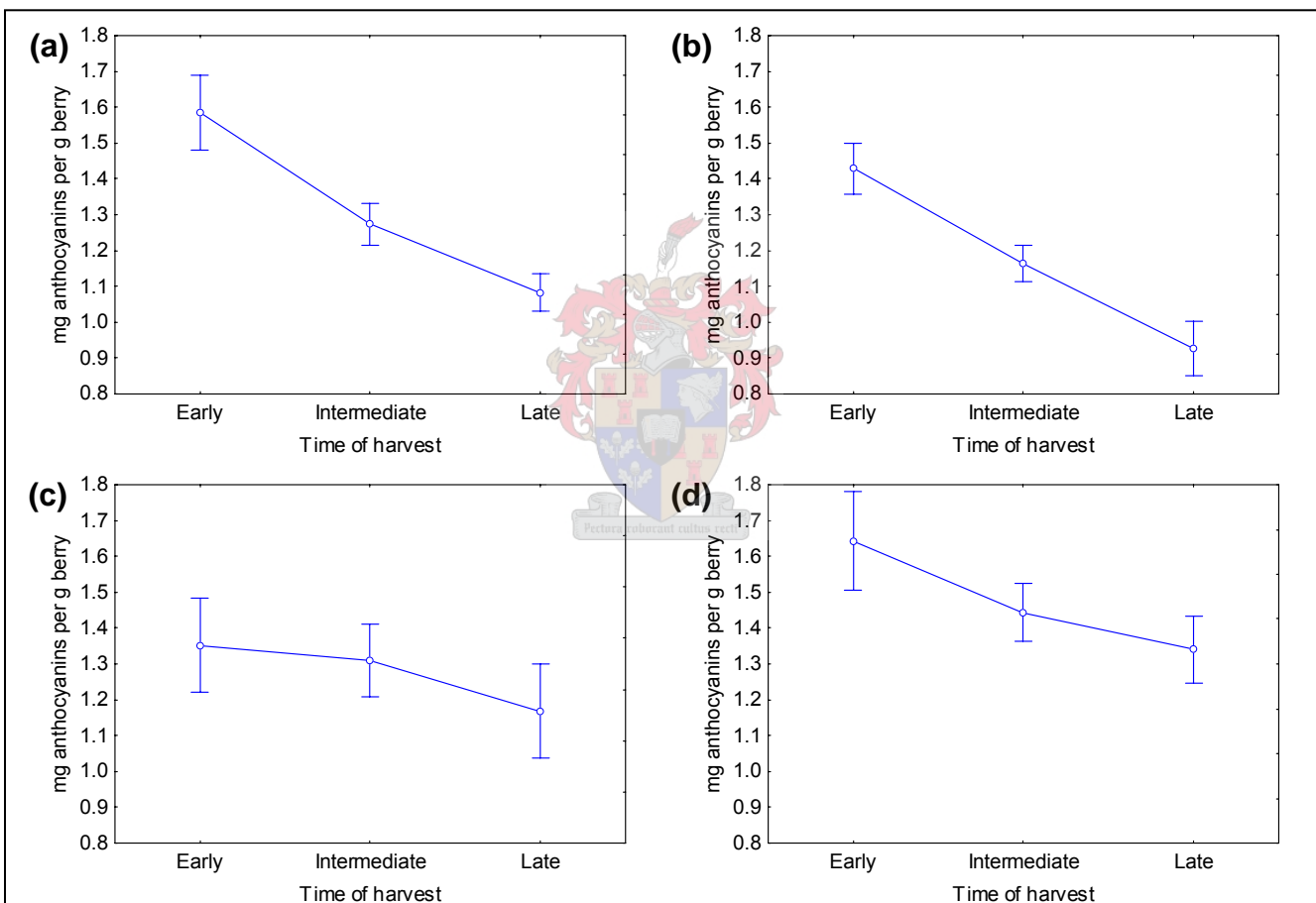


Figure 6. Significance of the relationship between time of maturity and harvest on total anthocyanins concentrations of four *Vitis vinifera* cultivars (a) Cabernet, (b) Merlot, (c) Pinotage, and (d) Shiraz.

Table 4. The effects of time of maturity and harvest on the total phenolics concentrations of *Vitis vinifera* cultivars.

Cultivar	Harvest Season period	Days from the 1 st day of harvest	Total n ^{*1}	Block grading n ^{*1}			Sugar (°Brix)	Total phenolics (OD280/g) ^{*4}
				A	B	C	Mean ^{*2} ± SD ^{*1}	Mean ^{*3} ± SD ^{*1}
Cabernet Sauvignon	Early	1-16	15	3	10	2	24.2 ^b ± 0.8	1.42 ^a ± 0.20
	Intermediate	17-32	39	10	23	10	25.7 ^a ± 1.0	1.29 ^{ab} ± 0.17
	Late	33-48	54	12	38	4	26.0 ^a ± 1.5	1.24 ^b ± 0.17
Merlot	Early	1-16	28	11	14	3	25.0 ^b ± 1.0	1.54 ^a ± 0.16
	Intermediate	17-32	39	13	22	4	24.9 ^b ± 0.9	1.34 ^b ± 0.14
	Late	32-50	22	3	16	3	25.8 ^a ± 1.0	1.26 ^b ± 0.15
Pinotage	Early	1-11	13	4	-	9	25.0 ^b ± 1.3	1.57 ^a ± 0.29
	Intermediate	10-19	20	11	6	3	26.7 ^a ± 1.3	1.54 ^a ± 0.17
	Late	20-35	7	6	-	1	25.5 ^{ab} ± 1.5	1.41 ^a ± 0.20
Shiraz	Early	1-16	13	6	2	5	25.7 ^{ab} ± 2.3	1.55 ^a ± 0.18
	Intermediate	17-32	39	18	14	7	25.7 ^b ± 1.5	1.46 ^a ± 0.21
	Late	32-49	31	5	15	11	27.4 ^a ± 1.8	1.45 ^a ± 0.15

Significance of least significant difference (LSD) test of treatment means at $p \leq 0.05$ and standard deviation. Means with the same superscript in the same column are not significantly different ($p > 0.05$).

*1 Abbreviation used: n, number of samples; SD, standard deviation

*2 As determined by the WineScan

*3 As determined by the reference method

*4 Expressed as OD280 per gram berry weight

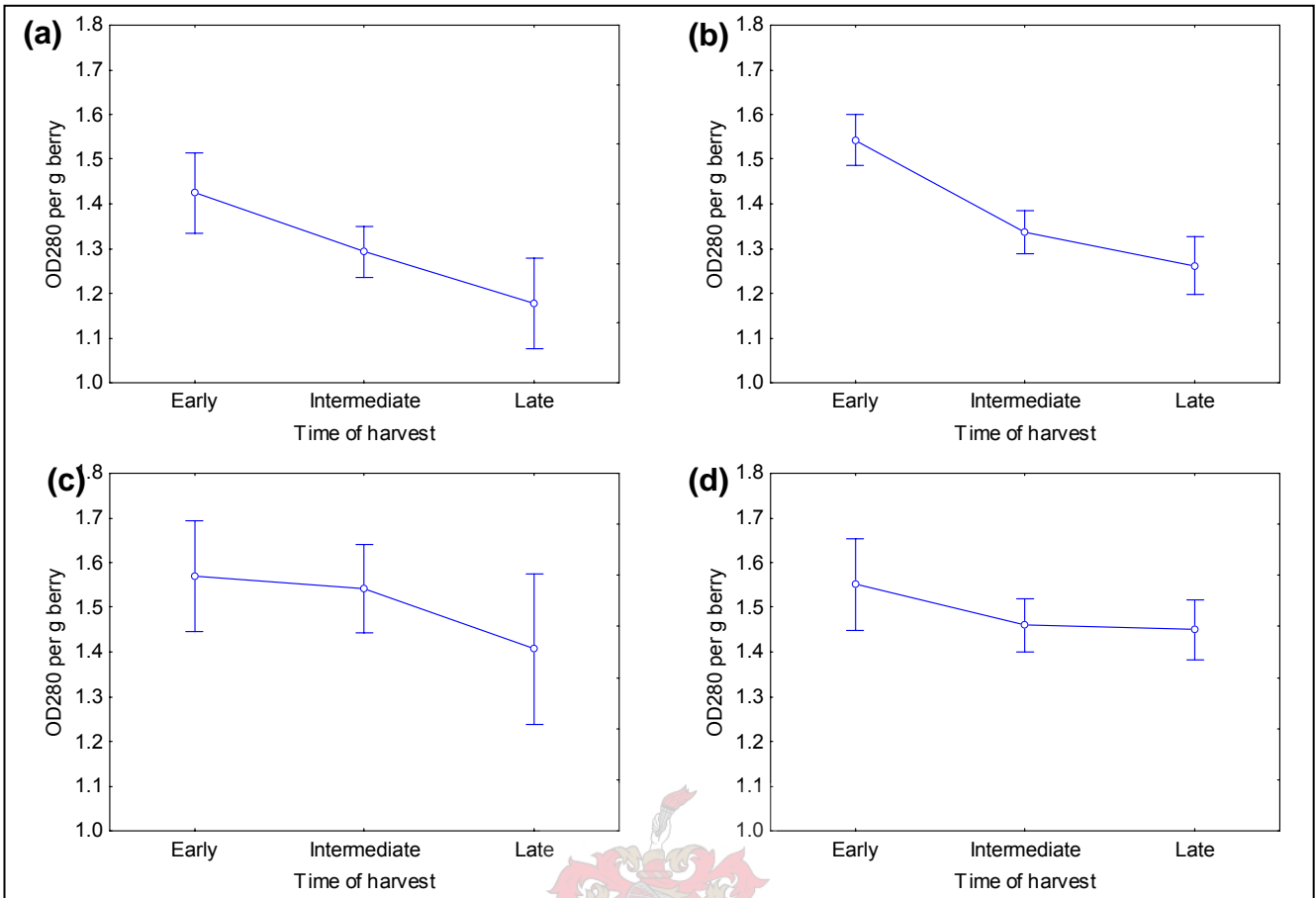


Figure 7. Significance of the relationship between time of maturity and harvest on total phenolics concentrations of four *Vitis vinifera* cultivars. (a) Cabernet, (b) Merlot, (c) Pinotage, and (d) Shiraz.

5.3.3.4 The effects of irrigation on the total anthocyanins and total phenolics concentrations of grapes

The division of sample sets according to irrigation was broadly based on grape homogenates originating from blocks irrigated on a regular basis (irrigated), blocks irrigated only when grapevines are showing signs of water stress (supplementary irrigation) and blocks with no irrigation system which obtain water via rain showers (dryland) (Table 2). Samples with sugar concentrations of $y < 23.5^\circ\text{Brix}$ were excluded from sample sets. This was done to eliminate the effects of grapes with low sugar concentrations on statistical analyses. In the Western Cape region of South Africa the climate is characterized by relatively warm, dry summers and annual winter rainfall. In recent years average summer day temperatures have increased. This might have been the reason why the most sampled blocks were irrigated ($n = 304$) or received supplementary irrigation ($n = 19$). Within the two sample sets of irrigated blocks irrigation systems, irrigation schedules as well as the amount of water supplied to different blocks differed but were not taken into account for this study. Only 10% of homogenate samples ($n = 37$) came from non-irrigated or dryland blocks.

No significant differences ($p > 0.05$) between the sugar concentrations and pH values of samples for different irrigation treatments were established. The TA of dryland samples was significantly higher ($p \leq 0.05$) than that of irrigated samples. There was no significant differences ($p > 0.05$) between the TA of supplementary irrigated and irrigated samples. There was also no significant association ($p > 0.05$) between the TA of supplementary irrigated and dryland samples. The tartaric acid and malic acid concentrations did not differ significantly ($p > 0.05$) between the three irrigation treatments but the mean values for irrigated samples were the lowest. Even though it was not statistically significant ($p > 0.05$), the mean potassium concentration of dryland samples was higher than the means for the other two treatments. There was statistically no significant differences ($p > 0.05$) between the average berry weight, anthocyanin concentration (Figure 8a) or total phenolics (Figure 8b) concentration of the three sets. Berry weights of dryland samples were lower than the berry weights of the two irrigated sets. Berries from supplementary irrigated blocks had the highest anthocyanin content but the lowest total phenolics. The mean total anthocyanin concentration of dryland samples was close to that of supplementary irrigated blocks. Dryland samples had the highest total phenolics concentration. Due to the uneven sample number distribution definite conclusions can not be made. However, samples receiving less water (dryland or supplementary irrigation) have slightly higher total anthocyanin and total phenolics concentrations which should result in wines of higher quality.

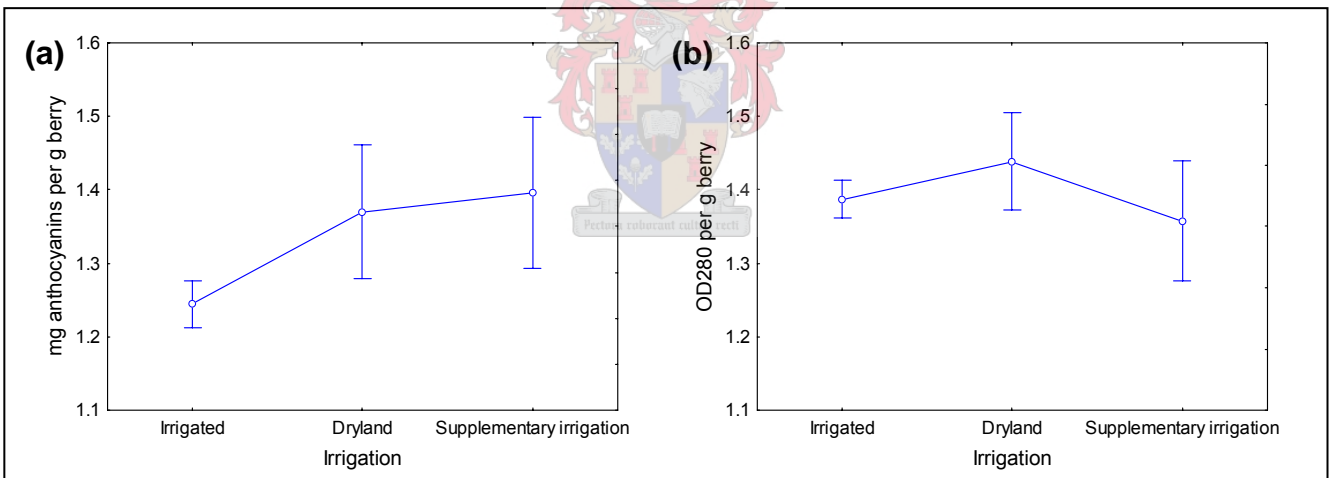


Figure 8. Significance of the relationship between irrigation treatment and (a) total anthocyanins concentrations, and (b) total phenolics concentrations.

5.3.3.5 Evaluation of the relationships between total anthocyanins, total phenolics and current viticultural block grading

Grape homogenates with sugar concentrations of $y \geq 23.5^\circ\text{Brix}$ were divided into 3 sets according to the viticultural quality grading (Table 2). Most samples were graded as A ($n = 118$) or B ($n = 179$) with only 17% of samples graded as C ($n = 61$). There were no significant differences ($p > 0.05$) between the sugar concentrations of the three sets. The mean sugar concentration of A graded samples was lower than that of the other two sets.

The mean pH value of A graded samples was significantly ($p \leq 0.05$) lower than that of B and C graded samples. The TA concentration for block grading B was significantly lower ($p > 0.05$) than that of the A and C graded sample sets. There were no significant differences ($p > 0.05$) between the tartaric acid concentrations of the three sets. The mean tartaric acid concentrations of C and B graded blocks was the highest and lowest respectively. The malic acid content of B graded samples was significantly lower ($p \leq 0.05$) than that of the other two sets. The potassium concentration of B graded samples was significantly higher ($p \leq 0.05$) than that of A graded samples. The potassium content of C graded samples did not differ significantly ($p > 0.05$) from that of the A or B graded samples. However, the mean potassium concentration of C graded samples was closer to the mean concentration of B graded samples. The berry weight of A graded samples was significantly higher ($p \leq 0.05$) than that of C graded samples. B graded samples did not differ significantly ($p > 0.05$) from neither A nor C graded samples regarding berry weight. The anthocyanin content of A graded samples was significantly higher ($p \leq 0.05$) than that of B graded samples (Figure 9a). C graded samples anthocyanin concentration did not differ significantly ($p > 0.05$) from that of A or B graded samples. The total phenolics concentration of B graded samples was significantly lower ($p \leq 0.05$) than that of A and C graded samples (Figure 9b). C graded samples had the highest mean total phenolics concentration. The total anthocyanins and total phenolics concentrations of grapes clearly did not correspond with the current viticultural block grading and the inclusion of these parameters could result in more accurate viticultural block grading.

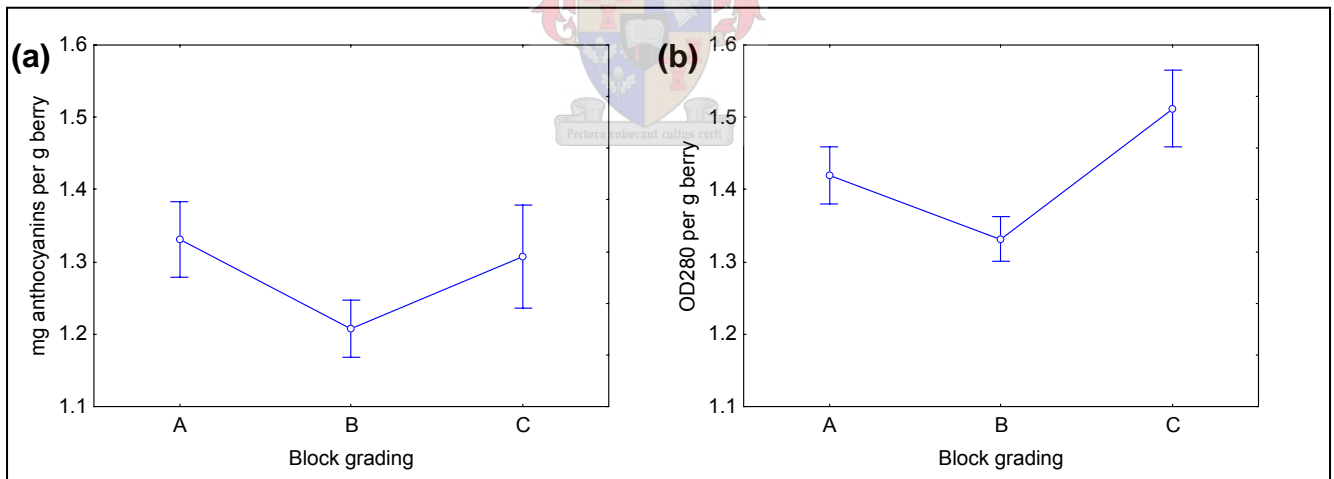


Figure 9. Significance of the relationship between the block grading and (a) total anthocyanins concentrations, and (b) total phenolics concentrations.

5.3.3.6 A comparison of grape chemical parameters of areas within the Stellenbosch region

The Stellenbosch district is an area well known for producing grapes and wines of high quality. Grape homogenates with sugar concentrations of $y \geq 23.5^\circ\text{Brix}$ from wards within the Stellenbosch district were divided into sample sets (Table 5). There were no significant

differences between the sugar concentrations, pH values, tartaric acid, malic acid, potassium content and berry weights of samples from different wards ($p>0.05$).

The TA of the Jonkershoek ward was significantly higher ($p\leq 0.05$) than that of the Helderberg ward. However, there were no significant differences ($p>0.05$) between the TA values of these two wards individually and those from the other wards. The anthocyanin concentration of Vlottenburg differed significantly from all wards ($p\leq 0.05$) except Jonkershoek ($p>0.05$) and Simonsberg ($p>0.05$). The anthocyanin content of samples originating from Stellenbosch Kloof differed significantly from that of Jonkershoek ($p\leq 0.05$) and Vlottenburg ($p\leq 0.05$) but not from the other wards ($p>0.05$). There were no significant differences ($p>0.05$) between the anthocyanin concentrations of Bottelary, Devon valley, Firgrove, Helderberg, Koelenhof, and Simonsberg. There were no significant differences ($p>0.05$) between the total phenolics content of Firgrove, Helderberg, Jonkershoek, Koelenhof, Simonsberg, and Stellenbosch Kloof. There were significant differences between the total phenolics concentrations of Bottelary and Devon valley from that of Vlottenburg ($p\leq 0.05$), but no significant differences between the total phenolics content of these two wards and the rest of the wards ($p>0.05$). The total phenolics content of Vlottenburg did not differ significantly from any of the wards ($p>0.05$) except from Bottelary ($p\leq 0.05$) and Devon valley ($p\leq 0.05$) as mentioned before. These results confirm that even within a geographic region such as Stellenbosch diverse terroir units can be distinguished that produce grapes with different chemical compositions.



Table 5. A comparison of the grape chemical parameters of areas within the Stellenbosch region.

Sample set	n* ¹	Sugar (°Brix)	pH	Titrateable acidity (TA)* ⁴	Tartaric acid (g/l)	Malic acid (g/l)	Potassium (mg/l)	Berry weight (g)	Total anthocyanins (mg/g)* ⁵	Total phenolics (OD280/g)* ⁶
		Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ² ± SD* ¹	Mean* ³ ± SD* ¹
Bottelary	24	26.3 ^a ± 1.6	3.88 ^a ± 0.09	4.3 ^{ab} ± 0.5	6.7 ^a ± 0.5	3.3 ^a ± 0.7	2458 ^a ± 309	1.54 ^a ± 0.12	1.24 ^{ab} ± 0.19	1.44 ^a ± 0.20
Devon Valley	70	26.0 ^a ± 1.8	3.92 ^a ± 0.15	4.1 ^{ab} ± 0.5	6.6 ^a ± 0.6	2.9 ^a ± 0.7	2592 ^a ± 449	1.45 ^a ± 0.19	1.28 ^{ab} ± 0.26	1.41 ^a ± 0.18
Firgrove	8	25.8 ^a ± 1.4	3.92 ^a ± 0.09	3.9 ^{ab} ± 0.3	6.8 ^a ± 0.5	2.7 ^a ± 0.7	2663 ^a ± 276	1.45 ^a ± 0.13	1.28 ^{ab} ± 0.15	1.43 ^{ab} ± 0.10
Helderberg	37	25.9 ^a ± 1.3	3.97 ^a ± 0.12	3.8 ^b ± 0.04	6.6 ^a ± 0.5	2.8 ^a ± 0.6	2687 ^a ± 361	1.45 ^a ± 0.18	1.25 ^{ab} ± 0.18	1.32 ^{ab} ± 0.16
Jonkershoek	13	25.1 ^a ± 1.0	3.92 ^a ± 0.19	4.5 ^a ± 0.6	6.7 ^a ± 0.4	3.2 ^a ± 0.6	2630 ^a ± 486	1.35 ^a ± 0.19	1.07 ^{bc} ± 0.24	1.27 ^{ab} ± 0.18
Koelenhof	11	24.8 ^a ± 1.1	3.96 ^a ± 0.11	4.4 ^{ab} ± 0.9	6.6 ^a ± 0.7	3.7 ^a ± 1.4	2685 ^a ± 363	1.48 ^a ± 0.24	1.23 ^{ab} ± 0.32	1.42 ^{ab} ± 0.24
Simonsberg	8	25.7 ^a ± 1.7	3.94 ^a ± 0.13	3.7 ^{ab} ± 0.5	6.5 ^a ± 0.3	2.8 ^a ± 0.3	2705 ^a ± 324	1.36 ^a ± 0.36	1.09 ^{abd} ± 0.18	1.35 ^{ab} ± 0.13
Stellenbosch Kloof	36	25.7 ^a ± 1.4	3.86 ^a ± 0.14	4.2 ^{ab} ± 0.7	6.6 ^a ± 0.5	3.0 ^a ± 0.8	2511 ^a ± 385	1.50 ^a ± 1.75	1.38 ^a ± 0.26	1.37 ^{ab} ± 0.23
Vlottenburg	9	25.4 ^a ± 1.1	3.91 ^a ± 0.06	3.7 ^{ab} ± 0.3	6.0 ^a ± 0.6	2.5 ^a ± 0.4	2274 ^a ± 288	1.64 ^a ± 0.26	0.79 ^{cd} ± 0.17	1.14 ^b ± 0.12

Significance of least significant difference (LSD) test of treatment means at $p \leq 0.05$ and standard deviation. Means with the same superscript in the same column are not significantly different ($p > 0.05$).

*¹ Abbreviation used: n, number of samples; SD, standard deviation

*² As determined by the WineScan

*³ As determined by the reference method

*⁴ Expressed as g/L tartaric acid

*⁵ Expressed as mg anthocyanins per gram berry weight

*⁶ Expressed as OD280 per gram berry weight

5.4 CONCLUSIONS

This study was a preliminary investigation of the implementation of total anthocyanins and total phenolics analyses at a commercial winery in South Africa. The investigation involved statistical analysis of the total anthocyanins and total phenolics concentrations present in a large number of grape homogenates from the 2005 vintage and from different areas within the Western Cape region of South Africa. All conclusions are preliminary due to the fact that the investigation only involved the information of one vintage. Several more seasons' information will be needed before definite conclusions can be made. Principal component analysis results highlighted the importance of measuring many chemical components for the purpose of capturing enough information to discriminate between samples. The analyses results confirmed that both the total anthocyanins and total phenolics concentrations of grapes increased with increasing sugar concentration ($^{\circ}$ Brix). There were significant differences between the total anthocyanins and total phenolics concentrations of the four *Vitis vinifera* cultivars investigated. More investigations are needed to establish the concentration ranges of the parameters for grapes produced in South Africa. For all *Vitis vinifera* cultivars investigated grapes harvested earlier in the season had significantly higher total anthocyanins and total phenolics concentrations than grapes harvested later in the season. These results imply that earlier harvested grapes will produce wines with deeper colour intensities and more tannins than wines from grapes harvested later in the harvest season. Ignoring irrigation volumes and schedules it does seem as if regularly irrigated vineyards have lower total anthocyanins and total phenolics concentrations than dryland vineyards. The total anthocyanins and total phenolics concentrations of grapes did not correlate with the viticultural block grading. These results highlight the need to include more objective measures of grape quality as part of grape quality control to ensure that grapes are "correctly" classified. Future aims include the analyses of more grape samples from various areas of South Africa. These results will be incorporated in the established data base to ensure the successful implementation of the two parameters total anthocyanins and total phenolics as part of routine grape quality control at South African wineries.

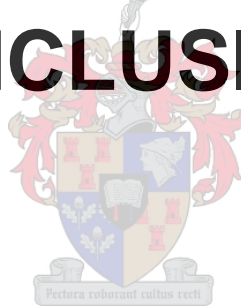
ABBREVIATIONS USED

PCA, principal component analysis; TSS, total soluble solids; TA, titratable acidity; SD, standard deviation; r, correlation; AVOVA, analysis of variance; PC, principal component; LSD, least significant difference; n, number of samples; SD, standard deviation; r, correlation coefficient.

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GENERAL DISCUSSION AND CONCLUSIONS



GENERAL DISCUSSION AND CONCLUSIONS

6.1 CONCLUDING REMARKS AND PERSPECTIVES

Modern-day winemaking is a profit driven business. Wineries invest large sums of money, by employing well trained personnel, the use of sophisticated technology and costly marketing campaigns to ensure maximum wine sales and subsequent economic viability. Presumably consumers are willing to pay more money for a wine of better and consistent quality. Wine quality fundamentally refers to the pleasure a wine provides to the consumer. In this context objective analytical measures that correlate strongly with wine quality are of utmost importance.

Apart from wine aroma and taste, wine colour is an important indication of red wine quality. Deeper coloured red wines are perceived to be of higher quality than lighter coloured red wines. It has been established that the anthocyanin concentration of grapes is strongly correlated to wine colour intensity (Somers and Evans, 1974; Iland, 1987) and therefore to wine quality. Anthocyanins are classified as phenolic compounds. Phenolic compounds cause sensory sensations such as colour, flavour, astringency, bitterness and hardness of wines (Somers, 1971; Noble, 1994; Gawel, 1998). Therefore the inclusion of total anthocyanins and total phenolics analyses as part of red grape quality control will provide important information regarding critical sensory aspects related to red wine quality. The aims of this study were firstly to develop an analytical method that is fast and accurate for the quantification of total anthocyanins and total phenolics of grapes and, secondly to use the generated reference analysis method data to do an evaluation of the possibility to implement total anthocyanins and total phenolics analyses as part of the grape quality control protocol at a commercial winery in South Africa.

Existing analyses for total anthocyanins and total phenolics concentrations in grapes are complex and time consuming. A major challenge for researchers today is to find simple and practical solutions for complex problems. Infrared spectroscopy is an attractive analytical tool because of its speed, reliability and simplicity of performance. Another important benefit of infrared spectroscopy is the reduction of chemical waste. The measurement of total anthocyanins concentrations in grapes using near infrared spectroscopy (NIR) has already been evaluated successfully (Damberg *et al.*, 2003). In South Africa the use of the Foss WineScan FT120 spectrometer (Foss Analytical, Denmark) is well established. Due to the availability of instrumentation the application of Fourier transform infrared spectroscopy (FT-IR) as an analytical tool for the quantification of total anthocyanins and total phenolics present in grapes was evaluated in this study since the commercial calibration models provided with the WineScan instrument have unsatisfactory prediction accuracy. New calibration models were developed and satisfactory results were obtained for both the prediction of total anthocyanins and total

phenolics concentrations of grapes. The South African wine industry strives to obtain calibration models with prediction accuracies of 0.10 mg/g for total anthocyanins and 0.10 OD₂₈₀/g for total phenolics concentrations of grapes respectively. Prediction results for the total phenolics concentrations of grapes were slightly less accurate than that obtained for total anthocyanins concentrations of grapes. This could suggest that absorbance at 280 nm is not specific enough for the quantification of total phenolics of grapes. The calibration results obtained in this study show that FT-IR can be used as an analytical tool for screening grapes for total anthocyanins and total phenolics concentrations. Further research and development of FT-IR calibration models will be aimed at lowering prediction errors by including more samples from a broad range of grape maturities and from several more vintages. To use the obtained FT-IR calibration models during grape quality control, the calibration model accuracy and robustness must be improved. Although the reference method accuracy was satisfactory, efforts must still be made to work even more accurately to ensure optimal FT-IR results. There is some concern about the large number of samples that had to be removed from sample sets based on poor repeatability of the reference method.

The use of principal component analysis (PCA), a multivariate statistical analysis tool, captured important FT-IR spectral information of grape homogenates. Clear differences between grape homogenates related to vintage and grape maturity were revealed during PCA investigations. A PCA investigation revealed that the water content of the samples was a major source of variation between the grape homogenates of the 2004 and 2005 vintages. It was also established that Pinotage has a spectral profile quite different opposed to Cabernet Sauvignon, Merlot and Shiraz. These findings highlighted the importance of detailed exploratory investigations of FT-IR spectra before eliminating wavenumbers “supposedly” only contributing to noise. Results also emphasize the need for the establishment of a FT-IR spectral library in conjunction with the chemical analyses results database. All future grape homogenates scanned will be included in the established FT-IR spectral database to evaluate spectral trends over several seasons.

In the process to develop the calibration models a database was established containing both spectral data and analytical results regarding the total anthocyanins and total phenolics concentrations of grapes delivered to Distell (Distell Group (Pty) Ltd). The generation of the reference data enabled a preliminary evaluation of the possibilities to implement total anthocyanins and phenolics values as additional grape quality control parameters. There were significant differences between the total anthocyanins and total phenolics concentrations of the four *Vitis vinifera* grape cultivars investigated in this study. Results showed that earlier harvested grapes had higher total anthocyanins and total phenolics concentrations than grapes harvested later in the season. The current grape grading system at Distell did not reflect the total anthocyanins and total phenolics concentrations of grapes emphasizing the need to include more objective measures during grape grading.

More data from several seasons must be included in the database to establish the concentration ranges of total anthocyanins and total phenolics concentrations of *Vitis vinifera* red cultivars produced in South Africa. It is important to obtain grape analyses results for each vineyard including grapes from all stages of maturity to build up a comprehensive vineyard history database. The inclusion of larger numbers of samples, from all areas of grape production, will provide important statistics to identify production areas with higher average total anthocyanins and total phenolics concentrations of grapes. These results will add great value to terroir studies. A comprehensive database will also provide valuable information to assist during decisionmaking regarding the optimum harvest dates of vineyards and could equip producers, viticulturists and winemakers with concrete analyses values for phenomena previously unaccounted for in South African vineyards. In conclusion, further development of the calibration models developed during this study will enable the application of FT-IR analyses for the prediction of total anthocyanins and total phenolics during routine grape quality control which will be beneficial to the South African wine industry.

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CHEMICAL COMPONENT DATA

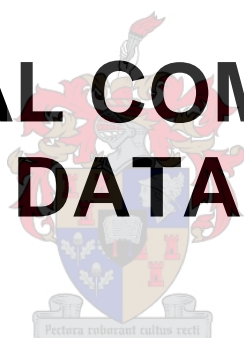
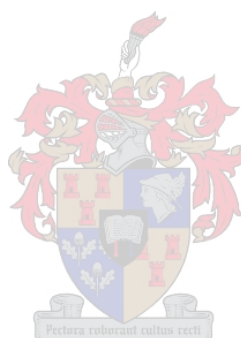


Table 1. Chemical component range of 2005 vintage samples (n = 603). Conditions of analysis are described in Chapter 5, sections 5.2.2 and 5.2.3.

Chemical component	Cabernet Sauvignon n ^{*1} = 239	Merlot n ^{*1} = 155	Pinotage n ^{*1} = 64	Shiraz n ^{*1} = 145
	Mean ^{*2} ± SD	Mean ^{*2} ± SD	Mean ^{*2} ± SD	Mean ^{*2} ± SD
Total anthocyanins (mg/g berry)	1.1 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.3 ± 0.3
Total phenolics (OD280/g berry)	1.2 ± 0.3	1.3 ± 0.3	1.4 ± 0.5	1.4 ± 0.3
Sugar (°Brix)	23.5 ± 2.6	24 ± 2.2	24.5 ± 2.5	24.1 ± 3.6
Titrateable Acidity (g/L)	4.5 ± 1	4.2 ± 0.8	5.1 ± 0.6	4.3 ± 1.2
pH	3.9 ± 0.2	3.8 ± 0.1	3.9 ± 0.2	3.8 ± 0.2
Tartaric Acid (mg/L)	6.3 ± 0.7	6.5 ± 0.6	6.8 ± 0.7	6.1 ± 0.8
Malic Acid (mg/L)	3.3 ± 0.8	2.7 ± 0.6	4.6 ± 0.9	3.4 ± 0.9
Potassium (mg/L)	2594.7 ± 451.8	2245.7 ± 331.8	2475.7 ± 463.7	2371.7 ± 602.5

*¹Abbreviations used: n, number of samples; SD, standard deviation

*²As determined by the reference method or by the WineScan



LYSOZYME

The solution for Bacterial
problems in Red Wine



Marius Lambrechts

Distell

CONTENTS

- Occurrence of LAB from grapes to wine.
- Why do we have pH problems?
- pH and SO_2
- How does pH influence LAB?
- Metabolism of LAB
- Lysozyme
- Application of lysozyme
- Other options



-
- **Why do we have bacteriological problems?**

- **Occurrence on grapes**



alcoholic fermentation

spontaneous, inoculated MLF

ageing

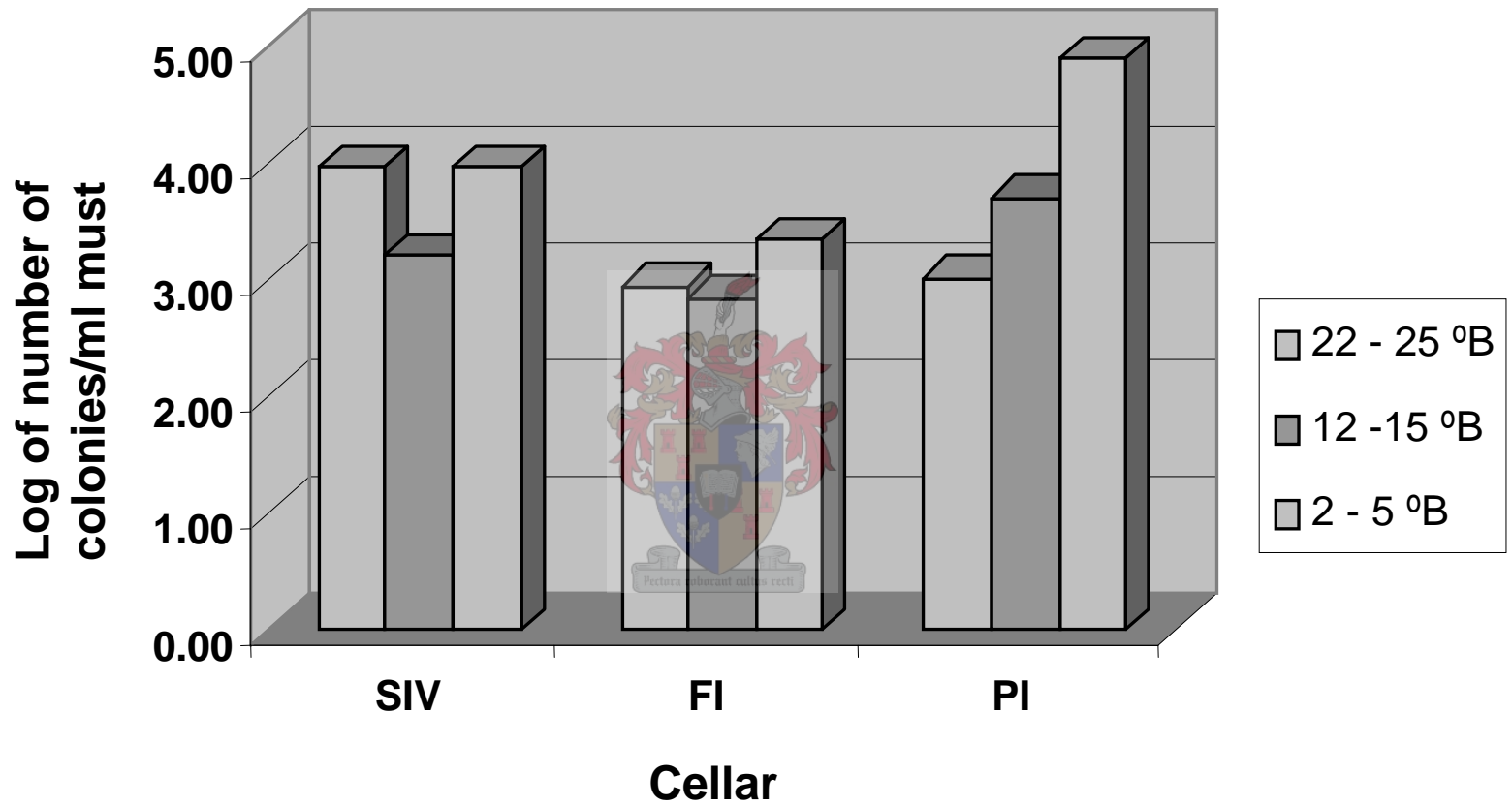
GRAPES

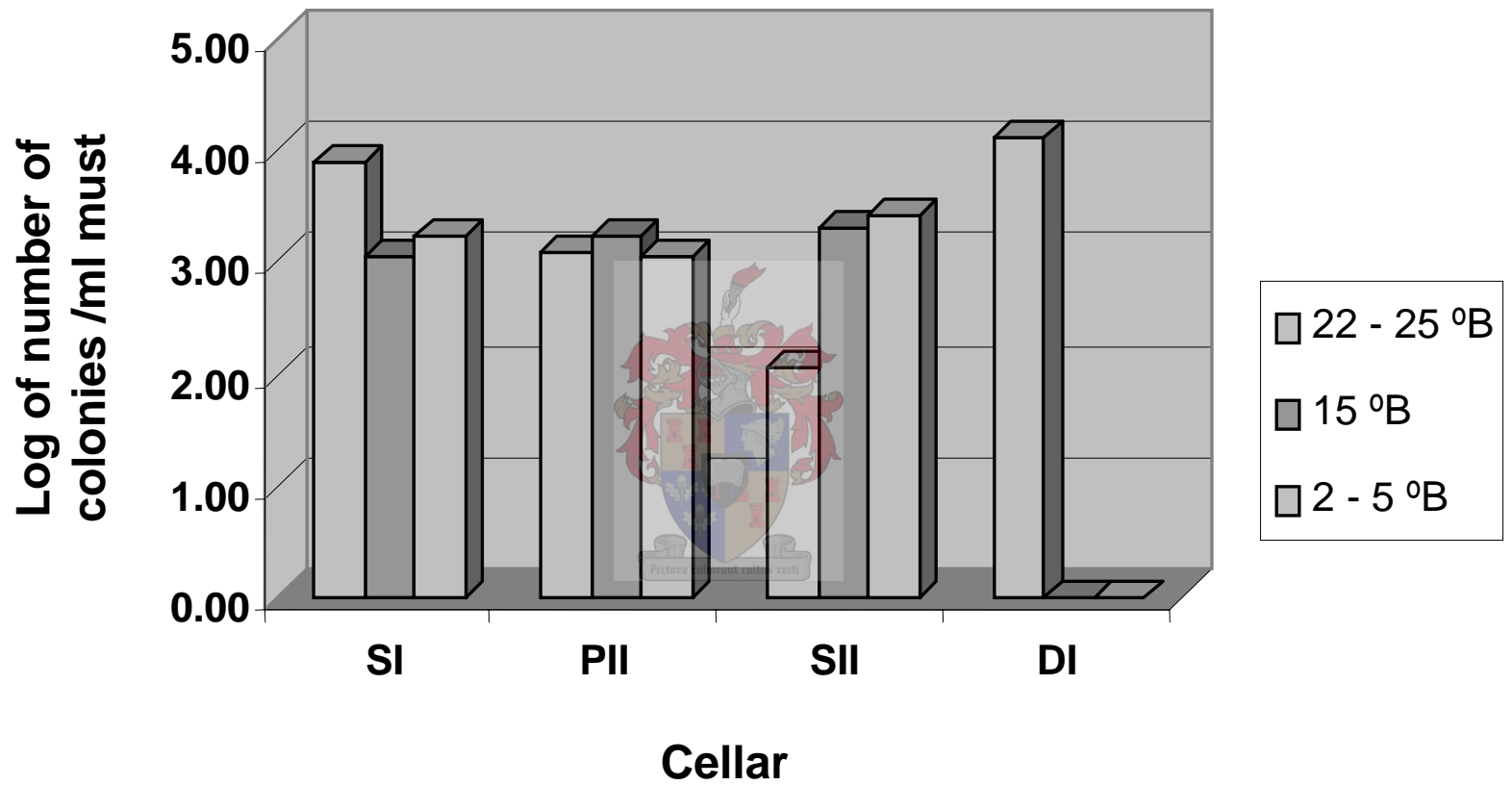
- Grapes - 10^2 ~ 10^5 cells/ml
 - seasonal variation
 - cold soaking



Must composition of samples during the 1999 harvest season

Year & Area	Cultivar	Sugar conc. (°B)	T.A. (g L⁻¹)	pH	SO₂ dosage (mg kg⁻¹)
Stellenbosch (SI)	Pinotage	24.3	5.7	3.5	50
Stellenbosch (SII)	Merlot	24.6	6.8	3.41	50
Stellenbosch (SIII)	Merlot	25.2	5.35	3.75	40
Stellenbosch (IV)	Pinotage	24	6.5	3.4	30
Paarl (PI)	Cabernet franc	24	4.0	3.75	30
Paarl (PII)	Pinotage	23	5.7	3.5	30
Franschoek (FI)	Merlot	25.2	5.8	3.71	40
Durbanville (DI)	Merlot	23.4	6.2	3.4	50





MALOLACTIC FERMENTATION

- Spontaneous
 - $10^2 - 10^5$ cells/ml \approx $10^6 - 10^8$ cells/ml
- Inoculated
 - $10^6 - 10^8$ cells/ml



Ageing

- SO_2 and pH
- Low pH - short survival time of LAB
- High pH - long survival, even years

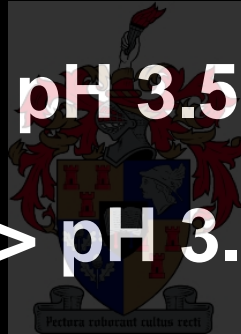
WHY DO WE HAVE HIGH pH?

- **What is pH?**
 - measurement of free proton (H^+)
- **Role of potassium in pH**
 - protons get replaced by potassium
 - potassiumbitrate
- **Why do we have high potassium concentration?**
 - high potassium concentration in our soil
 - incorrect viticultural practices
 - harvest at higher sugar levels



Effect of pH on LAB

- *Oenococcus* < pH 3.5
- *Lactobacillus* > pH 3.5
- *Pediococci* >> pH 3.5



- pH and SO₂ relationship

The percentage of sulfur dioxide forms in solution at various pH values. Solution conditions are 14% v/v ethanol and 80 mM ion strength

pH	Molecular SO₂	Bisulfite ion	Sulfite ion	Free SO₂ for 0.825 mg/L molecular
2.7	10.5	89.5	0.00283	7.85
2.8	8.54	91.5	0.00364	9.66
2.9	6.90	93.1	0.00467	12.0
3.0	5.56	94.4	0.00596	14.8
3.1	4.47	95.5	0.00759	18.5
3.2	3.58	96.4	0.00964	23.1
3.3	2.87	97.1	0.0122	28.8
3.4	2.29	97.7	0.0155	36.0
3.5	1.83	98.2	0.0196	45.1
3.6	1.46	98.5	0.0248	56.6
3.7	1.16	98.8	0.0312	71.1
3.8	0.924	99.0	0.0394	89.3
3.9	0.736	99.2	0.0497	112.0
4.0	0.535	99.4	0.0627	141.0



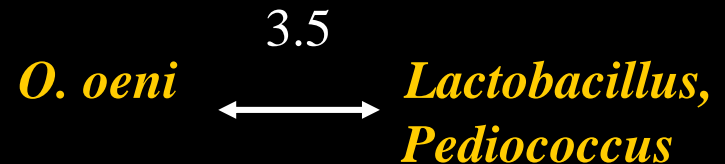
WHAT IS IMPORTANT TO REMEMBER ABOUT LAB?

- **Several genera**
 - *Oenococci oeni*, *Lactobacillus*, *Pediococcus*, *Leuconostoc mesenteriodes*

- **Spontaneous versus inoculated**

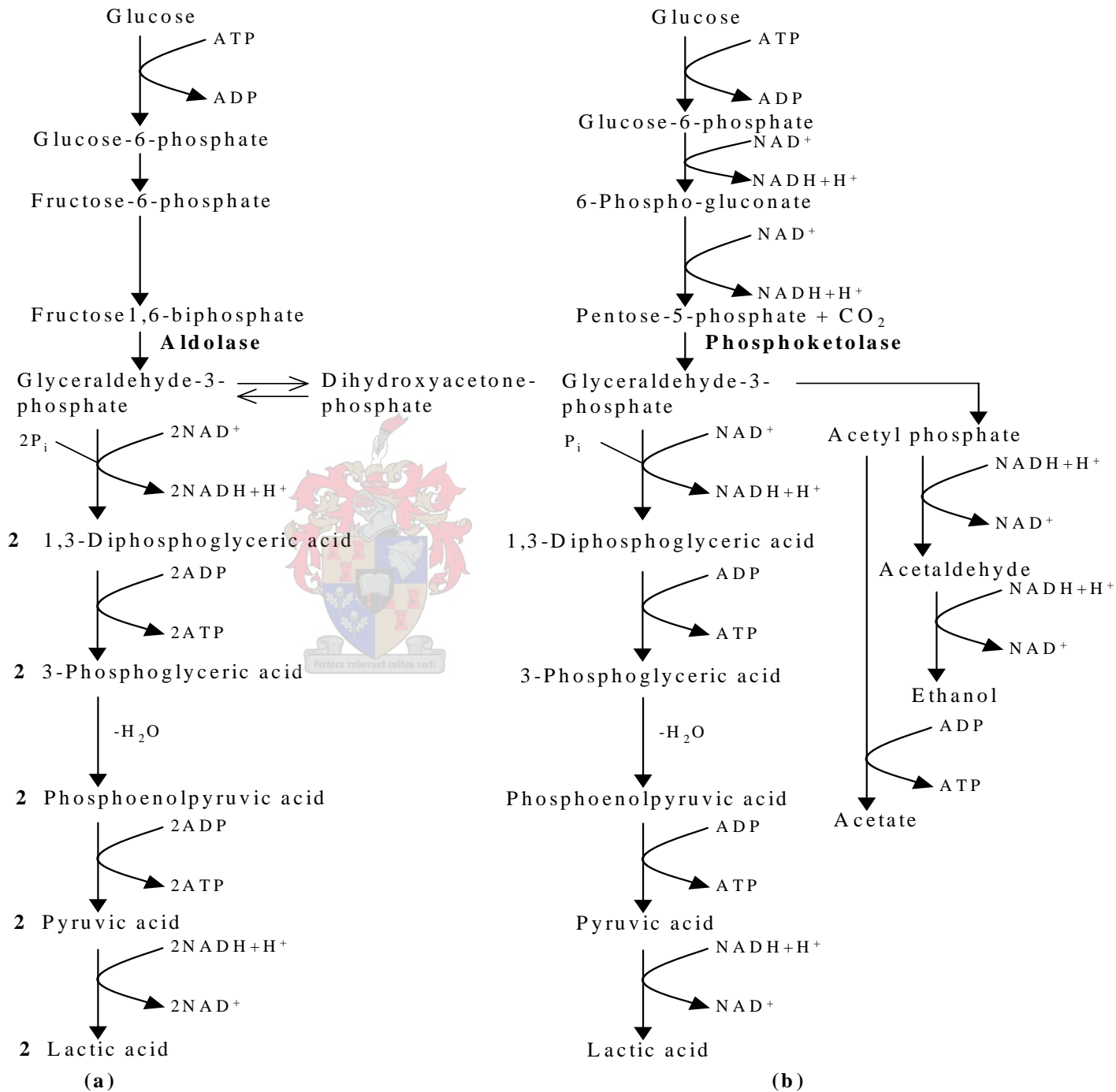
- **pH**

- 3.5 growth speed
- 3.4 sugar → acetic acid
- ease of MLF

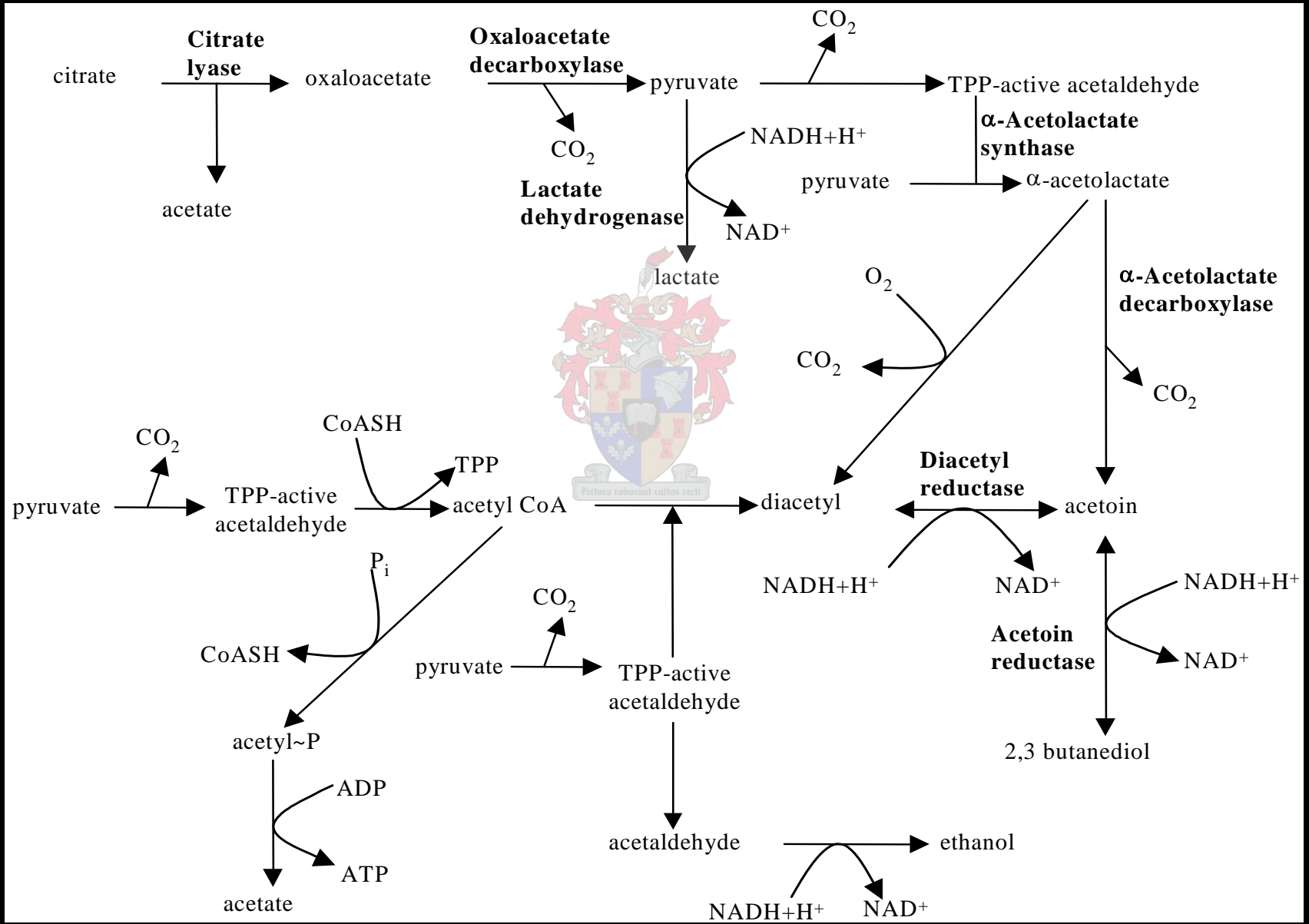


- **Need low substrate**
- **Off-flavours**
- **Biogenic amines**

ACID FORMATION



CITRIC ACID DEGRADATION



LYSOZYME

- **Lysozyme is an enzyme with bactericidal properties**
- **Approved for use as biopreservative in the food industry**
- **Non-toxic and has GRAS status**
- **Widely distributed in nature**
 - **Animals, plants, insects, phages**
- **Commercial source of lysozyme is hen egg white**
- **Toothpaste, cheese etc.**



LYSOZYME

- **OIV approved use of lysozyme in winemaking**
- **Mainly used in winemaking for**
 - inhibition of MLF
 - control of MLF
 - microbial stabilisation after MLF

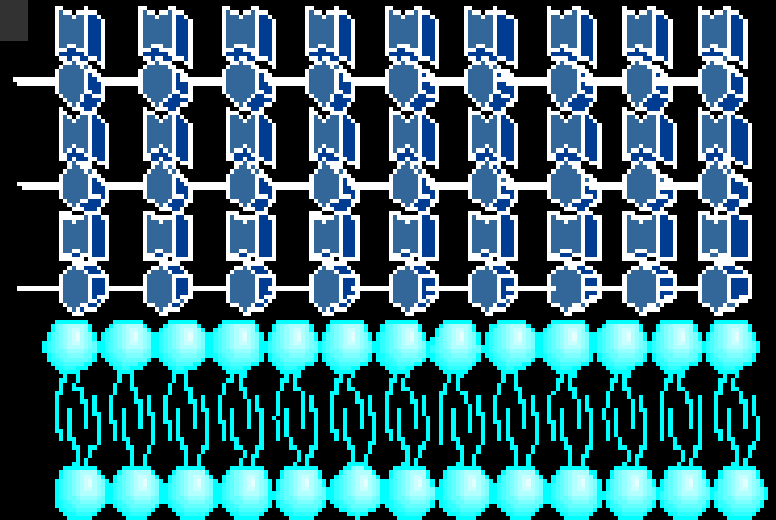
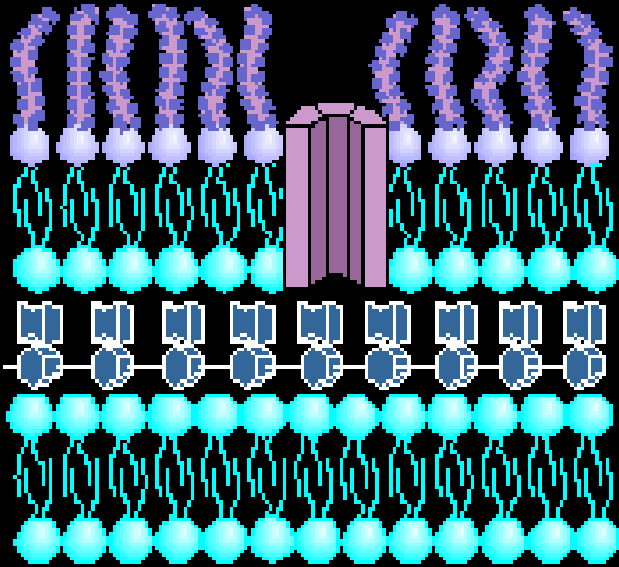


FUNCTION

- **Lysozyme is defined as 1,4- β -N-acetylmuramidase**
 - cleaves β -1,4-glycosidic bonds in the peptidoglycan layer of cell walls
- **Lysozyme has muramidase and chitinolytic activity**
- **Effective only against Gram-positive bacteria**
 - Gram-(+) have a thick peptidoglycan layer
 - Gram-(-) thin peptidoglycan layer
- **Outer cell membrane**
- **Gram-(-) can be sensitised**
 - addition of EDTA



Gram negative



Gram positive



ACTIVITY

- Action limited to Gram(+) bacteria
- Lysozyme is not effective against yeast
- Active in wine pH and not affected by alcohol
- Winemaking compounds affecting lysozyme activity
 - tannins, meta tartaric acid
 - pigments, oak chips, carbon
 - bentonite, silica
- No permanent preservation
- No antioxidant activity
- Effect of LAB cell numbers (→ 10⁵ cfu/mL ←)



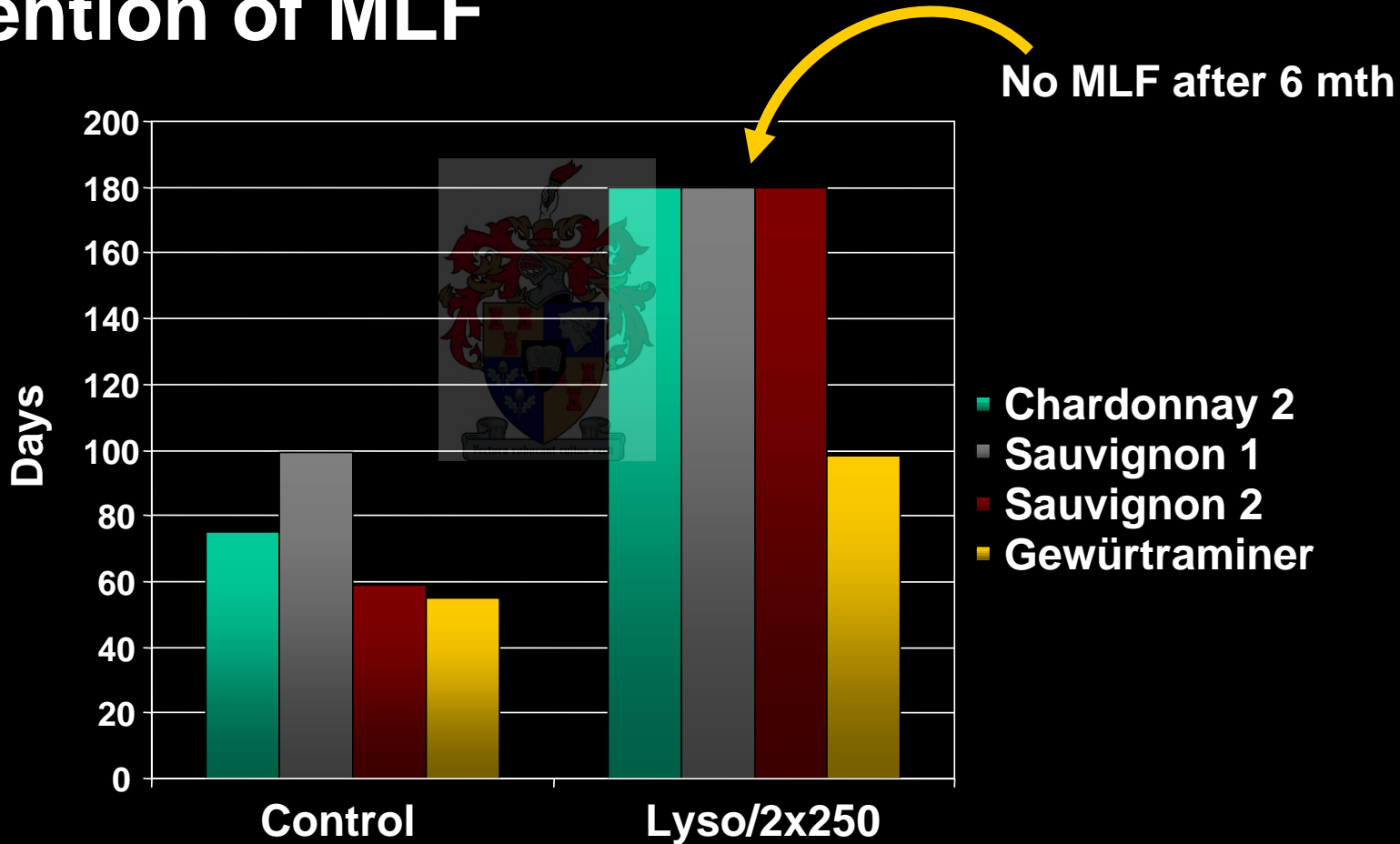
APPLICATIONS

- **Delay MLF**
- **Maceration**
- **Carbonic maceration (100 mg/L → 200 mg/L) inhibits spontaneous MLF**
 - presence of high [sugar] – volatile acidity



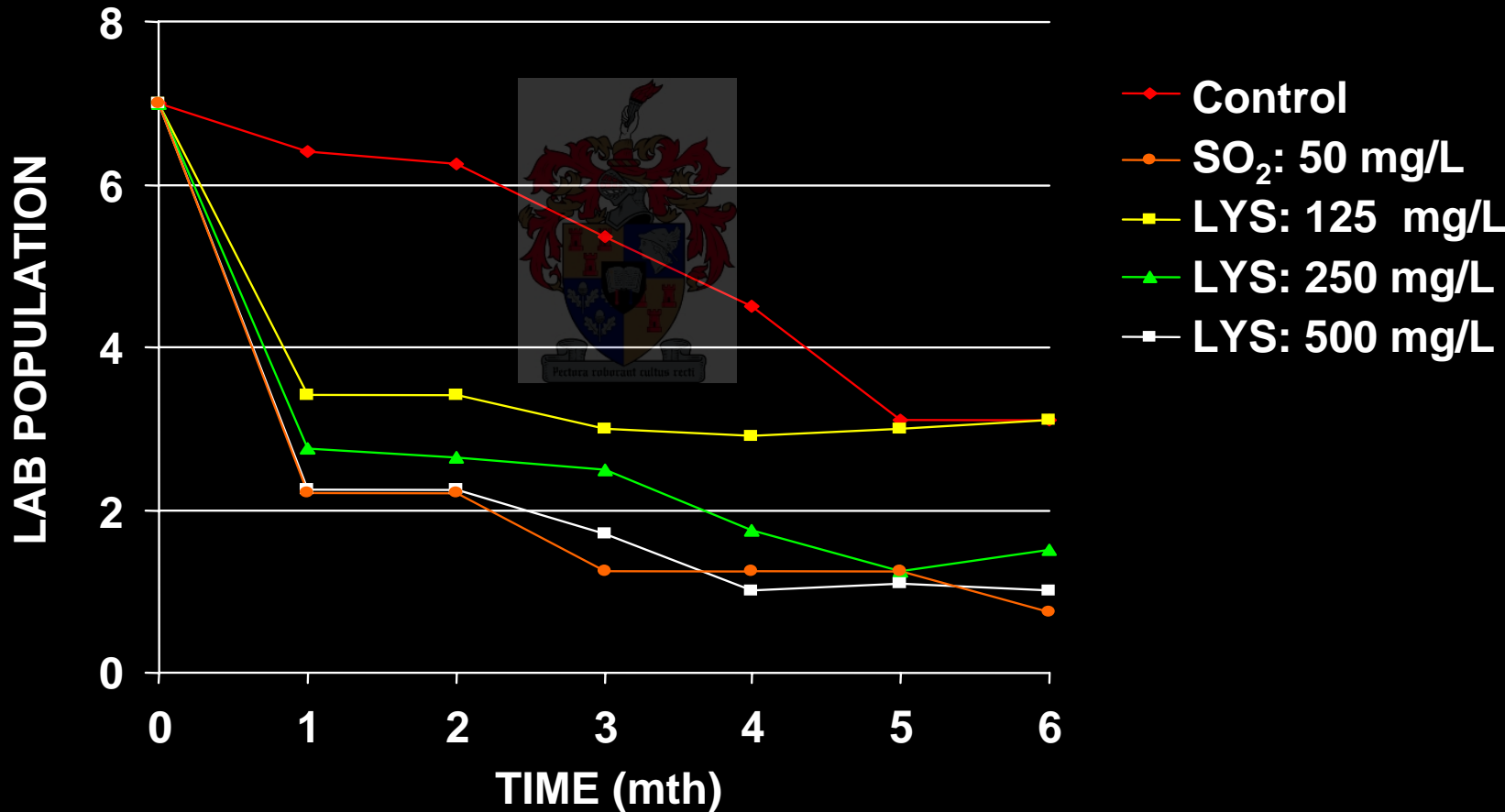
APPLICATIONS (continue)

- **Prevention of MLF**

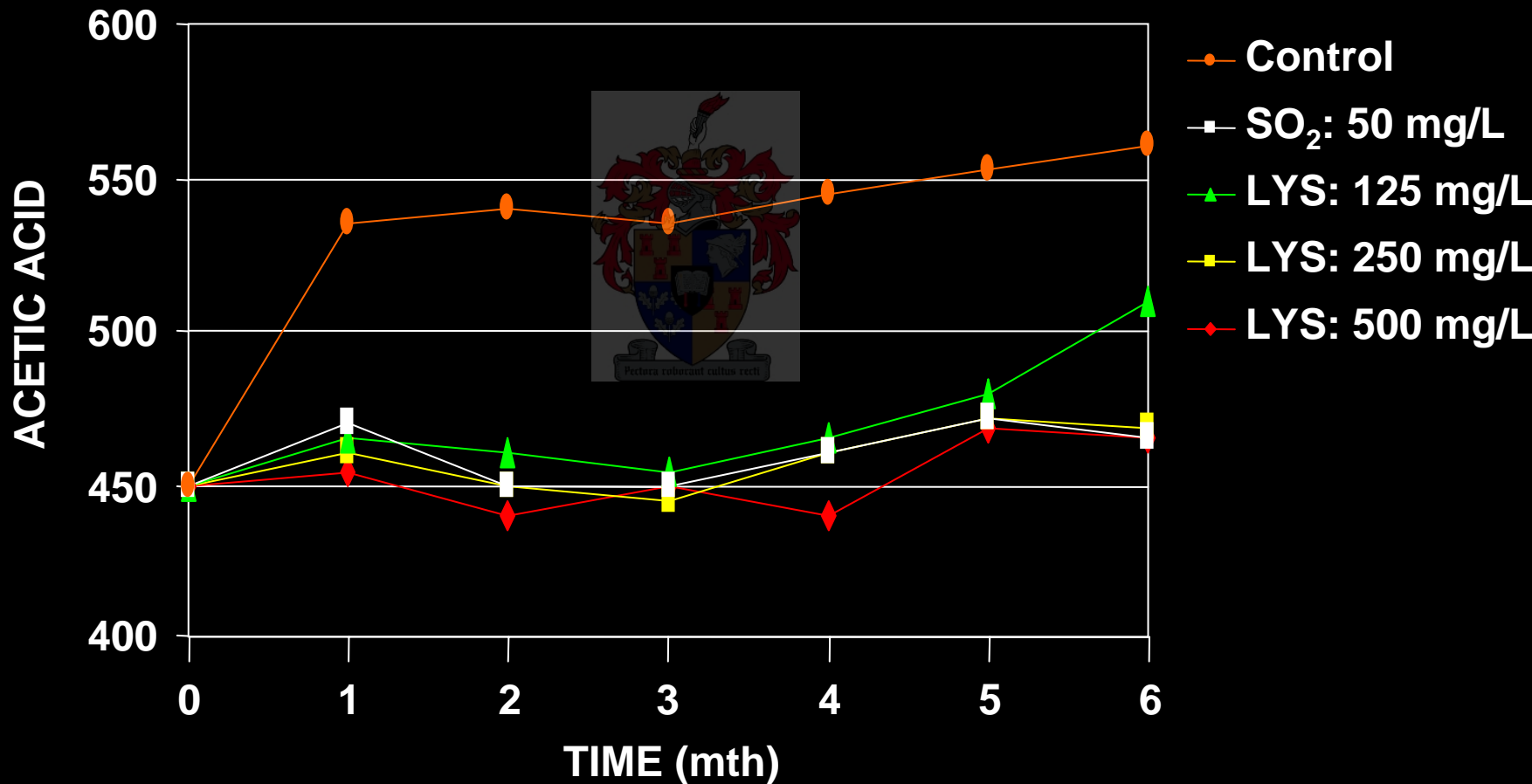


THE EFFECT OF LYSOZYME ON MLF

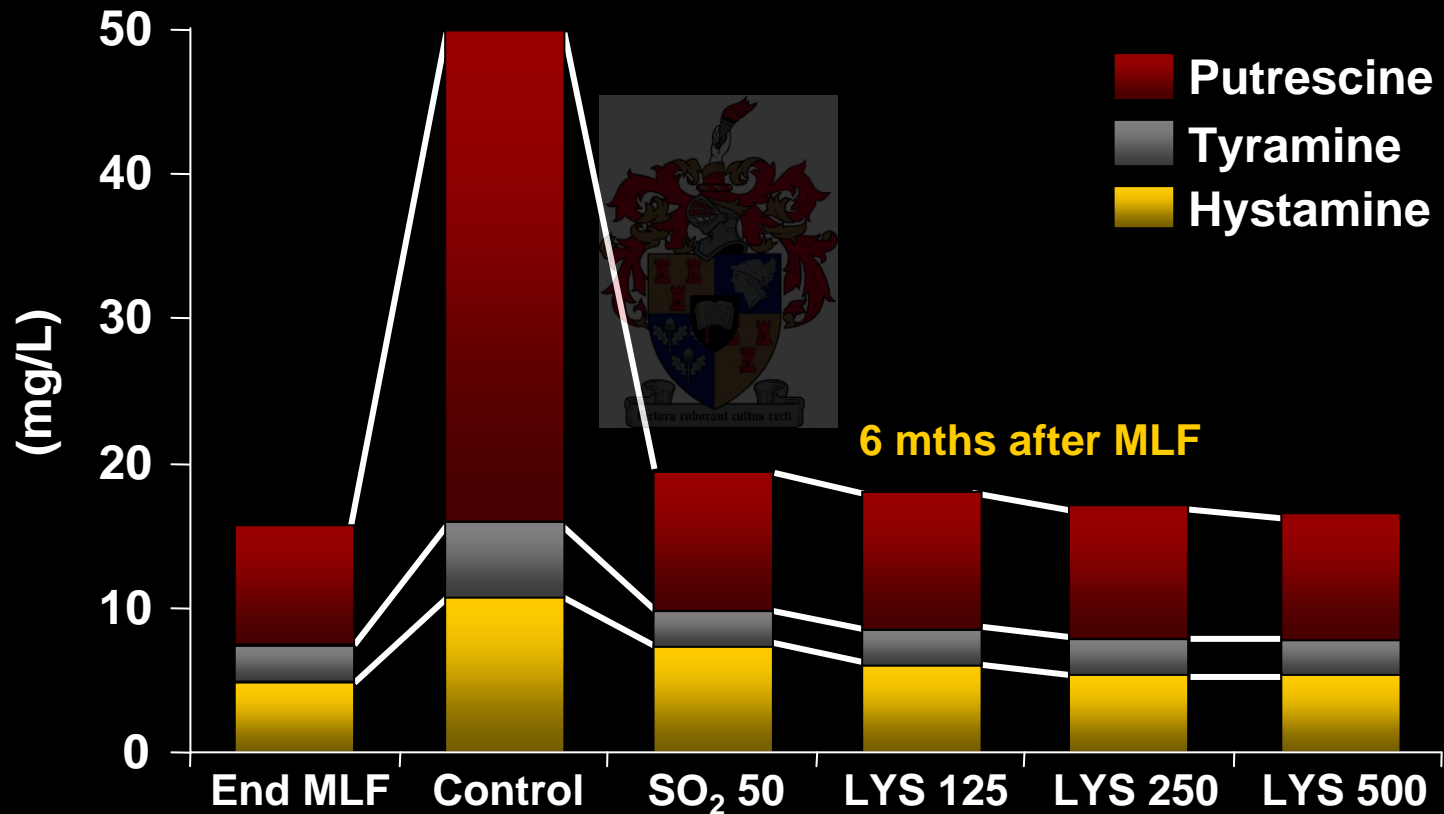
MICROBIOLOGICAL STABILIZATION OF PINOT NOIR WINES IN BARRELS AFTER MLF



ACETIC ACID PRODUCTION



FORMATION OF BIOGENIC AMINES IN BARRELS AFTER MLF



STUCK / SLUGGISH FERMENTATIONS

- **Sometimes caused by LAB**
 - sugar → VA
 - eliminate LAB (500 mg/L)
- **LAB not the cause**
 - prevent VA formation
 - preventative dose (200-300 mg/L)



STUCK / SLUGGISH FERMENTATIONS (continue)

- **SO₂ treatment**
 - can influence yeast
 - total SO₂
- **MLF already started**
 - monitor VA carefully
 - high dosage to eliminate



APPLICATION IN SA WINE INDUSTRY

- **Maceration/delayed alcoholic fermentation**
- **Vineyard with a history of problems**
- **High pH grapes**
 - add after crush
 - SO₂ addition (< 10-20%, if any)
 - prevent growth of spontaneous LAB
 - lysozyme denatured during alcoholic fermentation
 - will have to inoculate for MLF
- **VA during ageing**



CONCLUSION

- **Additional aid**
- **Does not replace SO₂**
- **No antioxidant activity**
- **LAB differ in sensitivity**
- **Short term effect**
- **Experiment**



OTHER OPTIONS

- **Viticultural practices**
- **Acid additions before alcoholic fermentation**
 - **tartaric, malic, potassium**
 - **homogenation of grapes**

