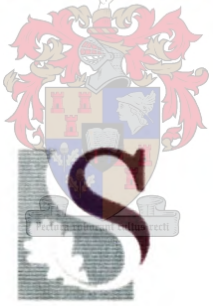
Manipulating the levels of ethyl acetate and isoamyl acetate formation during the production of wine and brandy

by

Jennifer Carr Bayly



Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at Stellenbosch University

Supervisor:
Dr M du Toit

Co-Supervisors:
Prof IS Pretorius
Prof MG Lambrechts

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.

Jennifer Carr Bayly

SUMMARY

The production of wine is a complex process, which involves the conversion of sugar in grape must to ethanol, carbon dioxide and other byproducts. The principal organism in winemaking is yeast, of which *Saccharomyces cerevisiae* is the most important due to its ability to survive winemaking conditions, its GRAS (Generally Regarded As Safe) status and the favourable flavours it imparts during the winemaking process. However, due to the demands of the consumer and the emergence of sophisticated wine markets, a demand is developing for specialised yeast strains with enhanced and new oenological properties. For these reasons, research into the contribution of wine yeast to the aroma bouquet as well the influence of wine or brandy maturation in wood on the aroma bouquet is important for consumer demands to be met.

The fruity aroma of wine is associated with esters, which are produced during the alcoholic fermentation by yeast. Important acetate esters in wine and brandy are ethyl acetate, which has a fruity, solvent-like aroma, and isoamyl acetate, which has a banana-like aroma. These esters are produced through the action of acetyltransferases (AATases), which catalyse the reaction between a higher alcohol and acyl Coenzyme A. Esters are mainly a product of alcoholic fermentation. However, their concentration changes during wood maturation and it has been found that the concentration of acetate esters can increase during the maturation period.

In this study, the aim was to investigate the influence of AATase I and AATase II, which are encoded by the *ATF1* and *ATF2* genes respectively, on the aroma bouquet of wine and brandy. Therefore, the first objective of this study was to clone the *ATF2* gene from a commercial wine yeast strain and to overexpress this gene in a commercial wine yeast strain and in a wine yeast strain that already has the *ATF1* gene overexpressed. Disruption cassettes were also designed in order to disrupt the *ATF1* and *ATF2* genes in a commercial wine yeast strain. The resultant recombinant wine yeast strains were used for the production of wine and brandy. GC analyses and tasting trials were conducted to determine the effect of the overexpression or disruption of these genes on the aroma bouquet of wine.

The results obtained indicated that there are differences in the aroma bouquet of wine and brandy when changes are made in gene expression. The results indicated that the *ATF1* gene plays a large role in the production of ethyl and isoamyl acetate. When this gene was overexpressed, the level of ethyl acetate was 5.6-fold more than that of the control and the level of isoamyl acetate was 3.5-fold higher than that of the control. However, no increase in ethyl acetate or isoamyl acetate was observed when the *ATF2* gene was overexpressed. An increase in 2-phenylethyl acetate and diethyl succinate was observed in brandy, although there was a decrease in total ester concentration. A decrease in acetic acid was also observed in the brandy produced, which could be an indication of ester production. Similarly, no increase in ethyl acetate

or isoamyl acetate was observed in the wine or brandy produced when both the *ATF1* and *ATF2* genes were overexpressed in a single yeast. Once again, a marked decrease was observed in acetic acid concentration in both the wine and brandy.

In conclusion, it is clear that changes in gene expression can change the aroma profile of wine or brandy. However, the role of the *ATF2* gene still remains unclear and further studies are needed to clarify its role in yeast. Future studies involving the effect of wood maturation on ester concentration will also be of importance, so that the winemaker or distiller can make a product that suits the ever-changing market.

OPSOMMING

Die produksie van wyn is 'n komplekse proses wat die omskakeling van die suiker in mos tot etanol, koolstofdioksied en ander byprodukte tot gevolg het. Die hooforganisme betrokke in die wynmaakproses is gis, waarvan *Saccharomyces cerevisiae* as een van die belangrikste geag word as gevolg van die vermoë daarvan om onder die wynfermentasietoestande te kan oorleef, die "GRAS"-status (Generally Regarded As Safe) daarvan en die invloed wat dit op die aroma van die uiteindelike produk het weens die werking daarvan gedurende alkoholiese fermentasie. Die behoefte aan wyn met nuwe, verbeterde eienskappe het die vraag na meer gespesialiseerde gisrasse deur beide die verbruiker en nuwe wynmarkte gedurende die afgelope paar jaar drasties laat toeneem. Dit is om dié redes dat navorsing oor die bydrae van wyngis en houtveroudering tot die aroma van beide wyn en brandewyn so belangrik geag word.

Die vrugtige aroma van wyn word geassosieer met die esters wat gedurende die alkoholiese fermentasie deur gis gevorm word. Die belangrikste asetaatesters in wyn en brandewyn is etielasetaat, wat vir 'n oplosmiddelagtige, vrugtige aroma bekend is, en isoamielasetaat, wat 'n piesangaroma veroorsaak. Die esters word geproduseer deur die werking van asetieltransferases (AATases), wat as katalis in die reaksie tussen 'n hoër alkohol en asetiel-Ko-ensiem A optree. Alhoewel esters hoofsaaklik 'n produk van alkoholiese fermentasie is, wissel die konsentrasie daarvan gedurende houtveroudering. Daar is gevind dat die konsentrasie van die asetaatesters gedurende die verouderingsproses kan verhoog.

Die studie het ten doel om die invloed van AATase I en AATase II, wat onderskeidelik deur die *ATF1*- en *ATF2*-gene geënkodeer word, op die aroma van wyn en brandewyn te ondersoek. Die eerste doelwit van die studie was vervolgens om die *ATF2*-geen vanaf 'n kommersiële wyngisras te kloneer en dit daarna te ooruitdruk in 'n kommersiële wyngisras, asook die geen te ooruitdruk in 'n kommersiële wyngisras wat reeds die *ATF1*-geen ooruitdruk. Disrupsiekassette is ook vir die disrupsie van die *ATF1*- en *ATF2*-gene in 'n kommersiële wyngisras ontwerp. Die rekombinante wyngisrasse wat gedurnde die studie gemaak is, is vir die produksie van wyn en brandewyn gebruik. Gas chromatografise-ontledings en sensoriese evaluerings is ook op die wyn en brandewyn uitgevoer.

Die resultate van die studie het bewys dat daar wel veranderings plaasvind wanneer 'n verandering in geenuitdrukking gemaak is. Die resultate het weereens bevestig dat die *ATF1*-geen 'n belangrike rol in die produksie van etiel- en isoamiel-asetaat speel. Wanneer die *ATF1*-geen ooruitgedruk is, is die etielasetaatproduksie 5.6 keer meer en die isoamielasetaatproduksie 3.5 keer meer as in die kontrole. Die ooruitdrukking van die *ATF2*-geen het geen verhoging in etielasetaat of isoamielasetaat of in totale esters in die wyn getoon nie, alhoewel die ras 2.7 keer meer diëtielsuksinaat geproduseer het. In die brandewyn wat geproduseer is met die gisras waarin *ATF2*

ooruitgedruk is, was daar wel 'n verlaging in die asynsuur, wat 'n aanduiding van estervorming kan wees, alhoewel die totale esters wat geproduseer is minder was as in die kontrole. 'n Verhoging in diëtielsuksinaat en 2-fenielasetaat is ook gevind. Daar is geen verhoging in etiel- of isoamielasetaat getoon wanneer die *ATF1*- en *ATF2*-geen saam ooruitgedruk is nie. Die ras het minder totale sure in wyn en brandewyn geproduseer en ook geen verhoging in totale esters getoon nie.

Uit die resultate is dit duidelik dat veranderings in geenuitdrukking 'n verandering in die aromaprofiel van wyn en brandewyn kan veroorsaak. Die rol van dié *ATF2*-geen is nog steeds onduidelik en verdere studies sal moet plaasvind om die rol van die geen te verduidelik. Studies wat konsentreer op die invloed van houtveroudering op esterkonsentrasie is ook belangrik vir die toekoms, want dit sal die wyn- of brandewynmaker meer beheer oor die uiteindelike produk gee en daardeur die wyn- of brandewynmaker help om 'n produk te vervaardig wat sy mark bevredig.

This thesis is dedicated to my family and husband for their continuous love and support.

BIOGRAPHICAL SKETCH

Jenni Bayly was born in Stellenbosch South Africa on 17 April 1977. She matriculated from Rhenish Girls' High School, Stellenbosch, in 1995 and enrolled at Stellenbosch University in 1996. She obtained a BSc degree in 1998, majoring in Microbiology and Biochemistry. In 1999, she enrolled as an honours student at the Institute for Wine Biotechnology and obtained her BScHons in December 1999.

ACKNOWLEDGEMENTS

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

Dr. Maret du Toit, who acted as my supervisor, for her continuous support and encouragement and critical reading of this thesis.

Prof. Isak Pretorius and **Prof. Marius Lambrechts,** who acted as co-supervisors for this project, for their critical reading of this thesis.

Jason Hignett and **Mariska Lilly,** for their assistance and support with this project.

Michelle Veenstra, for all her help in the editing and formatting of this manuscript.

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

My **parents,** for their support and encouragement throughout my studies.

My **husband,** for his patience and encouragement.

PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the journal *Yeast*. Chapter 3 will be submitted for publication.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

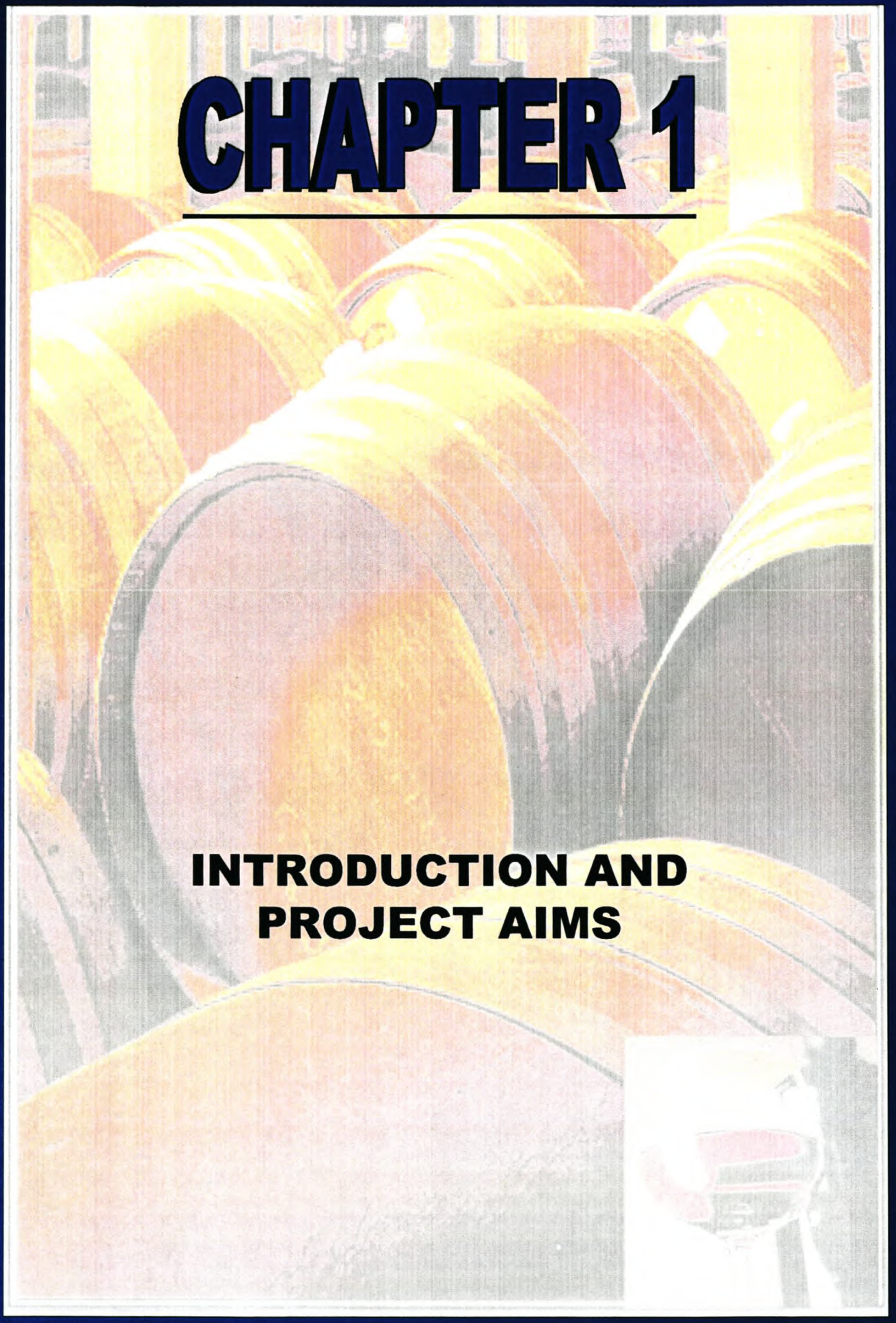
Chapter 2 **LITERATURE REVIEW**
The effect of wood maturation on the aroma of wine and brandy

Chapter 3 **RESEARCH RESULTS**
The manipulation of the levels of ethyl acetate and isoamyl acetate during the production of wine and brandy

Chapter 4 **GENERAL DISCUSSION AND CONCLUSIONS**

Chapter 5 **ADDENDUMS A, B, C AND D**

CONTENTS



CHAPTER 1

INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The fermentation of grape juice by yeast has been used for centuries to produce wine, a complex natural product (Rankine, 1989). The yeast *Saccharomyces cerevisiae* has been identified as the most important yeast in wine, as it is able to survive under the stresses of winemaking conditions and because of the favourable aroma products it produces during alcoholic fermentation. The composition of the final product of an alcoholic fermentation is influenced by numerous factors, such as the grapes themselves, by the direct and indirect reactions that occur during alcoholic fermentation of the grape must, the winemaking process and the ageing methods, for example wood maturation (Amerine and Singleton, 1977). Due to all these processes, wine and other alcoholic beverages, such as brandy, are chemically complex beverages, in which over 1300 volatile compounds have been identified (Nykänen, 1986).

The odours that result from the alcoholic fermentation process are mainly due to ethyl alcohol, higher alcohols and volatile compounds, such as esters. These odours give wine its grape-like and vinous character (Amerine and Singleton, 1977). The characteristic fruity flavours of wine are due to a mixture of various esters, most importantly hexyl acetate, ethyl caproate, ethyl caprylate, isoamyl acetate and 2-phenylethyl acetate (Pretorius, 2000). The fruity flavour of esters is usually associated with young wines (Lambrechts and Pretorius, 2000). However, studies by Aznar *et al.* (2001) showed that esters are still found in wines that have been aged in wood and still contribute to the aroma bouquet after ageing. Therefore, esters are not only important in young wines, but can be important in older wines too. The influence of wood on the aroma of wine and brandy therefore was researched.

The compounds extracted from the wood maturation process result in a compositional change in the wine and it therefore develops new and more complex aromas (Amerine and Singleton, 1977). Wood maturation results in the extraction of volatile and non-volatile compounds, which influence both the aroma and flavour of wine and brandy (Sefton, 1991). During wood maturation, the aromas formed during the alcoholic fermentation decrease. The concentration of esters tends to decrease during barrel maturation. However, the decrease will depend on the type of wine aged and the conditions of ageing (Cerdán *et al.*, 2002). In general, it has been found that the acetate esters of fusel oils decreased more rapidly than the ethyl esters from fatty acids (Ramey and Ough, 1980).

Many winemaking processes remain non-specific, such as the levels of compounds formed during the fermentation process as well as the extractions that take place during the wood maturation stage. Although many studies have been conducted on wood maturation, the exact extraction of compounds that can take place remains uncontrolled. However, science and new technologies can be used to

reduce the level of unpredictability in the winemaking process at the fermentation stage. The production of various compounds produced during alcoholic fermentation can also be increased or decreased through the use of genetic modification, thereby giving the winemaker a choice with regard to the degree of control over the aroma bouquet of the wine and brandy produced.

Due to globalisation and easy access to information, consumers have become more knowledgeable with regard to product value and quality (Bisson *et al.*, 2002). Through the genetic modification of genes involved in the production of aroma compounds and a thorough knowledge of winemaking processes that influence aroma, such as the wood maturation stage, winemakers and distillers may be able to produce wines and brandies specific for niche markets.

In this thesis, the effects of wood maturation on wine flavour are discussed in detail and the effect of the genetic modification of wine yeast is researched with regard to the changes in wine composition and sensory characteristics.

1.2 PROJECT AIMS

This study forms part of the ongoing research at the Institute for Wine Biotechnology concerning the influence of yeast on the aroma of wine and brandy, in particular the role played by ester-producing genes in wine and brandy aroma. Previous research showed that the overexpression of the native yeast *ATF1* gene had a significant effect on the levels of certain acetate esters, thereby dramatically changing the aroma profiles of the wine and brandy produced (Lilly *et al.*, 2000). This project is a continuation of this research and attempts to disrupt the *ATF1* gene and overexpress and disrupt a related gene, the *ATF2* gene, in wine yeast.

The *ATF2* gene shows only 37% homology to the *ATF1* gene and the exact role in ester synthesis is unknown, but it seems to play a role in the production of isoamyl acetate (Nagasawa *et al.*, 1998). The *ATF2* gene has also been identified as playing an important role in the detoxification of certain steroids in the cell through esterification (Cauet *et al.*, 1999). The regulation of the *ATF2* gene is still not completely understood, but it is thought to be repressed by oxygen at a transcriptional level and unaffected by the presence of unsaturated fatty acids (Mason and Dufour, 2000). Unlike the *ATF2* gene, the *ATF1* gene is repressed at a transcriptional level by unsaturated fatty acids (Mason and Dufour, 2000) and, similarly to the *ATF2* gene, it is repressed at a transcriptional level by the presence of oxygen (Fujiwara *et al.*, 1999).

The specific aims of the research conducted were:

i. to amplify the *ATF2* gene from the industrial wine yeast strain VIN13, using PCR primers;
ii. to clone the ATF2 gene from the industrial wine yeast strain VIN13 into a vector containing an overexpression cassette;

iii. to overexpress the *ATF2* gene in the industrial wine yeast strain VIN13;

iv. to overexpress the *ATF2* gene in the industrial yeast strain VIN13 in which the *ATF1* gene is already being overexpressed (Lilly *et al.*, 2000);

v. to disrupt the *ATF1* and *ATF2* genes in the industrial wine yeast strain VIN13;

vi. to determine the changes in aroma profiles of wine and brandy produced using these modified wine yeast strains; and

vii. to sensorially evaluate the wine and brandy produced using these modified wine yeast strains.

1.3 LITERATURE CITED

Amerine MA, Singleton VL. 1977. Alcoholic fermentation. In *Wine, an introduction*. University of California Press, Ltd.: London; 74.

Aznar M, López R, Cacho JF, Ferreira V. 2001. Identification and quantification of impact odorants of aged red wines from Rioja. GC-olfactory, quantitative GC-MS, and odor evaluation of HPLC fractions. *J Agric Food Chem* **49:**2924-2929.

Bisson LF, Waterhouse AL, Ebeler SE, Walker MA, Lapsley JT. 2002. The present and future of the international wine industry. *Nature* **418:**696-699.

Cauet G, Degryse E, Ledoux C, Spagnoli R, Achstetter T. 1999. Pregnenolone esterfication in *Saccharomyces cerevisiae*: a potential detoxification mechanism. *Eur J Biochem* **261:**317-324.

Cerdán TG, Mozaz SR, Azpilicueta CA. 2002. Volatile composition of aged wine in used barrels of French oak and of American oak. *Food Res Int* **35:**603-610.

Fujiwara D, Kobayashi O, Yoshimoto H, Harashima S, Tamai Y. 1999. The molecular mechanism of the multiple regulation of the *Saccharomyces cerevisiae ATF1* gene encoding alcohol acetyltransferase. *Yeast* **15:**1183-1197.

Lambrechts MG, Pretorius IS. 2000. Yeast and its importance to wine aroma – a review. *S Afr J Enol Vitic* **21:**97-129

Lilly M, Lambrechts MG, Pretorius IS. 2000. Effect of increased yeast alcohol acetyltransferase acyivity on flavor profiles of wine and distillates. *Appl Environ Microbiol* **66:**744-753.

Mason AB, Dufour JP. 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* **16:**1287-1298.

Nagasawa N, Bogaki T, Iwamatsu A, Hamachi M, Kumagai C. 1998. Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (*ATF2*) from *Saccharomyces cerevisiae* Kyokai No.7. *Biosci Biotechnol Biochem* **62:**1852-1857.

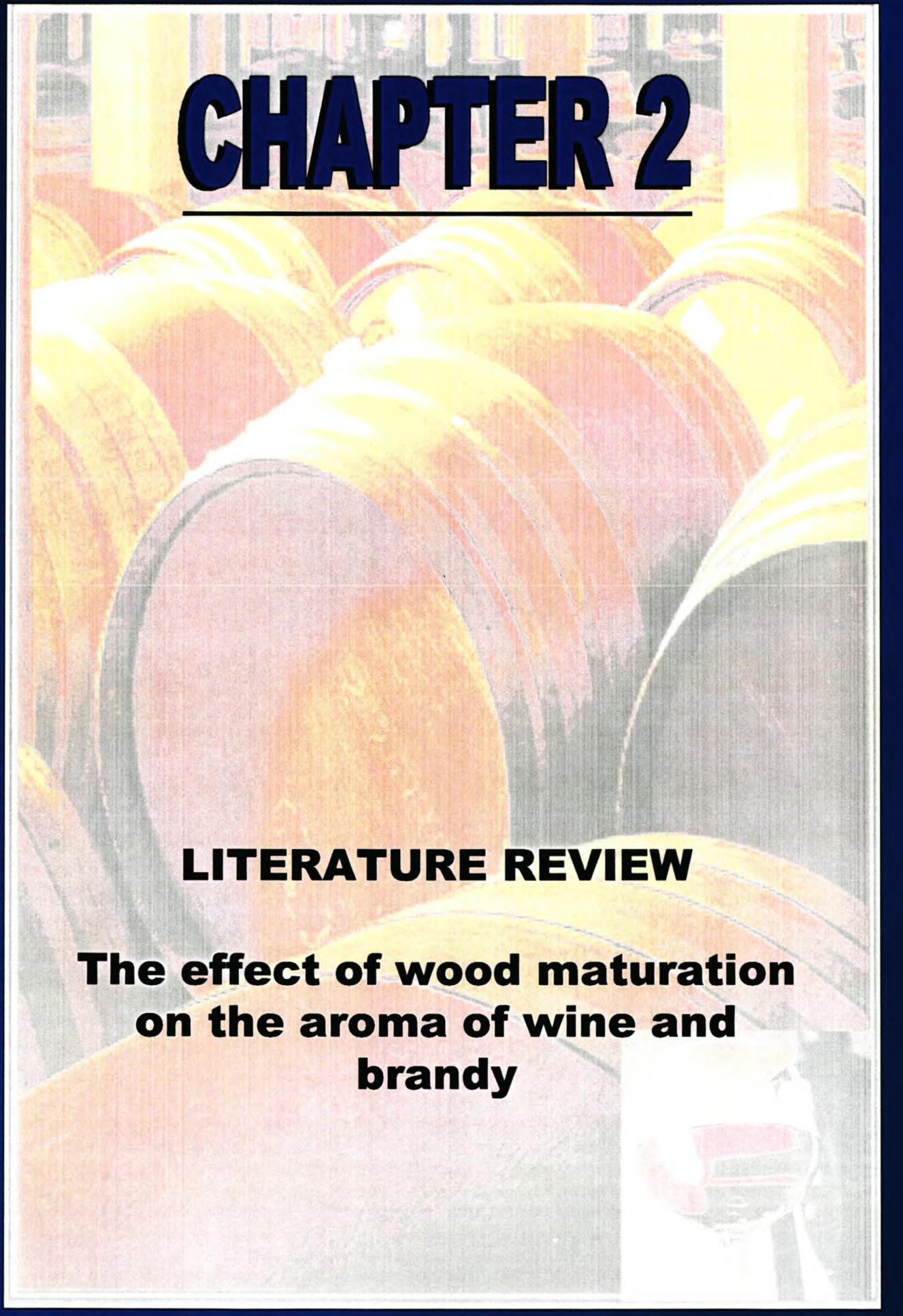
Nykänen L. 1986. Formation and occurrence of flavor compounds in wine and distilled alcoholic beverages. *Am J Enol Vitic* **37:**84-96.

Pretorius IS. 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16:**675-729.

Ramey DD, Ough CS. 1980. Volatile ester hydrolysis or formation during storage of model solution and wines. *J Agri Food Chem* **28:**928-934.

Rankine B. 1989. The composition of wines. In *Making Good Wine*. Pan Macmillan Australia Pty Ltd.; 293.

Sefton MA. 1991. How does oak barrel maturation contribute to wine flavor? *Wine Ind J* **2:**69-72.



CHAPTER 2

LITERATURE REVIEW

The effect of wood maturation on the aroma of wine and brandy

2. LITERATURE REVIEW

2.1 INTRODUCTION

Since ancient times, wine has been stored in some sort of container. At first, clay pots were used for storage and only much later, in Gaul, did they start to use wooden containers. This was due to a lack of clay in the area (Chatonnet, 1999). The use of the wooden container has changed since then. Instead of being used only to store wine, it is now used for its ability to enhance the flavours of the product that is aged in it (Swan *et al.*, 1997).

Wood is not inert and it plays an important role in the oxidation of wine and brandy, as well as contributing to their organoleptic qualities. Both volatile and non-volatile compounds are extracted from the wood. The degree to which these compounds are extracted is affected by various factors, which are discussed in section 2.4 of this review.

Wood maturation is also important for newly distilled spirits, because spirits are usually colourless and have a harsh and overpowering alcoholic flavour. The maturation of spirits in wood provides spirits with a richer, more golden colour, as well as a smoother flavour and aroma. Both the flavour and aroma become more complex and richer after barrel maturation (Maga, 1989) and this provides the style and uniqueness of each distilled product.

In this review, the compounds extracted from wood are discussed, as well as their effect on the aroma and flavour of wine and brandy. With the knowledge of wood extraction and the flavour and aroma of these compounds, a better understanding can be obtained of the aroma and flavour profile of wines and brandy aged in barrels.

2.2 PROPERTIES OF WOOD USED FOR THE MATURATION OF WINE AND BRANDY

2.2.1 Origin and species of wood used for the maturation of wine and brandy

The most common wood used for the maturation of wine and brandy is oak, belonging to the genus *Quercus*, which generally grows in temperate regions of the Northern hemisphere and at high elevations in some parts of the tropics (Schahinger, 1991). Oak is broadly classified as white or red oak, of which only a few of the species of white oak are suitable for cooperage. Redwood is sometimes used for large containers, but not for the barrels used for maturation, due to its harsh flavours and permeability (Chatonnet and Dubourdieu, 1998).

The species of oak used for cooperage in Europe are *Quercus robur* (pedunculate oak) and *Quercus petraea* (sessile oak). In North America, the predominant species of oak is *Quercus alba* (Schahinger, 1991). Chestnut wood has

also been used, although not often for cooperage (Swan *et al.,* 1997). Oak used in cooperage and for the maturation of wine and distilled spirits is usually from France or North America. The French oak is more expensive, about two to four times more than American wood (Waterhouse and Towey, 1994). Oaks from other countries, such as Spain, Yugoslavia, Hungary and Russia, are also used. However, the supply of oak from these countries is too small for foreign markets and it is used mostly for their own maturation of wine or distilled beverages (Schahinger and Rankine, 1992).

Q. petraea and *Q. robur* are found in different regions of France. *Q. petraea* is found in the plateau regions in the centre of Northern France and in the Vosges region (close to the forests of the central region). This species also grows well in areas with poor soil, such as the Allier forests and in the Never region of France. *Q. robur* is found in the South-western plains of France, in the plateaus of the Limousin region and on the western border of the Massif Central (Remy, 1997). French oak is often named after the forest from which it comes, which means that one can find Limousin oak, Tronçais oak, Nevers oak and Burgundian oak (Graff and Tchelistcheff, 1969; Schahinger and Rankine, 1992).

Q. alba, the common oak used for cooperage in North America, grows between 30° and 65° latitude north. In the United States of America, white oak is grown in the area east of the Mississippi River, through to the Appalachian mountains, and from Alabama in the south to the Great Lakes in the north (Schahinger and Rankine, 1992). Major oak forestry operations are located in Missouri, Ohio, Wisconsin, Illinois and Iowa (Chatonnet and Dubourdieu, 1998). Oregon oak (*Quercus garryana*) can also be used for cooperage and is grown in Oregon and North Carolina (Chatonnet and Dubourdieu, 1998). Other white oaks found in America include *Quercus marcocarpa* and *Quercus lylata*. This wood may also be used for wine storage, but, at this stage, very little is known about their composition and their influence on wine quality (Chatonnet and Dubourdieu, 1998).

The Australian wine industry has tried to use other wood for maturation, such as jarrah (*Eucalyptus marinata*), Australian "oak" (*Eucalyptus regnans*), Karri (*Eucalyptus diversicolor*), Blackwood (*Acacia melanoxylin*), Red gum (*Eucalyptus rostrata*), Kauri (*Agathis robusta*), Redwood (*Sequoia sempivirens*) and Douglas Fir (*Pseudotsuga taxifolia*). However, none of these have proved to be suitable for the maturation of wine or brandy. This is due to the undesirable flavours they give to the wine, particularly the resinous flavour that is typical of softwoods (Schahinger and Rankine, 1992).

For the maturation of wine and brandy, different oak is selected by the producer in order to obtain the desired characteristics. In Europe, most brandies are traditionally aged in barrels made from *Q. robur* (Guichard *et al.,* 1995). The most popular wood for the maturation of brandy in brandy-producing countries, such as South Africa, Germany, Mexico and Australia, is *Q. robur* from the Limousin region in France. In France, oak from the Limousin and Tronçias areas is used for the maturation of cognac (Guymon and Crowell, 1970).

2.2.2 Importance of wood structure for wine and brandy maturation

The wood used for the maturation of wine and brandy has not always exclusively been oak. In the past, other woods, such as chestnut, beechwood, acacia and ash, have been used. However, due to its physical and chemical characteristics, oak has been chosen as the wood used mostly in the vinification and ageing of vintage white and red wines, as well as for brandy (Remy, 1997). Hardwoods, such as oak, are more suitable for maturation than softwoods because they are tougher and more resistant to decay and fungal and insect attack. Softwoods tend to leak and need to be sealed, usually with wax, to prevent leakage. Once the softwood has been treated, it can be used for inert storage (Schahinger and Rankine, 1992). Other features that have made oak particularly suitable for barrel maturation are the wide medullary rays and thyloses, its durability, toughness and pliability and the favourable aromatic contribution it adds to the beverage aged in it (Singleton, 1995). The multiseriate rays and thyloses are of particular importance, as they are structural features that make oak suitable for cooperage (Schahinger and Rankine, 1992).

Medullary rays form part of the anatomy of all woods. These rays are much harder than other parts of the wood and are impermeable. Oak is different to most other woods in respect of these rays, as the cell layer is usually two or more cells wide (multiseriate), whereas this layer is only one cell wide (uniseriate) in other woods (Schahinger and Rankine, 1992). In *Q. alba*, this part of the wood makes up about 28% of the wood volume, whereas it ranges from 19-32% in other oak wood. These rays are the structure that gives oak its strength, flexibility and its watertightness (Schahinger and Rankine, 1992).

When oak trees grow, annual rings are formed consisting of two different types of wood: early (spring) wood and late (summer) wood (Hale *et al.,* 1999). Together, the early wood and the late wood make up the heartwood of the oak. The two rings differ in makeup, with the early wood consisting mainly of large vessels and the late wood mainly of wood fibres (Hale *et al.,* 1999). It is the presence of the large vessels in the early wood that makes oak ring-porous. The late wood is more dense than the early wood due to the lack of major vessels (Singleton, 1995). Thyllosis occurs within the early wood. During this process, thyloses are produced and form thin membranes, the thyllae, which stops liquid from moving through the heartwood of the tree (Chatonnet and Dubourdieu, 1998). The thyllae block the large conducting vessels in the early wood (Singleton, 1995). These structures are also important for keeping the oak watertight as, without them, the barrel contents would leak out at the stave ends (Singleton, 1995).

The heartwood of oak is a complex mixture of about 45% cellulose, 25% hemicellulose, 23% lignin and only a small percentage of extractives, 3-5%, which includes the wood tannins (Schahinger and Rankine, 1992). While the celluloses make up a large percentage of the wood, it seems that only the lignin and extractive material contribute to the barrel quality (Singleton, 1995). The hemicelluloses influence the wine and brandy indirectly, as it is only after pyrolysis (heating) that this

section is chemically transformed and then serves as a base for odourous compounds other than those from the lignin, extractive material and tannins (Schahinger and Rankine, 1992). The hemicellulose part of the wood is made up of heterogeneous polymers, including xylose and pentoses, which can be acetylated (Singleton, 1995).

Oak lignins are three-dimensional polymers formed by the co-polymerisation of two phenylpropenoic alcohols, hydroxy-4-methoxy-3-cinnamic alcohol (which has a guaiacyl structure; coniferyl alcohol) and hydroxy-4-dimethoxy-3,5-cinnamic alcohol (which has a syringyl structure; synapylic alcohol) (Vivas *et al.,* 1998). During the maturation of wines and spirits, the degraded part of the lignin releases phenolic aldehydes, such as vanillin, syringaldehyde, coniferaldehyde and sinapaldehyde (Vivas *et al.*, 1998).

2.2.3 Different methods of providing wood character to wine or brandy

Exposure of an alcoholic product to oak does not necessarily have to take place by means of a barrel. Today, many winemakers use wooden staves or chips in their stainless steel tanks in order to obtain a woody character and to reduce the costs of obtaining this "woody" character without the use of new barrels. The simplest way to add wood flavour to wine is through the addition of wood chips (Puech *et al.,* 1989). Other methods have also been used in order to extend the life of a barrel, including shaving out the barrels, renewing the heads and adding oak staves to the barrel (Schahinger and Rankine, 1992). However, Heraty *et al.* (1999) commented that the replacement of the head of the barrel was costly and impractical, due to the dislike of coopers working on site and working on barrels that they had not made themselves.

Oak chips are available in many forms. The cheapest way of obtaining these chips is from the shavings produced as a waste or byproduct during coopering. However, chips are available commercially and these are graded to uniform sizes. The commercial chips can be toasted to a standard degree. Fine chips, almost the texture of sawdust, are available and can be used as "tea bags" to give the product more "oakiness" (Schahinger and Rankine, 1992). It is generally found that European oak chips are harder to use on semi-finished wines than American oak chips, due to their higher tannin content (Singleton, 1995).

Oak extract may also be used instead of chips, and this method allows the winemaker or distiller a certain degree of control regarding the amount of oak extract added to the product (Puech *et al.,* 1989). These extracts are available in both a liquid and powder form and seem to be unpopular among winemakers (Schahinger and Rankine 1992) due to research showing that the final product is less complex than that produced by barrel maturation. In order to obtain the oak extracts, the chips are treated with 40% (v/v) and 20% (v/v) alcohol-water solutions and then in water at a desired temperature (higher temperatures for greater extraction). Other ways to increase the extraction are chemical treatments of the wood, such as sulphuric acid

or sodium carbonate treatments. Heating the wood can also increase the extraction yield (Puech *et al.*, 1989).

Oak staves have become popular in winemaking practices, both to extend the barrel life and to add wood in combination with stainless steel tanks (Swan *et al.*, 1997). These staves are inserted into the stainless steel tank itself. The staves may be toasted to a desired degree and different kinds of oak can be used. The winemaker can also decide on the amount of staves and, through this, he can control the exposure time of the wine to wood. However, this method of using oak staves does not seem to be as economical as chips and is actually relatively expensive (Schahinger and Rankine, 1992).

Although many forms of wood ageing exist, Swan *et al.* (1997) showed through both sensory and chemical analysis that the use of the oak barrel for maturation results in a much more complex product. Differences are found between wine with no wood ageing, wine aged in the barrel, wine aged in the presence of a high amount of oak staves, wine aged in the presence of a low concentration of oak staves and wines aged in the presence of oak chips. Sarni *et al.* (1990) found that wine that underwent barrel maturation was more complex than wine matured in the presence of oak chips or oak staves. However, Spillman (1999) found discrepancies in the comparative studies between maturation in oak barrels and the maturation in the presence of oak chips, as they had not been standardised. Spillman (1999) suggests that these comparative studies should be repeated, using the same methods for the treatment of the oak barrels and the oak chips. Previous studies have used inferior preparations for the oak chips and therefore the wine produced was not as complex. Thorough standardised studies should be conducted before any conclusions are made.

2.3 EXTRACTIONS FROM WOOD

2.3.1 Volatile fractions

2.3.1.1 Oak lactones

Masuda and Nishimura (1971) were the first to identify the two diastereoisomers of β-methyl-γ-octalactone, 4-hydroxynonanoic acid-γ-lactone and eugenol, as oak components. They found these components present in various woods, including the white oak, *Q. alba* (Masuda and Nishimura, 1971). Four oak lactone diastereoisomers have been identified in pure solutions, the *cis* (3S, 4R) and (3R, 4S) and the *trans* (3S, 4R) and (3R, 4S), of which only the *cis* (3S, 4S) and *trans* (3S, 4R) isomers, represented in **Figures 2.1a** and **b**, are present in oak extracts (Masson *et al.*, 1995a).

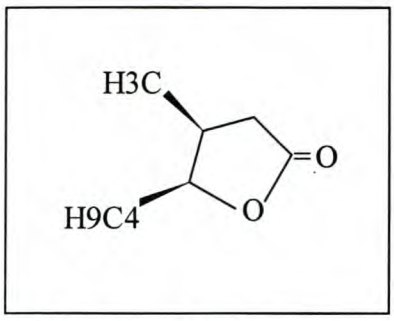


Figure 2.1a. The chemical structure of *cis* (3S, 4S) β-methyl-γ-octalactone (Masson *et al.*, 1995a)

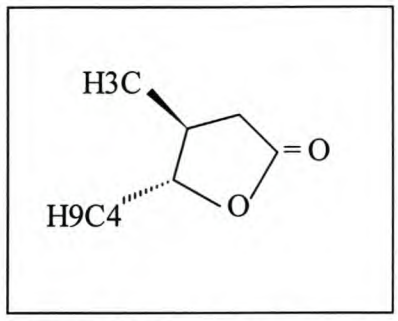


Figure 2.1b. The chemical structure of *trans* (3S, 4R) β-methyl-γ-octalactone (Masson *et al.*, 1995a)

The lactones found in oak are referred to as the aromatic oak lactones and are often called the oak or whiskey lactones (Waterhouse and Towey, 1994). The oak lactones are specific to the *Quercus* genus and are largely responsible for oak's aromatic quality (Chatonnet, 1999), contributing to the "oakiness" of wines and other alcoholic distillates aged in barrels (Singleton, 1995). The *cis* and *trans* isomer of β-methyl-γ-octalactone have different aroma threshold values, with the *cis* lactone having an aroma threshold of 96 μg/L and the *trans* isomer having a threshold of 460 μg/L in white wine (Chatonnet *et al.,* 1991). Not only does the *cis* isomer have a lower sensory threshold than the *trans* isomer, but it has also been found to be more aromatic than the *trans* isomer (Otsuka *et al.*, 1974). The *cis* isomer has a sensory threshold 12 times lower than that of the *trans* isomer and this difference can even be as great as 20 times when using GC-sniffing analysis (Masson *et al.,* 1995a). The *cis* isomer is well known sensorially for its oaky, vanilla flavour at low concentrations and coconut or varnish-like aroma at high concentrations (Towey and Waterhouse, 1996a). Other authors state that the *cis* isomer may also have "slightly musty and earthy" notes.

The *trans* isomer, on the other hand, is described as having a "fragrant celery" note, with a "weak coconut" aroma and a little "green walnut" character (Sefton *et al.,* 1993a). In alcoholic solutions, such as wine or brandy, the lactones occur in three different forms, as lactones themselves, in an open acid form, or as ethyl esters (**Figure 2.2** and **Figure 2.3**). However, Chatonnet *et al.* (1991) state that too much β-methyl-γ-octalactone can have a negative impact on white wines, with the optimal concentration being between 150-375 μg/L, as above this the wines taste too woody and resinous (Chatonnet *et al.,* 1991). The levels of lactones extracted may be manipulated through the toasting process during cooperage (Chatonnet *et al.,* 1991).

Straight chain gamma-non-alactone is another lactone extracted from wood, with an aroma threshold of 30 μg/L. This lactone contributes to a sweeter, more pleasant and fruitier bouquet. However, this lactone is found at levels lower than its threshold in most commercial wines (Singleton, 1995).

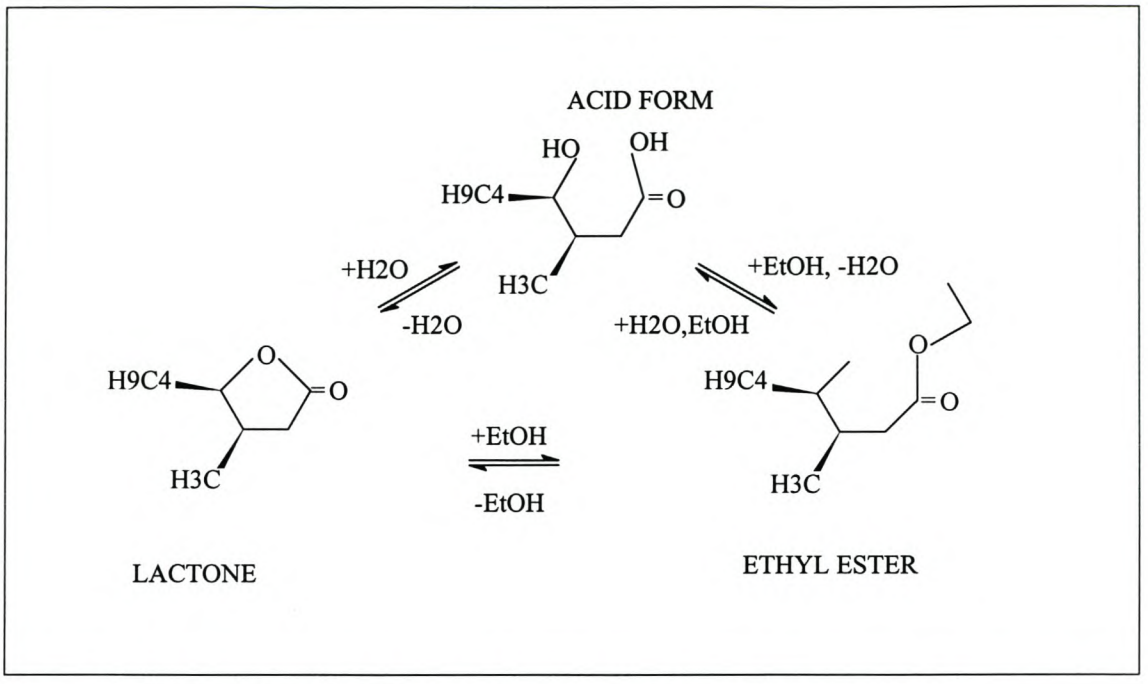


Figure 2.2. The various forms of the *cis* isomer (Waterhouse and Towey, 1994)

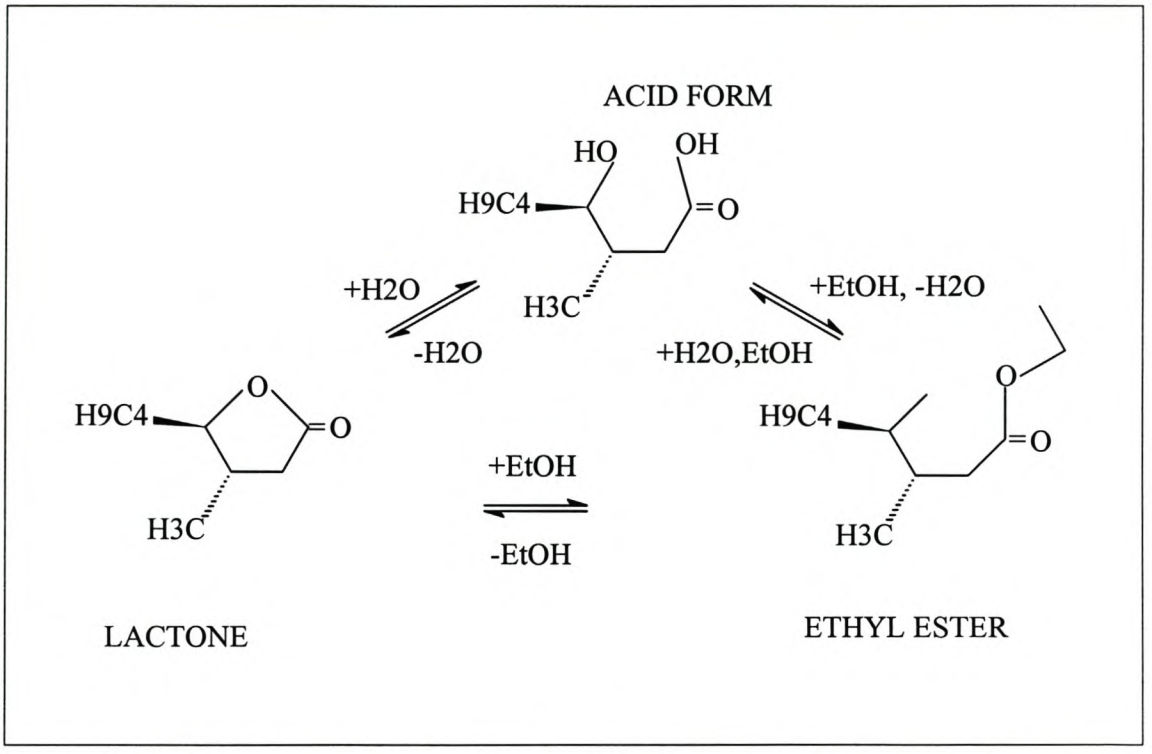


Figure 2.3. The various forms of the *trans* isomer (Waterhouse and Towey, 1994)

2.3.1.2 Volatile phenols

The majority of phenols found in wine come mainly from the grapes. However, during the maturation of wines and other alcoholic distillates, quantitative and qualitative changes occur. The increase in the amount of phenols is due to the

extraction of phenols from the wood. Phenols extracted from wood include low to medium molecular weight colourless compounds, flavonoids, pigments and high molecular weight tannins (Jindra and Gallander, 1987). Phenolic compounds are obtained through the degradation of lignin during the ageing process, through hydrolysis, pyrolysis (subjection to heat), or oxidative breakdown (Sefton, 1991). Not all phenols are derived from pre-existing phenols in the wood, as some are generated by carbohydrates during pyrolysis (Singleton, 1995). Phenols are most commonly lignin degradation products (Puech, 1987).

The main volatile phenol extracted from oak is eugenol (1-propenyl-4-methoxy-2-phenol), shown in **Figure 2.4** (Ribéreau-Gayon *et al.,* 2000). Eugenol is unlike other volatile phenols, as it is present in untoasted wood and is not generated solely through toasting (Towey and Waterhouse, 1996a). Eugenol has a sensory threshold of 11 μg/L in wine conditions (10% ethanol solution) and about 50 μg/L in 20% ethanol solutions (Singleton, 1995). The aroma of eugenol is perceived as clove-like. However, Singleton (1995) states that this is not possible at the concentration found in wine and brandy and that eugenol contributes to the general "oakiness" of the wine or brandy. Other phenols extracted from wood are nonflavonoid compounds, such as lignins, hydrolysable tannins, gallic acid, ellagic acid as well as aromatic acids and aldehydes (Jindra and Gallander, 1987). These are non-volatile phenols and will be discussed later.

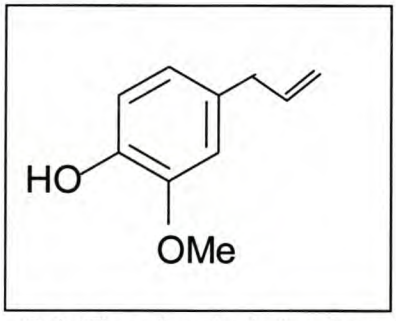


Figure 2.4. The chemical structure of eugenol (Sefton, 1991)

The degraded lignin-derived aromatic acids and aldehydes extracted from wood include cinnamic and benzoic aldehydes, as well as vanillin, syringaldehyde, sinapaldehyde and coniferaldehyde (Puech, 1987). Most research on volatile phenols has been done on vanillin and related compounds, such as syringaldehyde, coniferaldehyde, sinapaldehyde, acetovanillone and acetosyringone (Singleton, 1995).

Vanillin (4-hydroxy-β-methoxy-benzaldehyde), (**Figure 2.5**) is a principle aromatic active component in natural vanilla and, in oak barrels, vanillin is formed as a degradation product of lignin during pyrolysis (Spillman *et al.,* 1997). The threshold value of vanillin differs in red and white wines. In white wines, the threshold value is 65 μg/L, whereas it is much higher in red wine, at 300-400 μg/L (Sefton *et al.,* 1993a). It has been found that more syringaldehyde than vanillin is usually extracted from wood, but vanillin is much more aromatic (Singleton, 1995). In spirits aged in

new barrels, the levels of vanillin can be much higher than their sensory threshold. It is thought that the aroma of vanillin is strengthened by the other compounds in the group (Singleton, 1995).

Research has shown that vanillin plays a larger role in spirits, where it is often above its threshold value, than in wines, where the concentrations are usually below the threshold concentration and where it can be subject to biological reduction (Spillman *et al.,* 1997). This occurs when the wines are aged on the lees, as the yeast converts the vanillin to vanillic alcohol, which is less aromatic than vanillin (Singleton, 1995).

Although the levels of vanillin increase during wood ageing, the vanillin levels begin to decrease after long periods of wood maturation due to chemical changes (Spillman *et al.,* 1997). The overall sensorial contribution of vanillin to wine is still a debatable topic. Spillman *et al.* (1997) agree with researchers, such as Chatonnet (1999), that vanillin is an important contributor to wine flavour, even though it is found at subthreshold values. However, they agree with other researchers that other oak derived compounds, including β-methyl-γ-octalactone, also contribute to the vanilla flavour of wine. Oak-extracted ketone phenyls, such as acetovanillone, propiovanillone and propiosyringone, may intensify the vanilla aroma (Naudin, 1990).

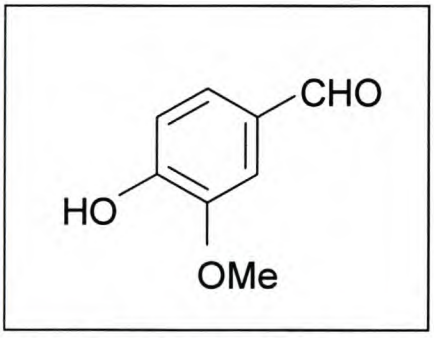


Figure 2.5. The chemical structure of vanillin (Sefton, 1991)

Volatile phenols based on guaiacyl (4-hydroxy-3-metyloxyphenyl) (**Figure 2.6**) or syringyl (4-hydroxy-3,5-dimethoxyphenyl) (**Figure 2.7**) nuclei are also extracted from oak (Sefton *et al.,* 1990). The guaiacyl derivatives generally have low aroma and taste thresholds compared to the syringyl derivatives, which have weak odours and make less of a contribution to the wine character (Sefton, 1991). Guaiacol and 4-methylguaiacol are extracted from oak, although at very small concentrations and only from untoasted or moderately heated wood. Larger quantities can be generated during lignin degradation occurring during barrel toasting at high temperatures (Sefton, 1991).

4-Ethylguaiacol and 4-ethylphenol are found in higher concentration in wines aged in used oak. However, very small amounts of these compounds are found in the extractive part of the oak (Sefton, 1991). High concentrations of 4-ethylphenol (medicinal, horse-like aroma) and 4-ethylguaiacol (smokey, spicy, medicinal-like

aroma) may be the result of bacterial or yeast contamination of the used oak barrels and not from the wood at all (Pollintz *et al.*, 2000).

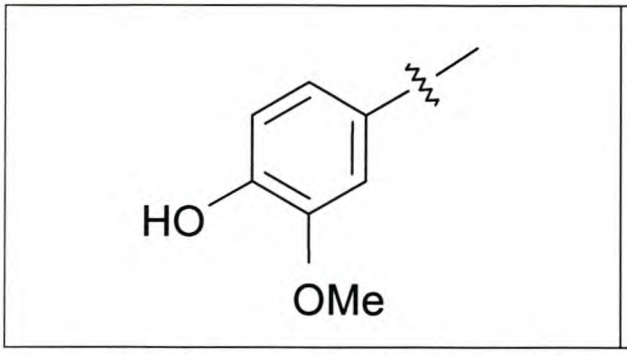


Figure 2.6. The chemical structure of the guaiacyl nucleus (Sefton, 1991)

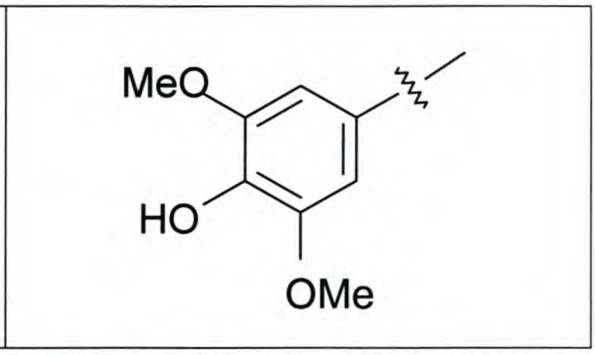


Figure 2.7. The chemical structure of the syringyl nucleus (Sefton, 1991)

The levels of phenols extracted from the wood depend on the length of time the wine or brandy has been in the wood, the number of fills the barrel has had, as well as the origin of the wood used to make the barrel (Jindra and Gallander, 1987). It is also interesting to note that Pocock *et al.* (1994) found that the total concentration of oak polyphenols was less in white wines than in model wine solutions after wood maturation. They thought that the fact that the white wines had less polyphenols could be attributed to the maturation of the wine on the yeast lees. The polyphenolic material may precipitate with yeast-derived protein. Therefore, the oak-derived tannins may have more of a sensory impact on white wines that have been racked off the lees and clarified before barrel maturation (Pocock *et al.,* 1994).

Pyrolysis of lignin can also result in the formation of free phenols, such as phenol itself, guaiacol, 2,3-dimethoxyphenol, catechol, resorcinol and hydroquinone. These components have characteristic medicinal aromas, but, their levels are too low in normal wines to have any affect on the flavour (Singleton, 1974).

2.3.1.3 Carbohydrate-derived volatiles

During pyrolysis, both the cellulose and hemicellulose in oak are degraded. The carbohydrate-derived volatiles are present in unheated oak, but at very low amounts (Sefton, 1991). The main carbohydrate-derived volatiles are furan aldehydes, of which the most dominant is furfural (**Figure 2.8**), which is produced from the pentoses in the lignin fraction of the wood. Rhamnose sugars in the wood are converted to 5-methyl furfural, while the hexoses in the wood are converted into 5-hydroxymethyl furfural (Singleton, 1995). Furfural plays a large role in the aroma of brandy, but it is enzymatically degraded to hydroxymethyl analogues in wine matured on the lees. These analogues have very high aroma and taste thresholds and are therefore unlikely to play a large role in the aroma and taste of wine (Sefton, 1991). Although furfural is below its flavour threshold value in wine, it may still influence the flavour of the wine indirectly, as synergistic effects between flavour and aroma compounds occur during wood maturation (Towey and Waterhouse, 1996a). Yeast

cells may also decrease the levels of furfural, as they are able to convert it to furfuryl alcohols, which have much less flavour (Naudin, 1990).

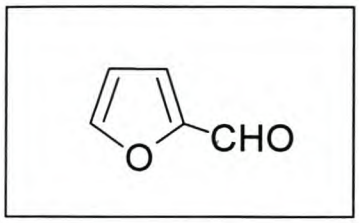


Figure 2.8. The chemical structure of furfural (Sefton, 1991)

In spirits, furfural contributes to a caramelised flavour and also gives "hotness" to the spirits (Singleton, 1995). It is important to note that the furfural found in brandy may not only be from oak extraction but may arise from distillation in a simple pot still as a result of the prolonged heating of the distilling material (Guymon and Crowell, 1972).

Polysaccharides also give rise to the presence of cyclotene (2-hydroxy-3-methyl-cyclopentenone) and maltol (3-hydroxy-2-methyl-pyranone), which are shown in **Figures 2.9** and **2.10** respectively (Schahinger and Rankine, 1992). Maltol is produced through the thermal degradation of 1,4-disaccharides in the wood and cyclotene is produced from less basic amadori intermediates and at a lower temperature than maltol. Aromatically, cyclotene and maltol contribute "burnt sugar" and "caramel" characteristics to the wine or brandy (Chatonnet, 1999). These compounds have been found only in toasted oak and are associated with furfural production (Singleton, 1995).

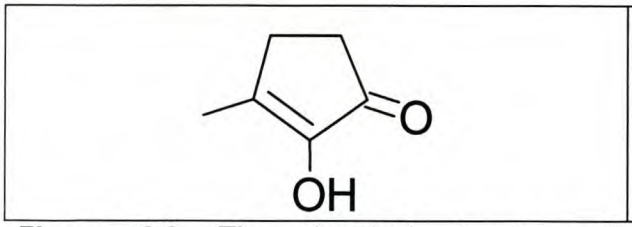


Figure 2.9. The chemical structure of cyclotene (Sefton, 1991)

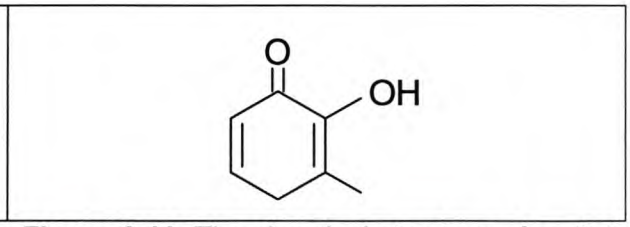


Figure 2.10. The chemical structure of maltol (Sefton, 1991)

Through the use of gas chromatography, new compounds have been identified in toasted oak. These new molecules have been identified as 2,3-dihydro-5-hydroxy-2-methyl-4(H)-pyran-4-one, contributing to a sweet vanilla odour, 4-hydroxy-2,5-dimethyl-3(2H)-furan-3-one, which has an intense "fruity-toasty" aroma and, lastly, 2,3-dihydro-3,5-dihydroxy-2-methyl-4(H)-pyranone, which has a "toasty", "fruity caramel" aroma. These molecules are found only in toasted oak (Chatonnet, 1999).

2.3.1.4 Terpene volatiles

Monoterpenes, sesquiterpenes and 9, 11 and 13-carbon norisoprenoids have been identified in extracts from American and Vosges oak (Sefton, 1991). Terpene derivatives are very odourous, especially the β-ionone, which has a violet aroma and

plays an important role in the flavour bouquet of red wine. The major norisoprenoic compounds found in oak include 3-oxo-α-ional, 4-oxo-β-ionol, blumenol C and the *cis* and *trans* isomers of 9-hydroxy-4,5-megastigmadiene-3-one (3-oxo-retro-α-ionol), which are unique to *Q. alba*. However, the role of monoterpenes, sequiterpenes and 9, 11 and 13-carbon norisoprenoids still remains unknown (Sefton, 1991). β-Carotene has been identified as a precursor of lutein in wood. The carotenoids may lead to the production of norisoprenoids (Masson *et al.,* 1997).

Many of the norisoprenoids found have been patented as flavour additives in the food, tobacco and perfume industries (Sefton, 1991). Bicyclic compounds of norisoprenoids, as well as tri-enone, have been found in oak. However, little research has been done with respect to the flavour thresholds of these compounds and their role in wine and brandy (Sefton, 1991).

2.3.1.5 Volatile acids

Volatile acids are among the extractives from oak and, together with other acids are responsible for the decrease in the pH of the wine and brandy aged in wood (Aiken and Noble, 1984). An increase in acetic acid, as well as ethyl acetate, is also found in wines and brandies aged in barrels. This is due to hydrolysis during the extraction process. Most of the acetic acid is derived from the alkaline hydrolysis of some of the hemicelluloses (Nishimura *et al.,* 1983). A large amount of acetic acid can be released through toasting and even more from charring of the oak (Singleton, 1995). The total acid concentration in wine and brandy is low at first and then increases after the first 12 months. Most of the acetic acid extracted from the wood is subjected to esterfication to ethyl acetate by the yeast due to the large excess of ethyl alcohol, especially in brandy (Onishi *et al.,* 1977).

2.3.1.6 Other volatiles

Triglycerides of unsaturated C_{12} and saturated C_{16} fatty acids, sterols and a ferulic acid ester with a C_{40} wax alcohol have been extracted from wood. These extracts have not yet been found to influence the flavour of the wine, but the sterols may produce haze in other products, such as spirits (Singleton, 1995).

Lyoniresinol (a lignin found in oak) (Sarni *et al.,* 1990), 2-phenylethanol, 4-vinylguaiacol (Towey and Waterhouse, 1996a) and pyrazines and pyridines (Sefton, 1991) are also compounds extracted from oak. However, their contribution to the aroma and flavour of wine is unknown.

Trans-2-nonenal, as well as *trans*-2-octanal and 1-decanal, can also be extracted from oak. Together, these compounds are responsible for the odour described as the "plank smell" that is acquired by some wines during ageing (Ribéreau-Gayon *et al.,* 2000). *Trans*-2-nonenal has a fairly low threshold value, at 180-200 ng/L, but it will alter the quality of red wine aroma only at about 600 ng/L (Chatonnet, 1999).

Diethyl succinate has also been reported as an oak extract in oak aged brandies. It is extracted from the wood as succinic acid. Under ageing conditions, the succinate esterifies with ethyl alcohol to form diethyl succinate (Onishi *et al.,* 1977). The

sensory effect of the diethyl succinate is negligible at the levels found in brandy (Guymon and Crowell, 1972).

2.3.2 Non-volatile fractions

2.3.2.1 Non-volatile phenols

The majority of phenolics extracted from oak are the hydrolysable tannins, which account for 5-15% of the dry weight of oak. These tannins are a complex mixture of oligomers of gallic acid and glucose, which are bonded by esters and oxidatively coupled linkages (Pocock *et al.,* 1994). The phenolic acids (gallic acid) contribute to the acidity of wine (Ribéreau-Gayon *et al.,* 2000). The exact origin of the gallic acid is unknown at this stage and it is thought that it is formed through the hydrolysis of galloyl esters. Galloyl esters have yet to be identified in oak and, at present, it is speculated that they are attached to some cell wall components (Viriot *et al.,* 1994).

In both wine and brandy, the increase in phenolics is due not only to the extraction, but also to the oxidation of aromatic aldehydes, which are considered to be degradation products of lignin (Jindra and Gallander, 1987).

Coumarins extracted from wood are found in two different forms: a glycosylated form (esculin and scopoline) found in green wood, or an aglycone form (esculetin and scopoletin) found in naturally seasoned wood. These two different forms of coumarins affect the wine differently. The glycosylated form gives a bitter taste, while the aglycone form contributes to the astringency of the resultant wine (Ribéreau-Gayon *et al.,* 2000).

2.3.2.2 Hydrolysable tannins

Tannins form part of a group of water-soluble plant polyphenols, which are characterised by their ability to bind proteins. Not all tannins are water soluble, as this characteristic is dependent on their molecular weight, e.g. they are not very soluble in water above 3000 Da. Tannins found in oak can be divided into two groups, the main group being the hydrolysable tannins and the other the non-hydrolysable tannins, which are also known as condensed tannins or proanthocyanidins (Puech *et al.,* 1999). Tannins are extracted into both wine and brandy during barrel maturation, as the tannins found after barrel maturation are not present in wine or brandy that has not been matured in barrels (Singleton, 1995).

The hydrolysable tannins, as well as the non-volatile tannins, form the major part of the nonflavonoids in oak extract (Quinn and Singleton, 1985), with the hydrolysable tannins being classified as either ellagitannins or as gallotannins (Puech *et al.,* 1999). These tannins are laid down in the heartwood at high concentrations during the growth of oak trees (Viriot *et al.,* 1994), with ellagitannins making up to 10% of the heartwood dry weight (Puech *et al.,* 1999) and being responsible for the durability of the oak (Masson *et al.,* 1994). They also aid the plant's resistance to microbial decay (Klumpers *et al.,* 1994) and defend the plant against fungal infection.

The chemical structure of ellagic acid, which is derived from ellagitannins, is shown in **Figure 2.11** (Viriot *et al.,* 1994).

Ellagitannins are esters of glucose with hexahydroxydiphenic acid (Bate-Smith, 1971). Eight ellagitannins have been identified in oak thus far. They are castalagin, vescalagin, grandinin and roburins A-E. Grandinin and roburins A-E are derived from castalagin and vescalagin and exist as dimers or with an additional pentose residue (Puech *et al.,* 1999). During tree growth, the ellagitannin content increases in the heartwood at first, but decreases constantly as the heartwood ages. The major ellagitannin in oak is castalagin; however, this ellagitannin decreases quickly during the growth of the tree (Viriot *et al.,* 1994). Vescalagin is also a common ellagitannin in oak (Puech *et al.,* 1999). As the ellagitannin content decreases in the wood, so the free ellagic and gallic acid increases (Viriot *et al.,* 1994).

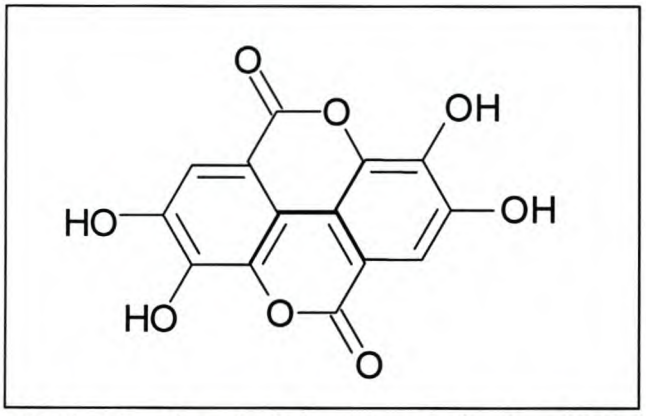


Figure 2.11. The chemical structure of ellagic acid (Sefton, 1991)

Wine pH encourages the hydrolysation of castalagin and vescalagin to produce castalin and vescalin respectively, due to the loss of a hexahydroxydiphenyl group. The hexahydroxydiphenyl group may be further hydrolysed to hexahydroxydiphenic acid and glucose, and hexahydroxydiphenic acid further to gallic acid. These reactions occur rapidly in water at wine pH, although these reactions are retarded in the presence of 10% ethanol, as in wine. Both gallic acid and hexahydroxydiphenic acid may contribute to oxidation reactions in wine (Chatonnet *et al.,* 1991).

Ellagitannins are involved in various reactions in wine. They have an effect on the structure of the phenolic compounds that are present in the wine, as well as on the colour of red wine. The ellagitannins also increase the rate of condensation of the procyanidins and limit the degradation processes, such as condensed tannin precipitation and anthocyanidin destruction (Vivas and Glories, 1996). During wine storage, the ellagitannins are easily oxidised and they produce hydrogen peroxide (H_2O_2) through oxidation with free radicals, such as oxygen, that have penetrated the wine. The reason for ellagitannins oxidising so easily, in comparison to other wine polyphenols, is because of their many *ortho* hydroxy functions. The H_2O_2 produced can now oxidise other wine components, in particular ethanol, which is transformed into acetaldehyde (Vivas and Glories, 1996). The ellagitannins also play a role as

antioxidants, due to their ability to oxidise easily and thereby prevent other compounds from oxidising (Puech *et al.,* 1999).

The ellagitannins are found in varying concentration in wines as a result of various factors, which will be discussed later. It is generally thought that high concentrations of ellagitannins contribute to the astringency and bitterness of wine (Quinn and Singleton, 1985). However, as in the case of the other extracts that have been discussed already, the concentration thresholds of ellagitannins in wine are higher than the concentrations at which they are extracted, therefore compounds other than ellagitannins are responsible for the astringent flavour of some wines that have been aged in oak (Puech *et al.,* 1999). The ellagitannins may have a synergistic effect with other phenolic compounds, thereby contributing to the astringency of wine. In distilled beverages, the ellagitannins may have an effect on the organoleptic quality of the distillate by affecting the solubility of volatile compounds and therefore their relative concentration in the solution and the headspace of the bottle (Puech *et al.,* 1999).

2.3.2.3 Non-hydrolysable or condensed tannins

This group of compounds is composed of C6-C3-C6 flavanol monomer units, which are linked by carbon bonds. This group is much less susceptible to hydrolysis than the hydrolysable tannins. Under intense heating conditions, the bonds of these tannins are broken in the presence of strong acids. When these bonds are broken, red-coloured anthocyanins are released, from which tannins derive the name, proanthocyanidins. The procyanidins (catechinic tannins) and the prodelphinidins (gallocatechinic tannins) form two of the most important groups of proanthocyanidins. Low amounts of these proanthocyanidins are found in oak heartwood. The condensed tannin concentration is much higher in grapes and their extraction from wood plays a very small role, if any, on the wine matured in the barrel (Puech *et al.,* 1999).

2.4 FACTORS INFLUENCING EXTRACTIONS FROM WOOD

Numerous factors can influence extractions from oak. This is due to the inherent differences in biological systems and the low reproducibility of traditional cooperage practices, such as the seasoning and toasting or charring of the oak (Towey and Waterhouse, 1996b).

2.4.1 Geographical origin and species of wood

The origin and the species of the oak influence numerous compounds extracted from oak. There are definite differences between the European oak (*Q. rubor* and *Q. petraea*) and American oak (*Q. alba*). Feuillat and Keller (1997) found that the influence of forest origin of European oak was comparatively lower than the species effect on the oak. However, the greatest difference is found between European and

American oak, with the former having a higher content of phenol and other extractables, whereas American oak is more odourous (Singleton, 1995).

With regard to the volatile compounds extracted from oak, the levels of β-methyl-γ-octalactone are significantly different in the different species of oak. Feuillat *et al.* (1997) found a marked difference in the levels of the *cis* and *trans* lactones, with pedunculate oaks (*Q. robur*) having much lower amounts than the wood from sessile oaks (*Q. pedunculata*) (Feuillat *et al.,* 1997). The levels of lactones can vary from 0.5 μg/g in dry wood (found in pedunculate oaks from the Limousin forests) to 77.9 μg/g (found in oak from the Tronçais forests) (Masson *et al.,* 1995a). However, when comparing the sessile and pedunculate oak to white oak, differences are found yet again. White oaks are even richer than the sessile oaks in lactone content (**Figure 2.12**).

It is important to note that the ratio of the *cis* to *trans* isomer is different in each tree, because of the difference in aromatic potential of the *cis* and *trans* lactone. Trees with a higher content of the *cis* isomer of β-methyl-γ-octalactone will have a higher aromatic potential than those with a high content of the *trans* isomer (Masson *et al.,* 1995a). In **Figure 2.12**, one can see the different levels of oak lactones extracted from the oak from various forests. This diagram clearly shows that pedunculate oaks have the fewest oak lactones and white oaks the most. There also is a difference between the levels of oak lactones from different forests, with oak from the Jupilles forest having a higher content of total lactones than oak from the Tronçais forest, and both of these are higher than oak from the Darney forest (Masson *et al.,* 1995a).

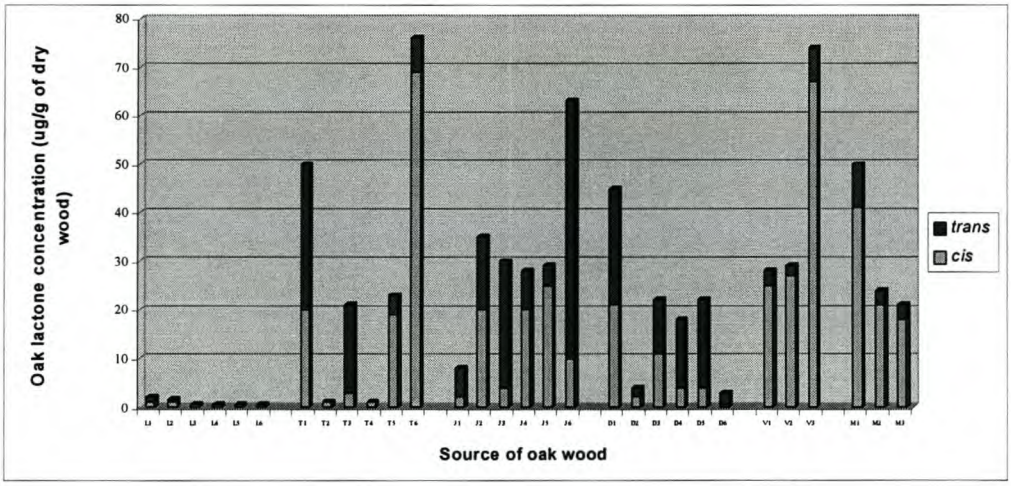


Figure 2.12. Differences in extraction of the *cis* and *trans* octalactone from oak from America and Europe (L=limousin region, T=Tronçais region, J=Jupilles region, D=Darney region, V=Virginian American wood and M=Missouri American wood) (Masson *et al.*, 1995a)

Masson *et al.* (1995a) reported that the high content of *cis* lactone in the American White oak contributes to an intense woody note. Guichard *et al.* (1995)

also found different levels of oak lactones in sessile, pedunculate and American white oak.

French oak barrels have a significantly higher total phenolic content than American oak barrels for the first barrel fill, with French barrels contributing up to 50% more total phenols than American oak. However, for the second fill, the extractable phenols are more or less the same (Towey and Waterhouse, 1996a). Differences in phenolic contents are also present between pedunculate and sessile oak. Pedunculate oaks have higher amounts of total phenols, total ellagitannins, vescalagin, castalagin and roburins A, B and D than sessile oaks (Feuillat *et al.,* 1997). Chatonnet and Dubourdieu (1998) measured twice as much extractable phenols in sessile oak than in white oak. Aiken and Noble (1984) found that there was a significant difference between the increase in total and nonflavanoid phenols in Cabernet Sauvignon aged in French versus American oak, as the extraction from the French oak was more than that from the American oak (Guymon and Crowell, 1972). Another study which compared oak tannin from American white oak and oak from the Tronçais, Vosges and Limousin regions in France (Sefton *et al.,* 1993b) measured the phenolic extracts as 35 g/kg (American oak), 45 g/kg (Tronçais and Vosges oak) and 55 g/kg for oak from the Limousin region. This shows, once again, that European oak is higher in total phenol extract than American oak.

Vanillin, syringaldehyde, coniferaldehyde, sinapaldehyde, vanillic acids and syringic acid are all found in higher concentrations in brandy aged in American oak. The level of these compounds in French oak is comparable with American oak that has already had one fill (Guymon and Crowell, 1970). Swan *et al.* (1997) compared vanillin levels extracted from Eastern European wood, American wood, French wood and chestnut wood. They found that there was a great difference between different barrels, but that American wood had the highest vanillin levels on average (**Figure 2.13**).

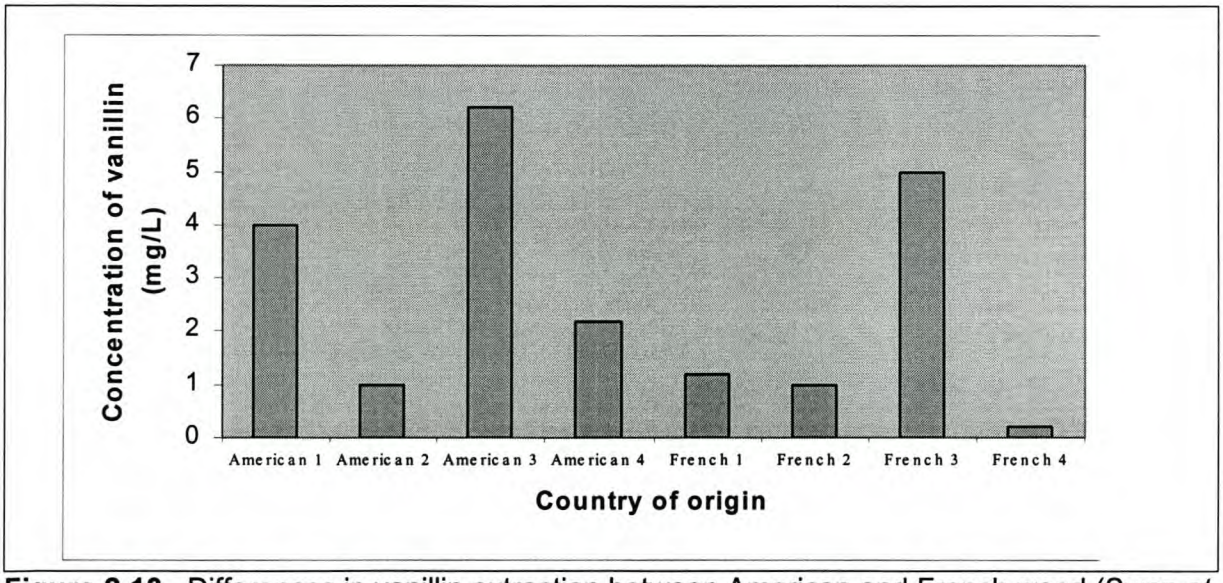


Figure 2.13. Differences in vanillin extraction between American and French wood (Swan *et al.,* 1997)

The carbohydrate-derived furfural has been found to be consistently higher in brandies aged in American oak (Guymon and Crowell, 1970). Pérez-Coello *et al.* (1999) also reported that American oak had higher amounts of furan derivatives than French oak.

When considering the aromatic aldehydes, Guymon and Crowell (1970) found that distillates aged in American oak were higher in aromatic aldehyde content than their French counterparts. However, they concluded that this difference was quantitative and did not result in a qualitative difference between the distillates aged in different wood.

Tannin extractions are higher from French oak than American oak, contributing to increased bitterness in brandy (Guymon and Crowell, 1970). Chatonnet and Dubourdieu (1998) found that, when comparing American white oak to sessile oak (*Q. petraea*) and pedunculate oak (*Q. robur*), the latter had almost double the amount of ellagic tannins than the other two.

Guymon and Crowell (1970) also found that there was a difference in the pH of brandy, depending on whether it was aged in French or American oak. The brandy aged in French oak had a lower pH. The reason for the lower pH was suggested as being an indication of differences in the composition of the acids in the oak.

Although there are many differences between the levels of extraction from American and French oak, it has been found that French oak seems to contribute more extract and colour than American white oak (Guymon and Crowell, 1972). This may be due to the higher levels of tannins in French oak (Chatonnet and Dubourdieu, 1998).

The origin of the wood is a determining factor when looking at the amount of extractable components. However, most of the recent studies on the effects of oak origin on the extractable components, have shown that the variability between individual trees is higher than the variability between species, when comparing European oak. It is also seen that the wood splitter, the cooper and the wine or spirit maker also play a role in influencing the structure of the wood and therefore the amount of extractable components (Masson *et al.,* 1995b). A spider graph in **Figure 2.14** gives the intensity of different aroma descriptors of wood from four different areas. As one can see, although the differences are not too great, they are present (Francis *et al.*, 1992).

2.4.2 Structural characteristics of wood

While studying the variability of oak anatomy, Feuillat and Keller (1997) found that there is a difference in the texture, number and width of multiseriate rays and the infradensity structure between pedunculate and sessile oak. They found that, for the same ring width, the pedunculate oak was more porous due to the difference in structure.

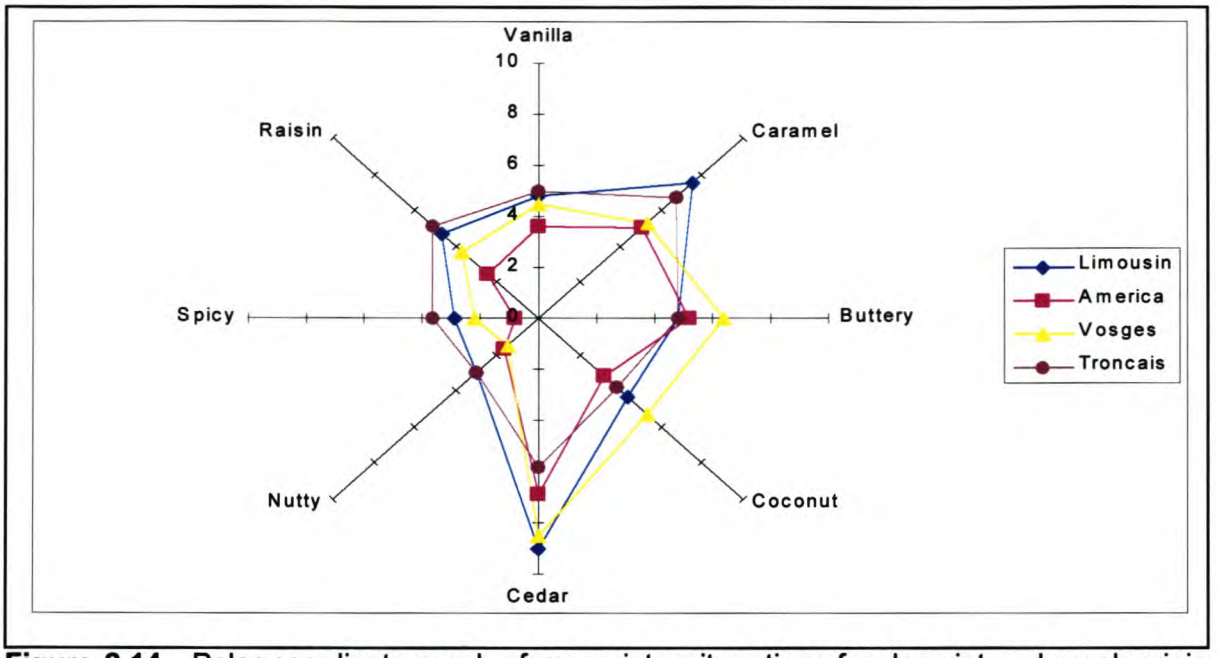


Figure 2.14. Polar coordinate graph of mean intensity ratings for descriptors by oak origin (Francis *et al.*, 1992)

When considering the structural effects on wood extraction, it is important to also note the age and the growth rate of the wood used for cooperage, as both these factors may have an influence on the level of phenols in the wood (Aiken and Noble, 1984). During the growth of the tree, extractables are laid down as the sapwood becomes heartwood (Singleton, 1995). The sapwood is the living part of the wood and has few polyphenols and ellagitannins, as these are toxic to the cells. The heartwood is no longer functional and therefore contains significant amounts of ellagitannins. As the heartwood becomes older, oxidation and polymerisation reactions most likely occur and this results in the insolubilisation of certain wood components, in particular the ellagitannins. Therefore, the oldest part of the wood will have the lowest amount of ellagitannins (Masson *et al.*, 1995b).

The rate of growth also affects the structural characteristics of oak. When a tree grows fast, it will have wide annual rings with a large proportion of summer (late) wood. As mentioned in 2.2.2, this is the part of the wood that is denser and has fewer pores (Singleton, 1995).

Today, wood structure is still used as a criterion to classify wood. However, recent research suggests that the effect of the wood structure and anatomy is low, showing very weak correlations with the quantity of extractives released into the wine or brandy. It has been suggested that the species of wood is a better indicator of wood quality and of cask properties (Feuillat *et al.*, 1997).

2.4.3 Seasoning of wood

Seasoning or drying of oak occurs after the tree is cut and usually takes place over two to three years (Graff and Tchelistcheff, 1969). Seasoning is done in order to dry the wood, which begins with a moisture content of about 41.2% that drops to about 8.7-12.4% at the end of the seasoning (Wilker and Gallander, 1989). Oak quality is

affected by the length of time allowed for seasoning, the rainfall and all climatic factors occurring in the country in which the wood is seasoned (Schahinger, 1991). The wood is able to absorb flavour and aromas during the seasoning process, so the actual site where seasoning takes place may also influence the wood quality (Schahinger, 1991).

Artificial seasoning may also be used to dry the wood, although the results are not as good as from natural seasoning. There are two main theories for this difference. Firstly, artificial drying may reduce the activity of enzymes involved in the condensation and polymerisation of wood tannins and, secondly, during natural seasoning most of the harsh tannins are leached by rain water, which does not occur under artificial conditions (Wilker and Gallander, 1989). Chatonnet (1999) also found that there was a constant decrease in ellagitannins during seasoning.

Artificial seasoning is much faster than the natural process and may result in longitudinal cracks in the wood. This is prevented in natural seasoning, during which the process is slow and uniform (Graff and Tchelistcheff, 1969). The artificial process may also result in small blisters forming on the surface of the wood during the manufacturing of the barrel. These blisters form pockets in the wood, which can be a place where microbial contamination may occur (Graff and Tchelistcheff, 1969).

Seasoning has an effect on the levels of oak lactones. Once again, different climatic regions affect the wood differently. Waterhouse and Towey (1994) found that the levels of oak lactones were higher in oak seasoned in Australia, where it is dry, while the levels of oak lactones were lower under the humid conditions in France. In **Figure 2.15**, one can see the different levels of the *cis* oak lactone of wood seasoned in Australia and wood seasoned in France. Oak seasoned in America, with similar seasoning conditions as France, also showed a loss in lactones of up to 80% (Sefton *et al.,* 1993b). This shows that dehydrating conditions increase the levels of oak lactones in oak (Waterhouse and Towey, 1994). Sefton *et al.* (1993b) also found that, under dry, artificial seasoning conditions, the levels of oak lactones increased up to five times over a period of six years.

The levels of eugenol decrease during seasoning, regardless of where the wood is seasoned (**Figure 2.16**) (Sefton *et al.,* 1993b). Francis *et al.* (1992) showed that, under wetter and high humidity summer climates, as in France, seasoning promotes the development of desirable wood flavour attributes, such as vanilla, caramel, butter and cedar. However, Chatonnet (1999) found that small amounts of phenolic aldehydes (such as vanillin) and volatile phenols (such as eugenol) were released by the oxidative breakdown of lignin during seasoning. This resulted in a decrease in these compounds, although Chatonnet (1999) also states that this effect is minimal compared to the effect that toasting has on increasing the amounts of these compounds.

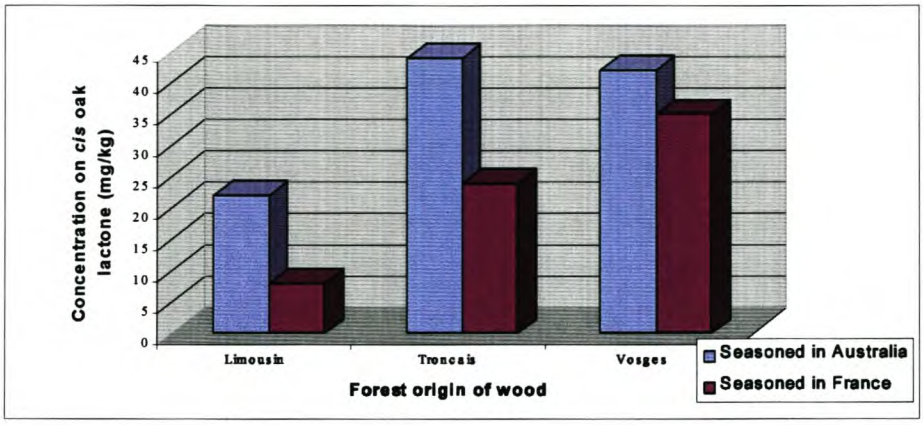


Figure 2.15. Influence of seasoning country on oak extractives (Sefton *et al.*, 1993b)

Microorganisms are also able to grow on the wood during seasoning. They penetrate the first couple of millimeters and are able to degrade ellagitannins at the surface of the wood, which is beneficial to the quality of the wine. Although the microorganisms grow, their contribution is low, if any at all, and the physical and chemical transformations that occur during seasoning are more important to the wood quality (Chatonnet, 1999). However, if wood is seasoned under very damp conditions, moulds and fungi may grow and cause the wood to rot (Schahinger, 1996).

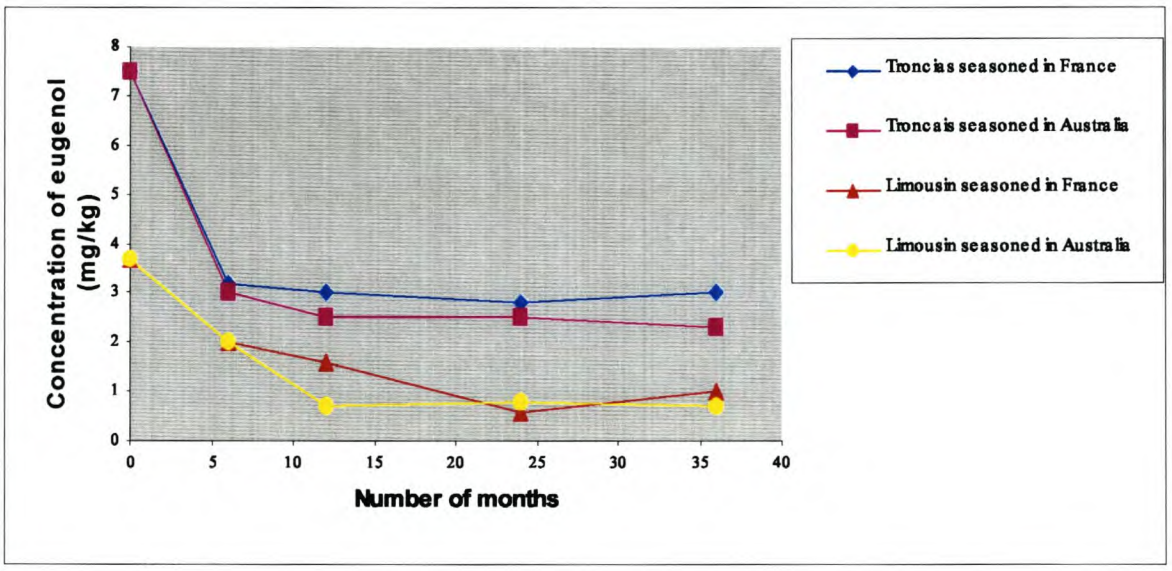


Figure 2.16. Graph showing the decrease in eugenol during seasoning (Sefton *et al.*, 1993b)

2.4.4 Thermal treatment (toasting) of wood

During barrel making, the wood is heated in order to bend it to make the barrel. Heating of the wood may be prolonged in order to get both physical changes and chemical changes, which will affect the organoleptic characteristics of the wine or brandy (Chatonnet *et al.,* 1991). Traditionally, oak can be toasted to different degrees, namely light, medium or heavy, all terms being based on the appearance of the inside of the staves. Toasting does not go as deep as charring, and many other degrees of toasting can be described as medium plus and very heavy (Hale *et al.,* 1999). Heating of the wood and different toasting levels have a significant effect on almost all the extractions from wood and are also responsible for the degradation of the lignin and making the lignin components of the wood available for extraction. Toasting aids the breakdown of carbohydrate polymers (part of the hemicelluloses in wood) and phenolic polymers (the lignin and ellagitannin part of the wood), thereby creating some compounds and destroying others (Chatonnet, 1999).

There are also differences in the thermal treatment of wood by European and American coopers. In Europe, the staves are bent and then heated with a wood-fired brazier without flaming the barrel, whereas, in America, steam bending is followed by charring with a gas burner (Guichard *et al.,* 1995).

Phenolic compounds decrease with an increase in toasting, as shown in **Figure 2.17** (Chatonnet *et al.,* 1991). As for the volatile phenols resulting from the degradation of the lignin component of the wood, their optimal synthesis is reached through heavy toasting, as illustrated in **Figure 2.17**. However, the volatile phenol eugenol is produced during heating and is increased by medium to heavy toast levels, but decreases when the wood is only lightly toasted (Sefton *et al.,* 1993b). The phenolic aldehyde concentration is also optimal between medium to heavy toasting levels, with vanillin showing a 50-fold increase when wood is lightly toasted (Sefton *et al.,* 1993b). At very heavy toasting levels, the vanillin concentration decreases due to thermal degradation (Chatonnet *et al.,* 1991). Another volatile phenol produced through toasting is guaiacol (Chatonnet, 1999). The ketone phenyls acetovanillone, propiovanillone and propiosyringone are produced during toasting (Naudin, 1990).

Furan compounds increase during heating and also increase with an increase in toasting level, with the highest concentration occurring at medium to heavy toasting levels (Chatonnet *et al.,* 1991). Together with the furans, other carbohydrate derivatives are also formed as a result of the thermal degradation, including, maltol, cyclotene, 2,3-dihydro-5-hydroxy-2-methyl-4(H)-pyran-4-one, 4-hydroxy-2,3-dimethyl-3(2H)-furan-3-one, furaneol and 2,3-dihydroxy-2-methyl-4(H)-pyranone (Chatonnet, 1999).

As for the oak lactones, the levels of both the *cis* and *trans* isomers of β-methyl-γ-octalactone increase with slight toasting and then decrease with an increase in toasting level (**Figure 2.18**) (Chatonnet *et al.,* 1991). Under intense heating conditions, the oak lactones are destroyed or evaporate and can be eliminated from

the wood completely. It has also been shown that the *cis* oak lactone dehydrates more quickly than the *trans* oak lactone and, as a result of this heating, increases the *cis* to *trans* ratio in wood (Waterhouse and Towey, 1994).

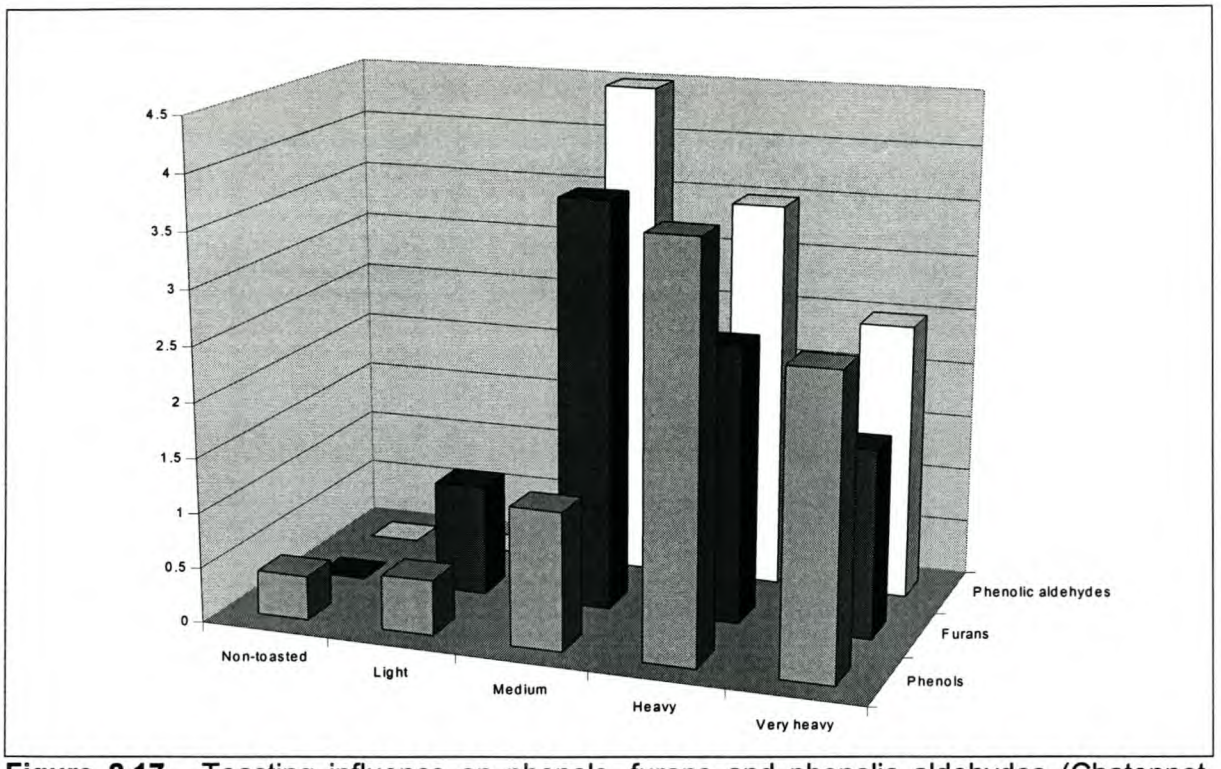


Figure 2.17. Toasting influence on phenols, furans and phenolic aldehydes (Chatonnet, 1997)

Studies have shown that heating of the oak results in an increase in ellagic acid and a decrease in the amount of ellagitannins (Hale *et al.,* 1999). It was also found that increasing the treatment time only accentuated this effect (Sarni *et al.,* 1990). However, the levels of other tannins in the wood decrease with an increase in the toasting level (**Figure 2.18**) (Chatonnet, 1997). Puech *et al.* (1999) found that even low heating reduced the vescalagin content of wood by 73% and the castalagin content in the surface layer of the wood by 46%. The compound *trans*-2-nonenal, which has an unpleasant sawdust aroma, also decreases with heavier toasting (Chatonnet, 1999).

Toasting makes it possible to improve the aromatic potential of wood due to the numerous effects it has on the extractable component of the wood, as well as the effect on lignin degradation (Chatonnet *et al.,* 1991).

2.4.5 Barrel size and age

The size of the barrel used for maturation also differs greatly. The majority of barrels used for wine maturation are usually between 190 L and 500 L in capacity, while 300 L to 500 L barrels are normally used for fortified wines, ports and sherries. For the maturation of spirits, the size of the barrel is usually 250 L to 340 L (Schahinger

and Rankine, 1992). In France, various barrel sizes are available and each region has its own name and capacity for the barrel (Graff and Tchelistcheff, 1969).

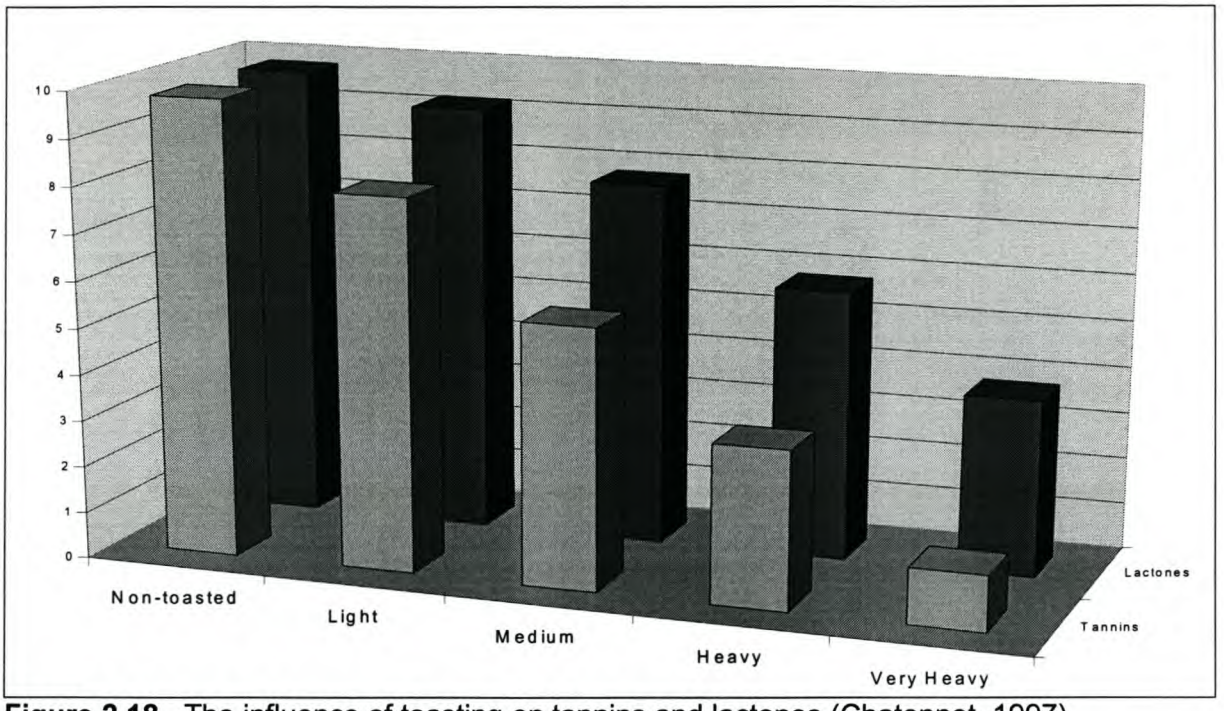


Figure 2.18. The influence of toasting on tannins and lactones (Chatonnet, 1997)

The four effects of barrel maturation, i.e. extraction, oxidation, evaporation and component reaction, would all be intensified by a larger surface to volume ratio between the barrel and the wine or distilled beverage (Singleton, 1995). The size of the barrel influences the extractions due to the surface to volume ratio. The smaller the barrel, the larger the surface to volume ratio and the higher the extraction (Guymon and Crowell, 1970). The barrel size also influences the rate of evaporation, as the larger the barrel, the less evaporation occurs (Guymon and Crowell, 1970).

The age or rather number of fills of the barrel has a significant effect on the amount of extractions. Since the number of extractable compounds is finite, the rate of extraction and the amounts of compounds extracted will diminish as the barrel is used in successive years (Towey and Waterhouse, 1996a).

Guymon and Crowell (1970) found that, although American oak was higher in oak lactone content than French oak, the American oak was mostly exhausted of lactones after its first fill. However, in more recent years, Towey and Waterhouse (1996a) found that the extraction of oak lactones actually increased from year one to year two, after which a decrease in extraction was seen in the third year. This is illustrated in **Figure 2.19a** and **b**. In this picture, it can also be seen that the increase for the *cis* isomer of β-methyl-γ-octalactone is greater from year 1 to year 2 than that of the *trans* isomer. The one exception in the diagram is US4, where the extraction of the *trans* isomer decreases from year 1 to year 2. It must be noted that this is a sample of Oregon oak, which is not used for commercial wine or brandy maturation.

This diagram also clearly shows the high extraction of the aromatic *cis* isomer of β-methyl-γ-octalactone from American oak, whereas the *trans* isomer is extracted in

greater quantities from French oak. It can be seen from these diagrams that the *cis* to *trans* ratio is about 5:1 for American oak and only 2:1 for French oak, which is why the American oak produces a more "oaky" wine or brandy (Towey and Waterhouse, 1996a).

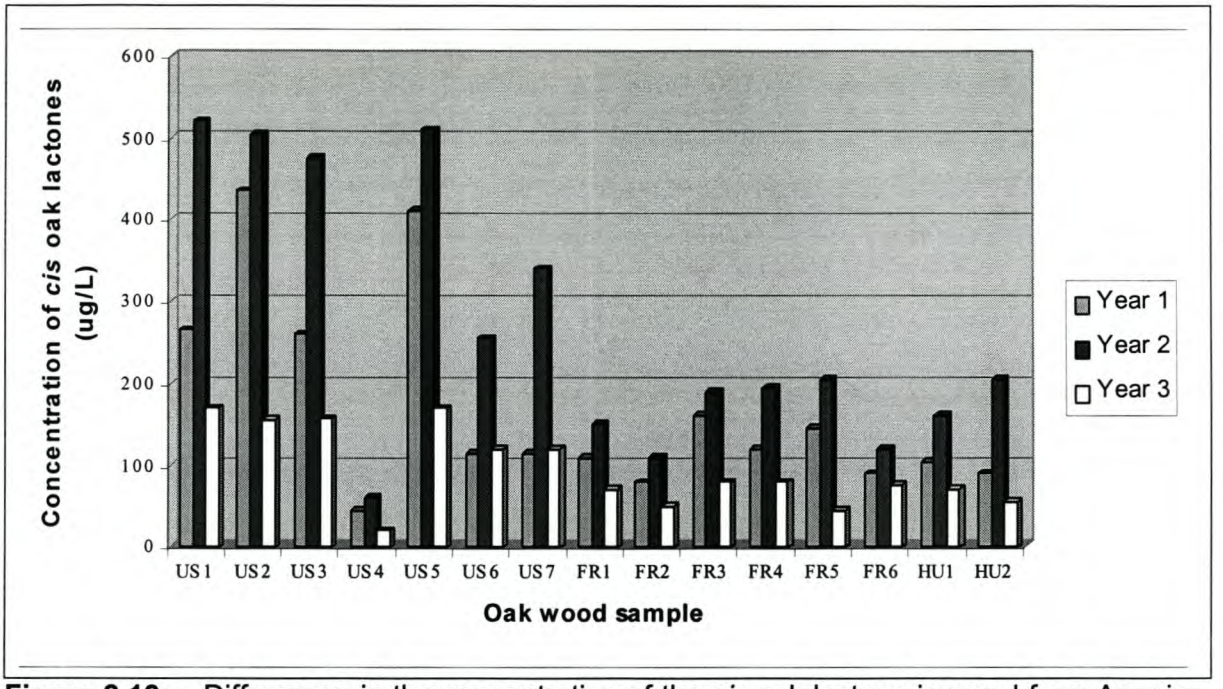


Figure 2.19a. Differences in the concentration of the *cis* oak lactone in wood from America, France and Hungary (Towey and Waterhouse, 1996a)

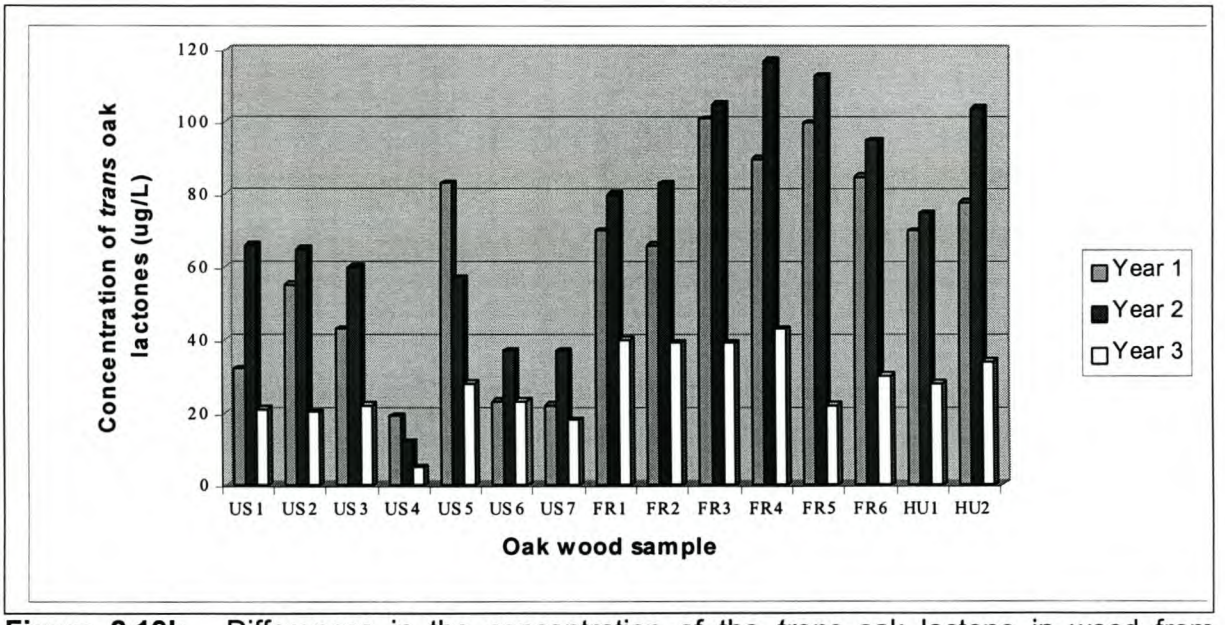


Figure 2.19b. Differences in the concentration of the *trans* oak lactone in wood from America, France and Hungary (Towey and Waterhouse, 1996a)

The extraction of volatile phenols and furfural (**Figure 2.20**) from oak decreases during successive fills. Significant decreases in the levels of guaiacol can be seen in **Figure 2.21**. Towey and Waterhouse also found that there was a decrease in the levels of eugenol, so much so that hardly any eugenol was detected in the second

year. As a result of the decreases in the phenols, wines that have been aged in older barrels may be less smoky and spicy in aroma (Towey and Waterhouse, 1996a).

Extraction levels for the first fill are the highest and these levels are never reached during the fills thereafter (Singleton, 1995). For this reason, wine aged in barrels that have had three fills or more should be shifted to less prestigious places in the market than those aged in a barrel that has had fewer fills (Singleton, 1995). Barrels that have had many fills may not yield enough gallic acid, which plays an important role as an oxidation catalyst. There is therefore a higher risk of unpleasant odours forming in old barrels (Chatonnet *et al.,* 1991).

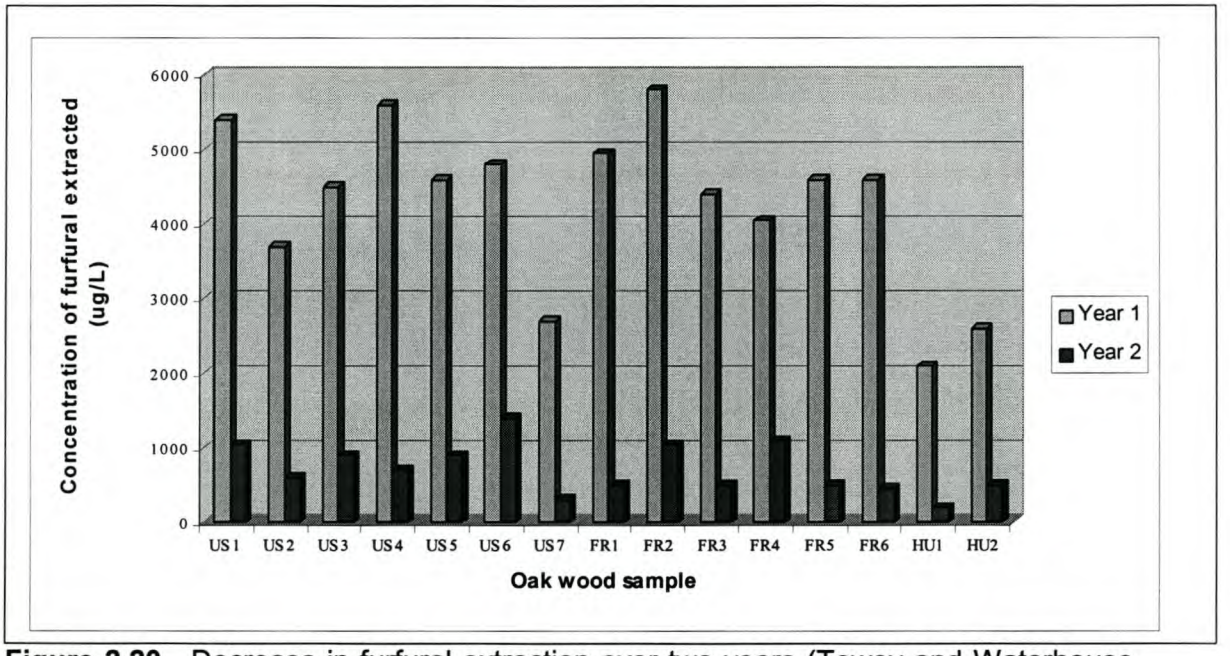


Figure 2.20. Decrease in furfural extraction over two years (Towey and Waterhouse, 1996a)

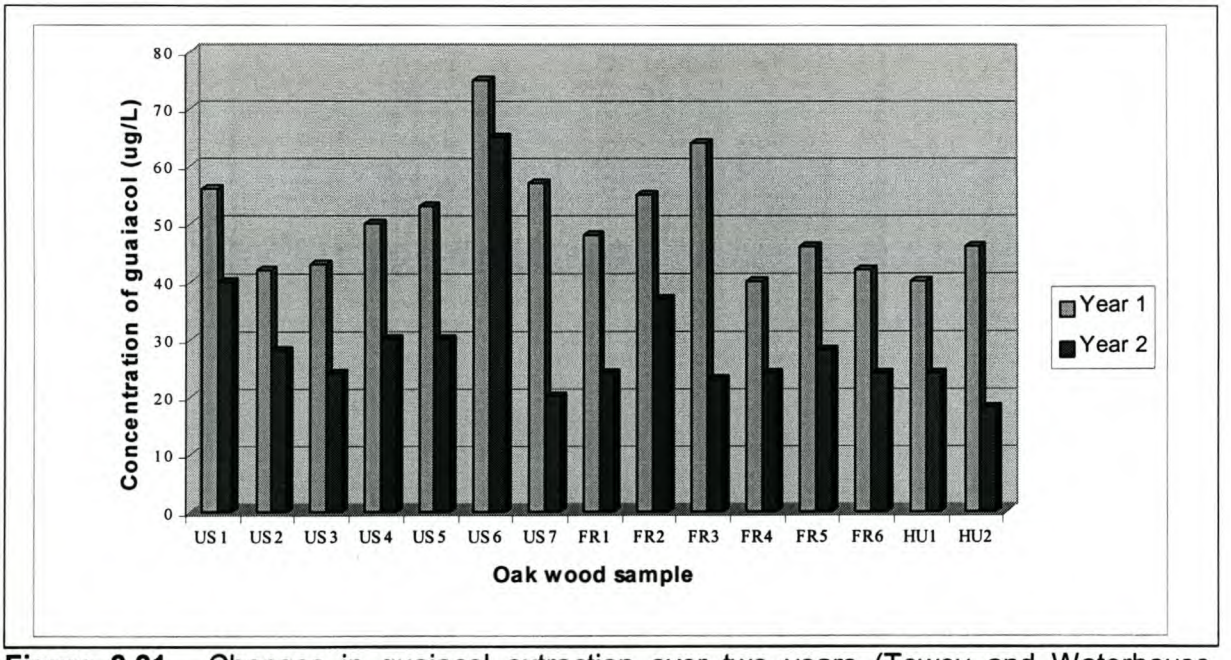


Figure 2.21. Changes in guaiacol extraction over two years (Towey and Waterhouse, 1996a)

The age of the barrel will also play a role if the barrel is not maintained correctly between fills. During this time, the barrels can become contaminated with undesirable microorganisms, which can affect the wine or brandy negatively. Due to this kind of growth, the useful life of the barrel is only five to six years (Towey and Waterhouse, 1996a).

2.4.6 Ethanol concentration

Ethanol has a definite effect on the amount of extractions from oak. Alcohol concentrations of 50-60% are the most effective for extracting oak components (Guymon and Crowell, 1970). The maximum extraction takes place at 55% alcohol (Singleton, 1995). This is also why brandy and other distillates have higher extractions than wine, as the extraction from oak is limited at the level of alcohol in wine (10-12% alcohol) (Guymon and Crowell, 1970). Differences in alcohol concentration shift the substances extracted from water solubles (low alcohol concentration), such as sugars, to poorly water soluble components of the wood, such as lignin (high alcohol concentration) (Singleton, 1995). A higher acetaldehyde concentration is found in brandies aged in wood, due to the oxidation of ethanol, and this is also directly linked to the ethanol concentration in the brandy (Onishi *et al.*, 1977).

Singleton (1995) reported that different aroma profiles were found at different alcohol concentrations. He found that it had been reported that brandy with 55% alcohol was described as "vanillin-floral", while at a lower alcohol concentration it was described as "oaky". Similar differences were noted in whiskey, with whiskey at 59% being described as flavoured and normal, at 63% as less mature and at 77% as "spicey, green oak".

2.4.7 Cellar temperature and humidity

Studies have shown that both water and ethanol can evaporate from intact barrels during the maturation period (Singleton, 1995). It has been demonstrated that, in a cellar with low humidity, the rate of water loss is greater than that of alcohol and the alcohol concentration therefore increases. However, if the cellar has a high humidity, the diffusion of water is decreased due to the vapour pressure of water at the barrel surface or in the air and the alcohol escapes, resulting in a decrease in the alcohol concentration (Guymon and Crowell, 1970). The same effect may occur in wine as well, although little data is available. For the maturation of brandy, a more humid cellar environment is preferable, as a greater ethanol loss takes place and the ethanol extracts and evaporates from the layer nearer the outer barrel surface (Singleton, 1995).

Due to the loss of water and ethanol, there is a decrease in the content of wine or brandy in the barrel. As the volume in the barrel decreases, the non-volatile constituents rise in concentration. The volatile components remain more or less the

same and do not diffuse as easily as the ethanol because of their molecular size (Singleton, 1995).

The evaporation of water through the barrel staves is influenced not only by the humidity of a cellar, but also by the water's liquid state, heat of vaporisation, self-association, adsorption to barrel carbohydrates and vapour pressure (as mentioned before). The effect of temperature is greater than that of humidity, with high temperature and greater air circulation increasing the rate of water loss (Singleton, 1995). It is generally found that wine aged in barrels in cellars that are humid and at a constant cool temperature can be aged for a long period, whereas, if the cellar is warm and dry, with fluctuating temperatures, the ageing process, as well as the extraction of aromatic compounds, is accelerated. This faster ageing is not beneficial to the wine (Graff and Tchelistcheff, 1969).

2.5 EFFECT OF BARREL MATURATION ON AROMATIC COMPOUNDS PRESENT IN WINE OR BRANDY

During the maturation period, the aromas that arise from the fermentation process tend to decrease (Cerdán *et al.*, 2002). It has been found that there is a decrease in the amount of fatty acid ethyl esters, in particular ethyl laurate and ethyl caprate, during the maturation of brandies in oak (Guymon and Crowell, 1972). One of the few esters that shows an increase during barrel maturation is ethyl lactate, an ester which is not produced during the fermentation process (Cerdán *et al.*, 2002).

Guymon and Crowell (1972) also noted an increase in hexyl alcohol and 2-phenethyl alcohol during brandy maturation. This was thought to be the result of an increase in concentration due to evaporation losses during ageing, as well as the hydrolysis of the corresponding acetic esters of these compounds.

2.5.1 Effect of alcoholic fermentation in the barrel

Fermentation on the yeast lees can result in considerable differences in the aromatic profile of the wine, as the yeast is able to change some of the oak extracts. Furan derivatives are among the compounds converted by the yeast. Although burnt wood contains furan aldehydes, these compounds are unstable during the alcoholic fermentation and are reduced into their corresponding alcohols by the carbonyl function of the yeast (Chatonnet *et al.*, 1991).

Reduction of the furan aldehydes still occurs in wines placed in the barrel after alcoholic fermentation and the removal of the yeast lees, although to a much lesser extent (Chatonnet *et al.,* 1991). Chatonnet *et al.* (1991) stated that these furan derivatives, except for 5-hydroxymethylfurfural, contribute to a grilled almond-like aroma, while the corresponding alcohols have more vegetative aromas, apparently resembling hay. As mentioned earlier, the aromatic influence of these compounds is low, due to their high odour thresholds (Chatonnet *et al.,* 1991). Furan aldehyde may

be enzymatically reduced by the yeast to furfurylic alcohol which is not aromatic at all (Naudin, 1990).

Barrel storage during fermentation results in a 20-30% increase in the volatile phenol, 4-vinylguaiacol. This increase is due to the decarboxylation of ferulic acid, which is derived from wood lignin degradation. This effect is only significant if the fermentation occurs in the barrel (Chatonnet *et al.,* 1991).

The vanillin concentration decreases when fermentation takes place in the barrel (Chatonnet *et al.,* 1991). This is because the yeast is able to convert vanillin to vanilla alcohol, which is much less aromatic than vanillin (Singleton, 1995). Barrel fermentation is indispensable for limiting the wine polyphenol content and allows for the fixation of oak tannins to the cellular walls and the mannoproteins released by the yeast lees. Wine stored with the lees will therefore have a lower tannin content and a lower proportion of free and reactive tannins as a result of the precipitation of the tannins (Sefton *et al.,* 1993b).

Fermentation of the wine in the barrel allows for a higher temperature during alcoholic fermentation and the wines that are made in this way are generally found to be fuller and more aromatically intense. This is due to these wines being richer in polysaccharides because of the higher fermentation temperature in the barrel (Chatonnet *et al.,* 1991). Barrel fermentation also aids in the precipitation of proteins that may cause instability in wines later (Chatonnet *et al.,* 1991).

2.5.2 Effect of barrel maturation on ester content of wine or brandy

Esters are important flavour compounds in wine and brandy and contribute to the fruity aroma of the product (Marais, 1978). The volatile esters found in wine and brandies are formed by the yeast during alcoholic fermentation (Ramey and Ough, 1980). Due to the high alcohol content of spirits, the esterfication of acetic acid to ethyl acetate is favoured, although the high water content in wine favours the hydrolysis of grape and fermentation esters (Singleton, 1995).

The total esters, mostly ethyl acetate, increase uniformly during the maturation of brandy in barrels. The concentration of esters can be two to three times more over four years of ageing (Onishi *et al.,* 1977). Various factors influence the formation of esters, including the initial composition of the brandy distillate and the activity of the barrel, i.e. the amount of acetic acid extracted, the amount of acetic acid available for extraction and the esterfication of acetic acid with alcohol (Onishi *et al.,* 1977). However, the increase or decrease in esters during the maturation period will depend on their initial concentration. If the initial concentration is greater than equilibrium amounts, the volatile esters will hydrolyse, thus leading to a decrease in their concentrations (Ramey and Ough, 1980).

Fatty acids and their esters may increase or decrease during barrel maturation (Singleton *et al.,* 1995). Onishi *et al.* (1977) found that the concentration of the acetate esters of isoamyl, *n*-hexyl and β-phenethyl alcohols decreased. They attribute this loss to the much larger concentration of ethyl alcohol compared to other

alcohols, the natural acidity and the pH (4.2-4.4) of brandy, which favours the esterfication reaction. This is in agreement with the results of Ramey and Ough (1980), who found that the acetate esters of higher alcohols generally hydrolysed more rapidly than the ethyl fatty acid esters. Onishi *et al.* (1977) came to the conclusion that the increase in esters during brandy ageing was largely due to an increase in ethyl acetate and not so much to the increase in ethyl esters of caproic, caprylic and capric acid.

Through the decrease in the esters of isoamyl, *n*-hexyl and β-phenethyl alcohols, ageing in oak subdues the fruity characteristics of brandy (Onishi *et al.,* 1977).

2.5.3 Effect of barrel maturation on higher alcohols in wine or brandy

The content of higher alcohols has been known to increase during barrel maturation. Chatonnet *et al.* (1991) found that white wines fermented in a barrel was affected less by fatty acid esters and fatty acids and affected more by higher alcohols and phenyl-ethanol than white wines fermented in tanks. Due to this, the wine fermented in barrels will be less fruity than that fermented in tanks (Chatonnet *et al.,* 1991).

2.6 CONCLUDING REMARKS

From this literature review it is clear that barrel maturation has a definite effect on wine and brandy quality. The barrel contributes to both the aroma and the flavour of the wine and brandy through the extraction of numerous compounds, which has been discussed in this review. Therefore, it is important for the winemaker or distiller to research all methods of wood maturation as so many factors influence the wood.

Wood maturation influences the amounts of esters in the wine or brandy. However, as discussed in this study, the type of wood used, as well as the conditions in the cellar (i.e. cellar humidity), will have an influence on the levels of esters. Esters are not extracted from the wood, although it has been found that ethyl lactate is formed only under barrel maturation. At present, there is little information about what happens to esters during maturation and more research can be done in this area.

Many studies have been conducted concerning the differences between oak wood from various countries, as well as on the factors that affect the extractable compounds in the wood. However, studies concerning the differences in the use of oak chips, oak staves or oak barrels in maturation still have to be conducted under standardised conditions so that the data can reflect a true comparison between the various methods. Studies can also be conducted on what happens to fermentation products so that the winemaker or distiller can have a clearer idea of what will happen to the components formed during fermentation in wood maturation.

In conclusion, it can be said that, although there is opportunity for research, it has been established that the effect of wood enhances the aroma and flavour of wine and brandy, increasing both the quality and enjoyment of the product.

2.7 LITERATURE CITED

Aiken JW, Noble AC. 1984. Composition and sensory properties of Cabernet Sauvignon wine aged in French versus American oak barrels. *Vitis* **23:**27-36.

Bate-Smith EC. 1971. Detection and determination of ellagitannins. *Phytochemistry* **11:**153-1156.

Cerdán TG, Mozaz SR, Azpilicueta CA. 2002. Volatile composition of aged wine in used barrels of French oak and American oak. *Food Res Int* **35:**603-610.

Chatonnet P. 1999. Volatile and odiferous compounds in barrel-aged wines: Impact of cooperage techniques and aging conditions. *American Chemical society.* p180-207.

Chatonnet P, Dubourdieu, D. 1998. A comparative study of the characteristics of American white oak (*Quercus alba*) and European oak (*Quercus petraea* and *Q. rubor*) for the production of barrels used in barrel aging of wines. *Am J Enol Vitic* **49:**179-185.

Chatonnet P. 1997. Barrel aging of red wines. In *The Barrel and the Wine, Scientific Advances of a Traditional Art*, Seguin Moreau, USA; 89-104.

Chatonnet P, Dubourdieu D, Boidron JN. 1991. Effects of fermentation and maturation in oak barrels on the composition and quality of white wines. *Wine Ind J* **2:**73-84.

Feuillat F, Keller R. 1997. Variability of oak (*Quercus robur* L., *Quercus petraea* Liebl.) anatomy relating to cask properties. *Am J Enol Vitic* **48:**502-508.

Feuillat F, Moio L, Guichard E, Marinov M, Fournier N, Puech JL. 1997. Variation in the concentration of ellagitannins and *cis*- and *trans*-β-methyl-γ-octalactone extracted from oak (*Quercus robur* L., *Quercus petraea* Liebl.) under model wine cask conditions. *Am J Enol Vitic* **48:**509-515.

Francis IL, Sefton MA, Williams PJ. 1992. A study by sensory descriptive analysis of the effects of oak origin, seasoning and heating on the aromas of oak model wine extracts. *Am J Enol Vitic* **43:**23-29.

Graff RH, Tchelistcheff A. 1969. The production and aging of wine in small oak cooperage. *Wines and Vines* **50:**26-30.

Guymon JF, Crowell EA. 1970. Brandy aging, some comparisons of American and French oak cooperage. *Wines and Vines* **51:**23-25.

Guymon JF, Crowell EA. 1972. GC-Separated brandy components derived from French and American oaks. *Am J Enol Vitic* **23:**114-120.

Guichard E, Fournier N, Masson G, Puech JL. 1995. Sterioisomers of β-methyl-γ-octalactone. I. Quantification in brandies as a function of wood origin and treatment of the barrels. *Am J Enol Vitic* **46:**419-423.

Hale MD, McCafferty K, Larmie E, Newton J, Swan JS. 1999. The influence of oak seasoning and toasting parameters on the composition and quality of wine. International Symposium on Oak in Winemaking. *Am J Enol Vitic* **4:**495-512.

Heraty E, McCord A, Skaanild L, Davenport M. 1999. The evaluation of alternative oak enhancements to extend barrel life. *Am J Enol Vitic* **50:**534-536.

Jindra JA, Gallander JF. 1987. Effect of American and French oak barrels on the phenolic composition and sensory quality of Sevyal blanc wines. *Am J Enol Vitic* **38:**133-138.

Klumpers J, Scalbert A, Janin G. 1994. Ellagitannins in European oak: polymerization during wood aging. *Phytochemistry* **36:**1249-1252.

Maga J. 1989. Wood role in alcoholic beverage flavor. *Food Rev Int* **5:**39-79.

Marais J. 1978. The effect of pH on esters and quality of Colombar wine during maturation. *Vitis* **17:**396-403.

Masson G, Baumes R, Puech JL, Razungles A. 1997. Demonstration of the presence of carotenoids in wood: Quantitive study of oak cooperage. *J Agric Food Chem* **45:**1649-1652.

Masson G, Guichard E, Fourinier N, Puech JL. 1995a. Stereoisomers of β-methyl-γ-octalactone. II. Contents in the wood of French (*Quercus robur* and *Quercus petraea*) and American (*Quercus alba*) oaks. *Am J Enol Vitic* **46:** 424-428.

Masson G, Moutounet M, Puech JL. 1995b. Ellagitannin content of oak as a function of species and of sampling position in the tree. *Am J Enol Vitic* **46:**262-268.

Masson G, Puech JL, Moutounet M. 1994. Localization of the ellagitannins in the tissues of *Quercus robur* and *Quercus petraea* woods. *Phytochemistry* **73:**1245-1249.

Masuda M, Nishimura K. 1971. Branched nonalactones from the *Quercus* species. *Phytochemistry* **10:**1401-1402.

Naudin R. 1990. A French view of barrel aging. *Wines and Vines* **71:**48-55.

Nishimura K, Onishi M, Masuda M, Koga K, Matsuyama R. 1983. Reactions of wood components during maturation. In *The Flavor of Distilled Beverages, Origin and Development*, Piggott JR. (ed). Ellis Horwood: Chichester, England; 241-255.

Onishi M, Guymon JF, Crowell EA. 1977. Changes of some volatile constituents of brandy during aging. *Am J Enol Vitic* **28:**152-158.

Otsuka K, Zenibayashi Y, Itoh M, Totsuka A. 1974. Presence and significance of two diastereoismers of β-methyl-γ-octalactone in aged distilled liquors. *Agri Biol Chem* **38:** 485-490.

Pérez-Coello MS, Sanz J, Cabezudo MD. 1999. Determination of volatile compounds in hydroalcoholic extracts of French and American oak. *Am J Enol Vitic* **50:**162-165.

Pocock KF, Sefton MA, Williams PJ. 1994. Taste thresholds of phenolic extracts of French and American oakwood: The influence of oak phenols on wine flavor. *Am J Enol Vitic* **5:**429-434.

Pollintz AP, Pardon KH, Sefton MA. 2000. 4-Ethylphenol, 4-ethylguaiacol and oak lactones in Australian red wines. *Aust Grapegrower Winemaker* **7:**45-52.

Puech JL. 1987. Extraction of phenolic compounds from oak in model solutions and evolution of aromatic aldehydes in wines aged in oak barrels. *Am J Enol Vitic* **38:**236-238.

Puech JL, Feuillat F, Mosedale JR. 1999. The tannins of oak heartwood: structure, properties and their influence on wine flavor. International Symposium on Oak in Winemaking. *Am J Enol Vitic* **50:**469-478.

Puech JL, Rabier P, Moutounet M. 1989. Principles of preparation and chemical composition of commercial oak extracts. In *Flavors and Off-Flavors*, Proceedings of the 6th International Flavor Conference, Rethymnon, Crete, Greece. Charalambous G. (ed). Elsevier Science Publishers B.B.: Amsterdam, Netherlands; 159-167.

Quinn MK, Singleton VL. 1985. Isolation and identification of ellagitannins from white oak and an estimation of their roles in wine. *Am J Enol Vitic* **36:**148-155.

Ramey DD, Ough CS. 1980. Volatile ester hydrolysis or formation during storage of model solutions and wines. *J Agric Food Chem* **28:**928-934.

Remy B. 1997. Selecting the oak wood for the use in cooperage. In *The Barrel and the Wine, Scientific Advances of a Traditional Art*. Seguin Moreau: USA; 9-26.

Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D. 2000. Aging red wines in vat and barrel: phenomena occurring during aging. In *Handbook of Enology,* volume 2. John Wiley & Sons Ltd: Chichester, England; 353-391.

Sarni J, Moutounet M, Puech JL, Rabier P. 1990. Effect of heat treatment of oak wood extractable compounds. *Holzforschung* **44:**461-466.

Schahinger G. 1991. The importance of oak origin. *Wine Ind J* **2:**65-89.

Schahinger G, Rankine B. 1992. Source, Types and Classification of Oak. In *Cooperage for Winemakers*. Ryan Publications: Adelaide, Australia; 4-15.

Schahinger G. 1996. Oak "seasoning" – what does it mean? *Aust Grapegrower Winemaker* **7:**17-19.

Sefton MA. 1991. How does oak barrel maturation contribute to wine flavor? *Wine Ind J* **2:**69-73.

Sefton MA, Francis IL, Pocock KF, Williams PJ. 1993a. The influence of natural seasoning on the concentrations of eugenol, vanillin and β-methyl-γ-octalactone extracted from French and American oakwood. *Sci Aliments* **13:**629-643.

Sefton MA, Francis IL, Williams PJ. 1990. Volatile norisoprenoid compounds as constituents of oaks used in wine and spirit maturation. *J Agric Food Chem* **38:**2045-2049.

Sefton MA, Spillman PJ, Pocock KF, Francis IL, Williams PJ. 1993b. The influence of oak origin, seasoning and other industry practices on the sensory characteristics and composition of oak extracts and barrel-aged white wines. *Aust Grapegrower Winemaker* **355:**17-25.

Singleton VL. 1995. Maturation of wines and sprits: comparisons, facts and hypotheses. *Am J Enol Vitic* **46:**98-114.

Singleton VL. 1974. Some aspects of the wooden container as a factor in wine maturation. In *The Chemistry of Winemaking*, American Chemical Society meeting (165th 1973: Dallas, Texas). American Chemical Society, Washington: DC.

Spillman P. 1999. Wine quality biases inherent in comparisons of oak chip and barrel systems. *Wine Ind J* **14:**25-33.

Spillman PJ, Pollnitz AP, Liacopoulos D, Skouroumounis GK, Sefton MA. 1997. Accumulation of vanillin during barrel-aging of white, red, and model wines. *J Agric Food Chem* **45:**2584-2589.

Swan JS, Newton J, Larmie E, Sayre R. 1997. Oak and chardonnay. *The Aust Grapegrower Winemaker* **403:**41-50.

Towey JP, Waterhouse AL. 1996a. The extraction of volatile compounds from French and American oak barrels in Chardonnay during three successive vintages. *Am J Enol Vitic* **47:**163-172.

Towey JP, Waterhouse AL. 1996b. Barrel-to-barrel variation of volatile oak extractives in barrel fermented Chardonnay. *Am J Enol Vitic* **47:**17-20.

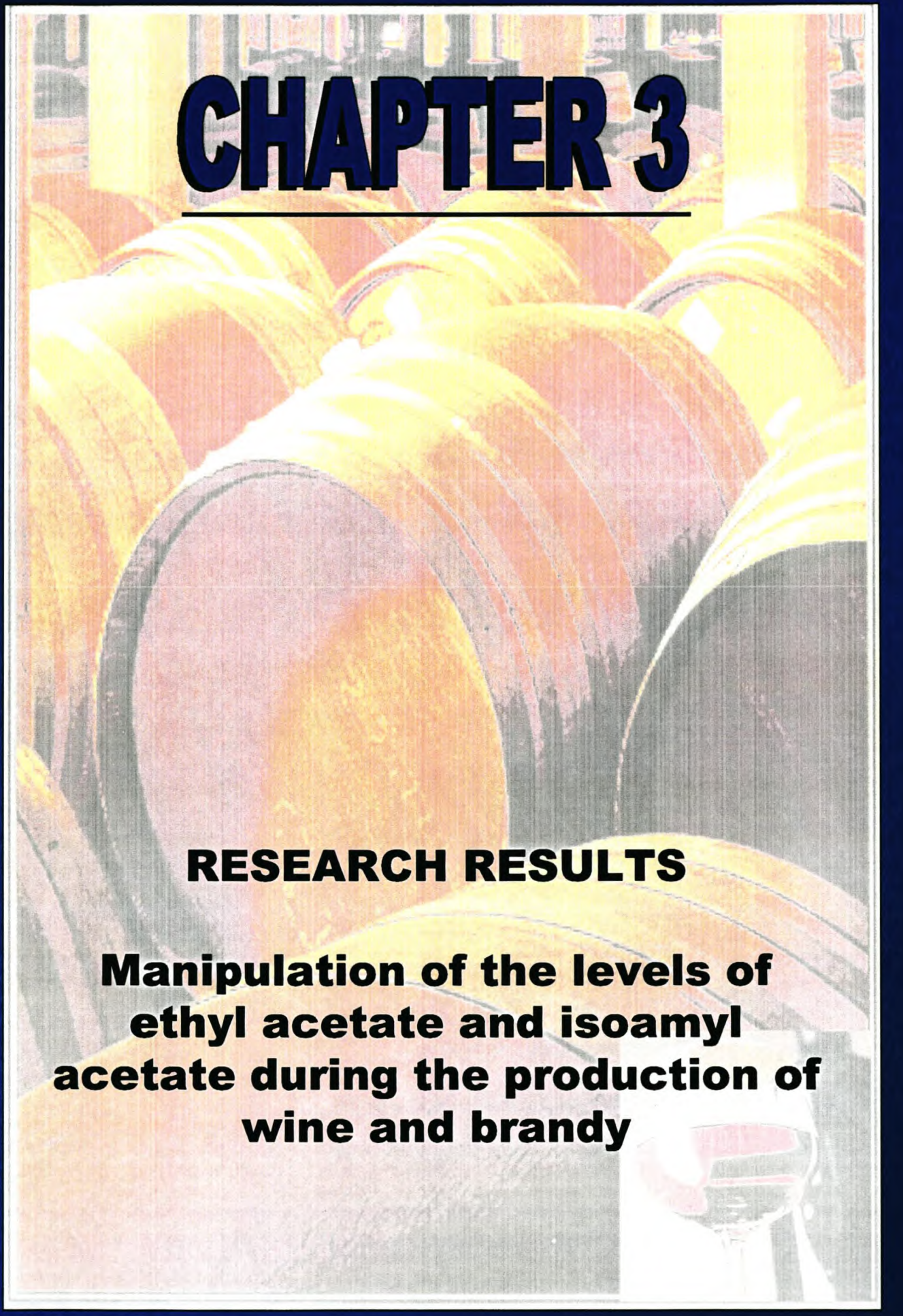
Viriot C, Scalbert A, Hervé du Penhoat CLM, Moutounet M. 1994. Ellagitannins in woods of sessile and sweet chestnut dimerization and hydrolysis during wood aging. *Phytochemistry* **36:**1253-1260.

Vivas N, Glories Y. 1996. Role of oak wood ellagitannins in the oxidation process of red wines during aging. *Am J Enol Vitic* **47:**103-107.

Vivas N, Pianet I, Bourgeois G, Vitry C, Servens C, Glories Y. 1998. Charaterization of heartwood lignin fractions from *Quercus robur* L. and *Quercus petraea* (Matt) Liebl., the main oak species used for barrel making. *Am J Enol Vitic* **49:**49-55.

Waterhouse AL, Towey PJ. 1994. Oak lactone isomer ratio distinguishes between wines fermented in American and French oak barrels. *J Agric Food Chem* **42:**1971-1974.

Wilker KL, Gallander JF. 1989. Comparison of Sevyl Blanc wines aged with air- and kiln dried American white oak. *Am J Enol Vitic* **40:**224-226.



CHAPTER 3

RESEARCH RESULTS

Manipulation of the levels of ethyl acetate and isoamyl acetate during the production of wine and brandy

3. RESEARCH RESULTS

MANIPULATION OF THE LEVELS OF ETHYL ACETATE AND ISOAMYL ACETATE DURING THE PRODUCTION OF WINE AND BRANDY

JENNIFER C. BAYLY, MARET DU TOIT, MARIUS G. LAMBRECHTS* AND ISAK S. PRETORIUS

Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, Stellenbosch, 7600, South Africa
**Distell, P.O. Box 184, Stellenbosch 7599, South Africa*

Esters, which impart fruity aromas to wine, are important contributors to the fermentation bouquet. It has been reported that acetate esters are produced mainly using acetyl coenzyme A and alcohol as a substrate by alcohol transferase (AATase) in *Saccharomyces cerevisiae*. It has been shown that the yeast has several types (at least three, probably four) of AATases that can produce acetate esters and thus influence the aroma of wine. The aims of this study are to construct wine yeast strains in which the *ATF1* and *ATF2* genes are individually, or as a set, either deleted or overproduced. The *ATF1* and *ATF2* genes have been cloned into the commercial wine yeast strain VIN13 individually or together. Both these genes have been placed under the control of the constitutive yeast phosphoglycerate kinase gene (*PGK1*) promoter and terminator. Southern blot analysis confirmed the integration of the modified copy of the *ATF1* and *ATF2* genes into the genome of the commercial wine yeast VIN13 and a reverse transcription polymerase chain reaction (RT-PCR) confirmed the expression levels. The recombinant wine yeasts were used in wine fermentations and the resulting wines and brandies were evaluated sensorially and gas chromatographic (GC) analysis was conducted to determine the aroma profile. GC analysis showed a decrease in the acetic acid concentration in the wine and brandy made with a yeast overexpressing the *ATF2* gene, as well as with a yeast strain overexpressing the *ATF1* and *ATF2* genes. However, unlike overexpression of the *ATF1* gene, no increase in ethyl acetate or isoamyl acetate was observed in wine or brandy made using a yeast overexpressing the *ATF2* gene and a yeast overexpressing both the *ATF1* and *ATF2* genes.

3.1 INTRODUCTION

Flavour compounds in wine and brandy not only play a large role in the aroma of the product, but also influence the final perception of the product. The aroma of wine and brandy is attributed mainly to the formation of aromatic compounds by the yeast, *Saccharomyces cerevisiae*, during alcoholic fermentation. Volatile esters formed by the yeast during fermentation have long been recognised as important flavour compounds in alcoholic beverages (Malcorps *et al.*, 1991). Acetate esters, in particular, play a very important role in the formation of the fermentation bouquet of wine and brandy, contributing to the "fruitiness" of the product. Two well known acetate esters are isoamyl acetate, which has a banana-like aroma, and ethyl acetate, which has a light-fruity, solvent-like aroma. Other esters associated with fruity flavours are ethyl caproate and ethyl caprylate, which have been associated

with an apple-like flavour, whereas flowery or honey-like flavours have been attributed to the ester 2-phenylethyl acetate (Engan, 1974).

Ester synthesis is an intracellular process that requires energy and the presence of two substrates: a higher alcohol and acyl Coenzyme A (acyl-CoA) (Malcorps *et al.*, 1991). The energy for this process is provided by the thioester linkage of the acyl-CoA co-substrate and is catalysed by an acyl transferase or ester synthase (Nordström, 1962). The acetyl-CoA is formed by the oxidative decarboxylation of pyruvate or by the direct activation of acetate with ATP (Mason and Dufour, 2000). Alcohol acetyltransferases (AATases) are used for the production of acetate esters. The genes coding for the AATaseI and AATaseII are *ATF1* and *ATF2* respectively. The *ATF1* is expressed under anaerobic conditions during the growth phase of yeast cells (Fujiwara *et al.*, 1999). The *ATF1* and *ATF2* genes have been successfully cloned and sequenced from *S. cerevisiae* (Fujii *et al.*, 1994; Nagasawa *et al.*, 1998). The Atf1p is responsible mainly for the production of isoamyl acetate and plays a minor role in the production of ethyl acetate (Fujii *et al.,* 1996). The Atf1p is also thought to play a role in fatty acid metabolism (Mason and Dufour, 2000). The exact role of the Atf2p is still to be determined, although it is known to play a role in isoamyl acetate production (Nagasawa *et al.*, 1998). Recent studies have also shown that Atf2p plays a role in prognenolone esterfication and therefore in detoxification in the yeast (Cauet *et al.,* 1999).

The AATase I enzyme is membrane bound and is inhibited by unsaturated fatty acids, as well as by certain lipids (Yoshioka and Hashimoto, 1981). Ester formation is also influenced by the fatty acid composition of the cell membrane (Yoshioka and Hashimoto, 1983). At particular concentrations, unsaturated fatty acids such as linoleic and linolenic acid reduce the amounts of isoamyl acetate, octanoic and decanoic acid, whereas an increase in caprylic, capric, lauric and palmitoleic acids can lead to an elevation in the levels of ethyl caprate, ethyl caprylate, ethyl acetate and isoamyl acetate (Rosi and Bertuccioli, 1992). The AATaseII gene is also repressed by oxygen, and is affected by unsaturated fatty acids. Both AATases are regulated at a transcriptional level (Mason and Dufour, 2000).

Problems in the levels of ethyl acetate and isoamyl acetate in the winemaking and brewing industries have led to research on the manipulation of the genes involved in their synthesis. Genetic studies on the *ATF1* gene in industrial yeast strains have shown that overexpression leads to a 3.5-fold increase in the levels of ethyl acetate and a 12-fold increase in the levels of isoamyl acetate (Lilly *et al.,* 2000). The null mutant of the *ATF1* gene in laboratory strains has shown an 80% decrease in isoamyl acetate production and a 30% decrease in the ethyl acetate production, in comparison to the wild-type strain (Mason and Dufour, 2000). Nagasawa *et al.* (1998) found that the double mutant of *atf1* and *atf2* showed no isoamyl acetate production, which has lead to the hypothesis that the *ATF2* gene is responsible for 20% of the isoamyl acetate production in the cell. However, this still remains to be proved (Mason and Dufour, 2000).

Therefore, the aim of this project was, firstly, to overexpress the *ATF2* gene in the industrial *S. cerevisiae* wine yeast strain VIN13, as well as to overexpress both the *ATF1* and *ATF2* genes in VIN13. Secondly, it was to disrupt the *ATF1* and *ATF2* genes in VIN13. The modified wine yeasts obtained were used for the production of wine and base wine for brandy production. Through these modifications, it was hoped to determine the effect of the genetic manipulation of the *ATF1* and *ATF2* genes on the aroma and flavour of the resultant wine and distillate. This will lead to a better understanding of the exact role played by these two genes in the fermentation bouquet and possibly lead to better control over the amount of ethyl acetate and isoamyl acetate produced during winemaking.

3.2 MATERIALS AND METHODS

3.2.1 Microbial strains and culture conditions

The bacterial and yeast strains used in this study, as well as the yeast transformants created in this study, are listed in **Table 3.1**. *Escherichia coli* DH5α was grown aerobically at 37°C in Luria-Bertani broth (Biolab, South Africa), supplemented with 100 μg/L ampicillin (Sigma) for the selection of positive clones (Sambrook *et al.*, 1989). The *S. cerevisiae* industrial wine yeast strain, VIN13 (Anchor Yeast, South Africa), was used for all yeast transformations. The VIN13 used for transformations was grown aerobically at 30°C in YPD Broth (Biolab, South Africa) and, after the transformation, was plated out onto YPD agar (Biolab, South Africa) supplemented with 400 mg/L of geneticin (Sigma) for the selection of positive transformants.

3.2.2 Plasmid construction and recombinant DNA methods

Standard isolation and DNA modification protocols were used in this study (Sambrook *et al.*, 1989). The plasmids used and constructed are listed in **Table 3.1**. The restriction enzymes, T_4 DNA Ligase and Expand Hi-Fidelity DNA polymerase, were used according to the instructions provided by the supplier, Roche. All primers used in this study are listed in **Table 3.2**.

For all PCR reactions in this study, a PCR-Biometra TRIO-Thermoblock machine was used. For 50 μl PCR reactions, 2 μl of DMSO, 200μM of dNTPs, 7μl of 25mM $MgCl_2$ (supplied with Expand, Roche) and 5 μl of a 2.5 μM primer stock solution were added. Amplification conditions included an initial DNA denaturation step at 95°C for 2 min, followed by cycles of denaturation at 95°C for 45 sec, primer annealing according to the specific primer melting temperatures and elongation at 68°C, allowing 1 min per 1 kb amplified. Reactions proceeded for 34 cycles.

Table 3.1 Microbial strains and plasmids used in this study

Strain or Plasmid	Genotype	Source/Reference
Bacteria		
Escherichia coli DH5α	F' *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF)U169 deoR* [F80d*lac* DE(*lacZ*)M15]	GIBCO-BRL/Life Technologies
Yeasts		
Saccharomyces cerevisiae wine yeast strain,VIN13	Commercial wine yeast	Anchor Yeast, South Africa
Transformants		
VIN13(pATF1-s)	*SMR1-410 PGK1$_P$-ATF1-PGK1$_T$*	Lilly *et al.*, 2000
VIN13(pATF2oe)	*loxP KanMX loxP PGK1$_P$-ATF2-PGK1$_T$*	This study
VIN13(pATF1-s, pATF2oe)	*SMR1-410 PGK1$_P$-ATF1-PGK1$_T$, loxP KanMX loxP PGK1$_P$-ATF2-PGK1$_T$*	This study
VIN13(Δ*ATF1*s*)	*loxP KanMX loxP δ-ATF1*	This study
VIN13(Δ*ATF2*s*)	*loxP KanMX loxP δ-ATF2*	This study
Plasmids		
pGEMT-easy	*bla LacZ*	Promega
pGEM-ATF2oe	*bla LacZ-ATF2*	This study
pJ	*bla loxP ENO2$_p$-KanMX loxP PGK1$_P$-PGK1$_T$*	J. Hignett
pJATF2oe	*bla loxP ENO2$_P$-KanMX loxP PGK1$_P$-ATF2-PGK1$_T$*	This study
pEG6	*bla loxP ENO2$_p$-KanMX loxP*	J. Hignett

* s = single disruption

3.2.2.1 Construction of an *ATF2* overexpression cassette

The following primers were designed for the amplification (with the use of a polymerase chain reaction (PCR)) of the coding region of the *ATF2* gene: ATF2OE'F and ATF2OE'R. A *Bgl*II site was incorporated in both the forward and the reverse primers. Genomic DNA isolated (Sambrook *et al.*, 1989) from the industrial yeast strain VIN13 was used as the template for the PCR amplification of the *ATF2* gene. The 1 627 bp fragment obtained was then cloned into the pGEMT-easy PCR cloning kit (Promega), yielding the plasmid pGEM-ATF2oe.

The pJ plasmid (J. Hignett, unpublished data) was used for the further construction of the overexpression cassette. This plasmid contains the promoter and terminator regulatory sequence of the yeast phosphoglycerate kinase gene (*PGK1$_P$*) and the terminator sequence of the yeast phosphoglycerate kinase gene (*PGK1$_T$*), with the unique restriction sites, *Eco*RI, *Xho*I and *Bgl*II, between these regulatory sequences, the *KanMX* gene, which was used for selection, as well as two *loxP* sites, which can be used in the *cre*-recombinase system (Güldener *et al.*, 1996). The plasmid pGEM-ATF2oe was digested with *Bgl*II and yielded a 1600 bp fragment.

This fragment was linearised using *Bgl*II and then sub-cloned into the pJ plasmid, thereby generating the plasmid pJATF2oe (**Figure 3.1**).

Table 3.2 Primers used in this study

Gene Primers			
Primer	**Sequence**	**Template**	**Restriction site (bold)**
ATF2OE'F	5'GATC**AGATCT**ATGGAAGATATAGAAGGATA 3'	VIN13	*Bgl*II
ATF2OE'R	5'GATC**AGATCT**TTAAAGCGACGCAAAT TCGC3'	VIN13	*Bgl*II
ATF'F	5'GATC**CTCGAG**ATGAATGAAATCGATGAGAA3'	VIN13	*Xho*I
ATF'R	5'GATC**CTCGAG**GTAAGGGCCTAAAAGGAGAG3'	VIN13	*Xho*I
Primers for disruption and overexpression cassettes			
Primer	**Sequence**	**Template**	**Recognition sequence**
ATF1'F	5'ATAAAAAACGGCACTTCATCAGTATCACAAATACCAT CAATTTATCAGCTCCTTCGTACGCTGCAGGTCGAC3'	pEG6	First 51 bp to 5' end of *ATF1* gene, last 21 bp to loxP site.
ATF1'R	5'GGTTATTTACACGACATAATCATATTGTCGAATAATAT CAGTCAAGCATCACCACTAGTGGATCTGATATCA3'	pEG6	First 51 bp to 3' end of *ATF1* gene, last 21 bp to loxP site.
ATF2'F	5'CTGAGACTTTCAAACGAATAATAACTTCAGCAATAAA AATTGTCCAGGTTACTTCGTACGCTGCAGGTCGAC3'	pEG6	First 51 bp to 5' end of the *ATF2* gene, last 21 bp to loxP site.
ATF2'R	5'CTTGCCTTTTGTACGACGTCCGCCGAGCTATACGAA GGCCCGCTACGGCAGCCACTAGTGGATCTGATATCA3'	pEG6	First 51 bp to 3' end of the *ATF2* gene, last 21 bp to loxP site
Sigma'F	5'ACCGGAGTGTCTTGACCATCCTAATATAAACAGTCTT AGGGAAGTAACCATTCGTACGCTGCAGGTCGAC3'	pJATF2oe	First 49 bp to Sigma sequence, last 20 bp to loxP site
Sigma'R	5'GAGATATGTCAGTATGACAATACGTCACCCTGAACGT TCATAAAACACATCGGCCGCCATAGGCCACTAGT3'	pJATF2oe	First 50 bp to Sigma sequence, last 22 bp to loxP site
URA'F	5'TCTTAACCCAACTGCACAGAACAAAAACCTGCAGAAC GAAGATAAATCATGTCGAAAGCTACATATAAGGAACGT GCTGCTACTCATCCTAGTCCTGTCATTCGTACGCTGCA GGTCGAC3'	pJATF2oe	First 100 bp to 3' end of the *URA3* gene, last 20 bp to loxP site
URA'R	5'TAATTTGTGAGTTTAGTATACATGCATTTACTTATACA GTTTTTTAGTTTTGCTGGCCGCATCTTCTCAAATATGCT TCCCAGCCTGCTTTTCTGTAGGCCGCCATAGGCCACT AGT3	pJATF2oe	First 97 bp to 3' end of *URA3* gene, last 21 bp to loxP site
Primers for probe production			
Primer	**Sequence**	**Template**	**Recognition sequence**
ATF1P'F	5'CCGAAGAAATGCAAAGAAGTAAG3'	VIN13	5' end of *ATF1* gene
ATF1P'R	5'CTTGTCTCGAATTTTTGTTGGGT3'	VIN13	5' end of *ATF1* gene
ATF2P'F	5'GAGTAAACGAATTCAACGAGTTTC3'	VIN13	5'end of *ATF2* gene
ATF2P'R	5'TACTGTTTGTACGCATTAGGAATG3'	VIN13	5' end of *ATF2* gene
Probe 1	5'CAATCAGGTGCGACAATCTA3'	*KanMX*	Within *KanMX* gene
Probe 2	5'CGAGCATCAAATGAAACTGC3'	*KanMX*	Within *KanMX* gene

The pJATF2oe plasmid was then used as a template for the PCR amplification of the integrating overexpression cassette. Primers were designed with homology to the *sigma* element corresponding to the terminal repeat of the retrotransposon Ty*3* and to the *loxP* sites on the pJATF2oe, namely Sigma'F and Sigma'R. A PCR fragment of approximately 3 350 bp was obtained, cleaned using a PCR purification kit (Sigma) and then used for the transformation of the industrial *S. cerevisiae* wine yeast strain, VIN13(pATF1-s), constructed by Lilly *et al.* (2000). Similarly, primers URA'F and UAR'R were designed with homology to the *URA3* gene in *S. cerevisiae* and to the *LOXP* site on the pJATF2oe plasmid. This PCR-generated fragment of ≈3 450 bp was cleaned using a PCR purification kit (Sigma) and then used for the transformation of the industrial *S. cerevisiae* strain VIN13. Transformations were carried out according to the Lithium Acetate method described by Gietz and Schiestl (1995). The following changes were made in the final steps: after the last heat shock, the cells were resuspended in 2 ml of YPD media and incubated at 30°C for 6 hours. Cells were then spun down and plated out in 200 µl volumes on YPD plates containing geneticin for the selection of positive transformants.

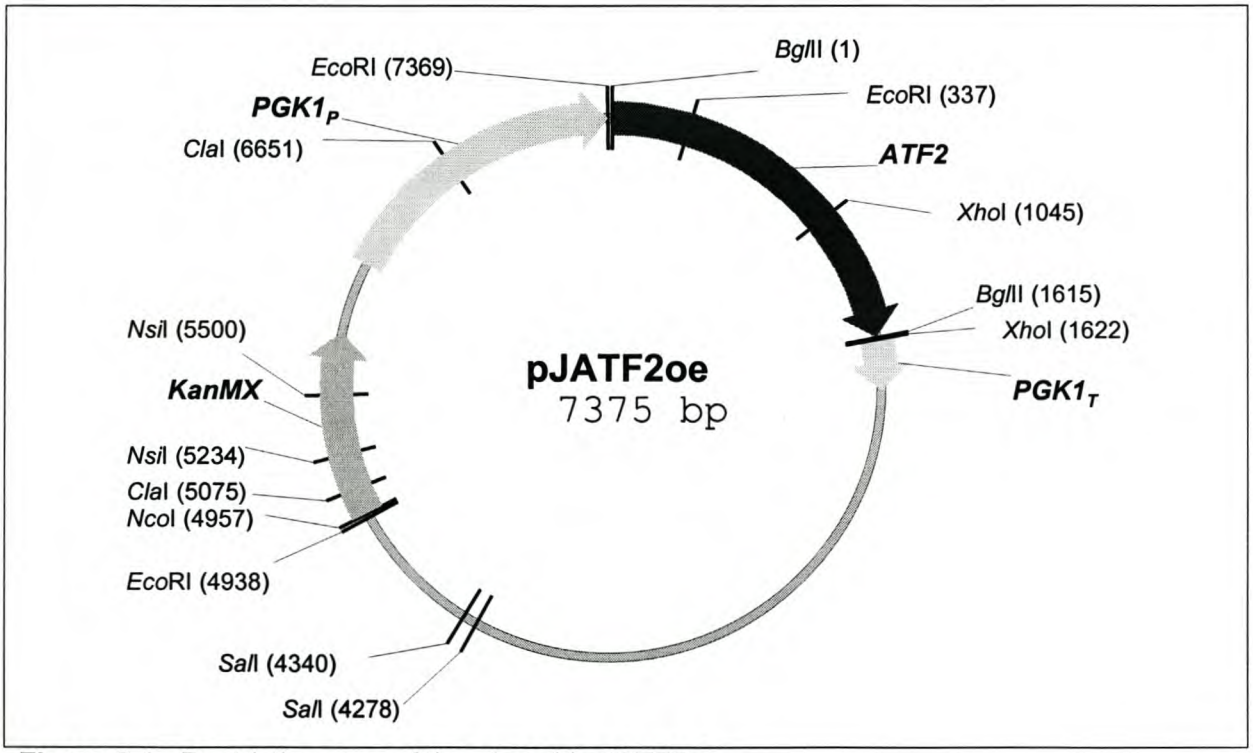


Figure 3.1. Restriction map of the plasmid pJATF2oe

3.2.2.2 Construction of disruption cassettes for the *ATF1* and *ATF2* genes

For the construction of the disruption cassettes, primers were designed with homology to the gene concerned and to the *loxP* site on the plasmid, pEG6 (J. Hignett, unpublished data). The following primers were designed for the disruption of the *ATF1* gene: ATF1'F and ATF1'R. These primers generated a 1 700 bp fragment, using pEG6 as a template. The PCR fragment was purified using a PCR purification

kit (Sigma) and the disruption cassettes that were obtained were then used for the transformation of the industrial *S. cerevisiae* strain VIN13. The same transformation method was used as described in 3.2.2.1.

3.2.3 Southern blot analysis

The isolation of genomic DNA from the control yeast VIN13, as well as from the transformed *S. cerevisiae* strains, VIN13(pATF2oe), VIN13(pATF1-s, pATF2oe), VIN13(Δ*ATF1*s) and VIN13(Δ*ATF2*s), was carried out as described by Sambrook *et al.* (1989). The genomic DNA isolated from each sample was digested with *Sal*I and *Xho*I, generating a 1 700 bp fragment. The digested genomic DNA was separated by agarose gel electrophoresis and transferred onto a Hybond-N nylon membrane (Amersham). The digoxigenin nonradioactive nucleic acid labelling and detection system (Roche) was used for Southern blot hybridisation for the confirmation of integration. Southern blots were performed using a PCR fragment generated with the primers Prob1 and Prob2, and with the use of the pEG6 plasmid as a template. The PCR reaction generated a 600 bp fragment homologous to a section of the *KanMX* gene.

Genomic DNA isolated from VIN13(Δ*ATF1s*) was cut with *Eco*RI and *Cla*I. The wild type strain yielded a 1 043 bp fragment, whereas the single disrupted strain yielded both a 1 043 bp fragment and a 1 325 bp fragment. A single disruption of the *ATF1* gene was confirmed using a 344 bp PCR-generated probe using primers ATF1P'F and ATF1P'R.

Similarly, genomic DNA was isolated from VIN13(Δ*ATF2s*) and cut with *Nco*I and *Nsi*I, with the wild type yielding a 2 365 bp fragment and the single disrupted strain a 2 365 bp and 1 375 bp fragment. The single disruption of the *ATF2* gene was confirmed with a probe generated through PCR using the ATF2P'F and ATF2P'R primers.

3.2.4 RNA isolation and RT-PCR

VIN13(pATF1-s), VIN13(pATF2oe), VIN13(pATF1-s, pATF2oe), VIN13(Δ*ATF1*s), VIN13(Δ*ATF2*s) and VIN13 were inoculated into 100 ml of YPD broth (Biolab, South Africa) and incubated at 30°C overnight. Total RNA was isolated from the cells using the FastRNA® Kit-RED product (BIO 101) according to the manufacturer's specifications. The RNA isolated was used for reverse transcription PCR (RT-PCR). RT-PCR was performed using the *C. therm.* Polymerase One-Step RT-PCR System (Roche). For the preparation of the RT-PCR, the RNA template and the gene specific downstream primer (ATF'R or ATF2OE'R) were denatured at 94°C for 5 min and then put on ice, while the rest of the PCR reaction components were added. Reverse transcription was carried out for 45 min at the annealing temperature optimal for the primer in use. This reaction was followed by a typical amplification reaction as described in 3.2.2. The PCR products obtained were then run on a 0.8% agarose gel and the intensity of the bands was quantified using the Alphalmager software v5.5

(Alpha Innotec Corporation, San Leandro, California, USA) program. The RNA isolated was also run on a denaturing formaldehyde gel and the differences in concentrations were included in the quantification of the RT-PCR bands.

3.2.5 Preparation of intact chromosomal DNA for pulse-field gel electrophoresis

The embedded agarose method (Carle and Olson, 1985) was used for the preparation of chromosomal DNA samples of the modified VIN13 strains made and of VIN13. Chromosomal DNA samples were separated using a contour-clamped homogeneous electric field (CHEF) electrophoresis, with the use of the CHEF-MAPPER (Bio-Rad Laboratories, Richmond, Va.) apparatus. CHEF separations were carried out according to the method of Van der Westhuizen and Pretorius (1992).

3.2.6 Small-scale white wine production

The wine yeast strains, VIN13, VIN13(pATF1-s), VIN13(pATF2oe) and VIN13(pATF1-s, pATF2oe), were each inoculated at $5X10^6$ cells/ml into 4.5 L of Colombard grape juice which had been frozen at -20°C and then defrosted for 3 days at 8°C. The Colombard juice was then allowed to ferment at 15°C until it was dry. The wines were cold stabilised, filtered and bottled according to standard white wine practices. All fermentations were done in duplicate.

3.2.7 Base wine production and small-scale distillation

The wine yeast strains, VIN13, VIN13(pATF1-s) VIN13(pATF2oe) and VIN13(pATF1-s, pATF2oe), were each inoculated at $5X10^6$ cells/ml into 15 L of Colombard grape juice which had been frozen at -20°C and then defrosted for 3 days at 8°C. The juice was then fermented until dry at 15°C. No sulphur dioxide was added to the juice at any stage. All fermentations were carried out in duplicate. For the distillation, 5 L round-bottomed flasks were used. These flasks were filled with 4.5 L of base wine and yeast lees from the original 15 L fermentation. To each 4.5 L, 3 g of copper sulfate and 2 copper plates were added in order to mimic the conditions of a copper pot still. The flasks were then heated on heating mantles and the distillate was collected at a flow rate of 5 ml per minute. The distillate was collected until 30% (vol/vol) alcohol was reached. The same procedure was used for the second distillation. However, the first 1% of the distillate (head) was collected at 2 ml/min and then discarded. The distillation was then continued at a flow rate of 5 ml/min, until the distillate (heart fraction) reached 70% (vol/vol) alcohol. Both the first and second distillates were kept at 4°C for GC analyses.

3.2.8 Gas chromatographic (GC) analysis

GC analyses were performed on the wine after stabilisation and bottling and base wine extractions were performed after alcoholic fermentation, after both the first distillation and the second distillation.

Ten millilitres was collected from each wine, rebate wine, 30% distillate and 70% distillate sample. To this 10 ml, 800 µl of 4-methyl-2-pentanol and 15 ml of dimethyl ether were added. The test tube was then mechanically rotated on a rotary mixer (Labinco) at 60 rpm for 30 min. The top ether layer was separated and analysed. All GC analyses were done on a Hewlett-Packard model 6890 series II gas chromatograph with a Lab Alliance capillary column (length, 60 m; inside diameter, 0.32 µm; film width, 0.5 µm). The method was that of Lilly *et al.* (2000).

3.2.9 Sensory evaluations and statistical analysis

Colombard wines were sensorially evaluated by a panel of eight experienced judges. The wines were evaluated on a scale of one to five for fruity, herbaceous, nutty, earthy, woody, caramel, chemical, pungent, oxidised, microbiological, floral or spicy characteristics, where one represented a low characteristic and five a high characteristic of the aroma. Tasting of the distillates was conducted in the same way, differing in aroma descriptions only.

The data collected from the sensory evaluation was then analysed statistically using the computer program of Statsoft, Inc. (2001), STATISTICA (data analysis software system), version 6.

3.3 RESULTS

3.3.1 Cloning and constitutive expression of the *ATF2* gene in the industrial yeast strains VIN13 and VIN13(pATF1-s)

The *ATF2* gene was successfully amplified from the genomic DNA of the industrial wine yeast VIN13 and cloned into the plasmid pJ, resulting in the plasmid pJATF2oe. Positive transformants of VIN13(pATF2oe) and VIN13(pATF1-s, pJATF2oe) were selected on YPD agar supplemented with 400 mg/L of the antibiotic geneticin. Possible positive transformants were restreaked twice onto geneticin and then onto YPD. The positive transformants obtained after restreaking were then verified for the integration of *ATF2* by Southern blot hybridisation. The strains VIN13(pJATF2oe) (**Figure 3.2a**) and VIN13(pATF1-s, pATF2oe) (**Figure 3.2b**) were produced and used for the production of wine and brandy.

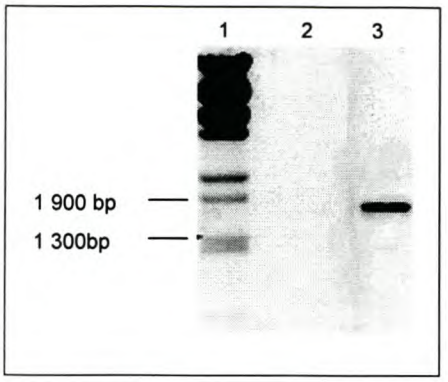


Figure 3.2a. Southern blot hybridisation with the *Bst*EII-digested λ DNA in lane 1, VIN13 in lane 2 and VIN13(pJATF2oe) in lane 3, showing a band of 1 700 bp obtained through *Sal*I and *Xho*I digest

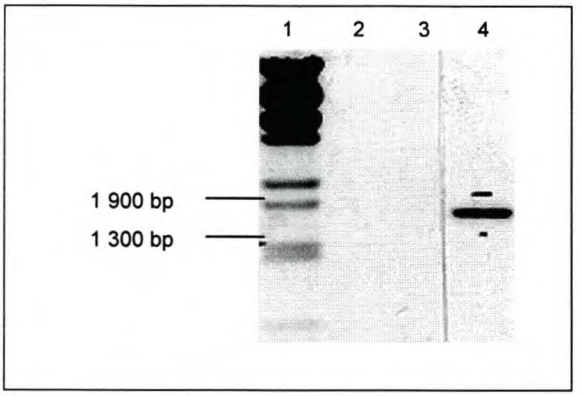


Figure 3.2b. Southern blot hybridisation with *Bst*EII-digested λ DNA in lane 1, VIN13 in lane 2 and VIN13(pATF1-s,pJATF2oe) in lane 4, showing a band of 1 700 bp obtained through a *Sal*I and *Xho*I digest

3.3.2 Disruption of the *ATF1* and *ATF2* genes in the industrial wine yeast strain VIN13

The disruption cassettes constructed and used in the transformations removed the whole coding region of a single copy of the *ATF1* gene and the *ATF2* gene. Positive transformants were selected in the same way as stated in section 3.3.1, and the results were verified by Southern blot hybridisation (**Figures 3.2a** and **b**).

A single copy of the gene concerned was disrupted, which was confirmed by Southern blot hybridisation (**Figures 3.3a** and **3.3b**), as two bands were observed, one the same as the wild type strain and the other confirming a single disruption. Attempts at marker recovery and disruption of the second copy of the gene were difficult and unsuccessful. The single disruptions yielded the following transformants: VIN13(Δ*ATF1*s) and VIN13(Δ*ATF2*s).

All yeast transformants obtained were positively identified as VIN13 strains through CHEF analysis, with VIN13 (Anchor yeast) as a positive control (data not shown).

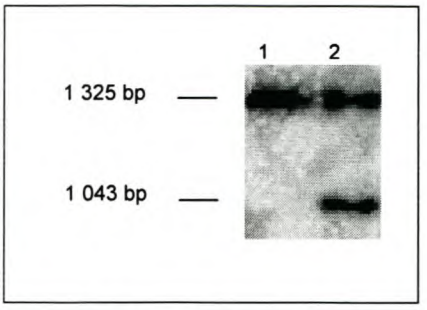


Figure 3.3a. Southern blot hybridisation confirming a single disruption of the *ATF1* gene. In lane1 the wild type VIN13 has only one band, whereas the single disrupted strain, VIN13(ΔATF1s), shows two bands of 1 325 bp and 1 043 bp

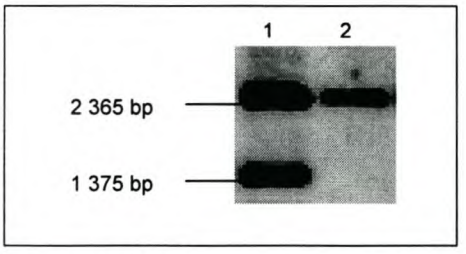


Figure 3.3b. Southern blot hybridisation confirming the single disruption of the *ATF2* gene. In lane 1, digested genomic DNA from VIN13(ΔATF2s) shows two clear bands of 2 356 bp and 1 375 bp, while the wild type, VIN13, in lane 2 has only one band of 2 356 bp

The RT-PCR obtained for VIN13, VIN13(pATF1-s), VIN13(pATF1-s, pJATF2oe) and VIN13(ΔATF1s) can be seen in **Figure 3.4**. In this figure, it is clearly seen that the *ATF1* gene is expressed at much higher levels for VIN13(pATF1-s) and VIN13 (pATF1-s, pJATF2oe). The RT-PCR for the *ATF1* gene from VIN13(ΔATF1s) was unsuccessful, as seen in **Figure 3.4**. The levels of expression are expressed as a percentage of the control and shown in **Figure 3.5**.

RT-PCR attempts using the gene-specific primers for the *ATF2* gene were unsuccessful, even when various concentrations of RNA and various temperatures were used.

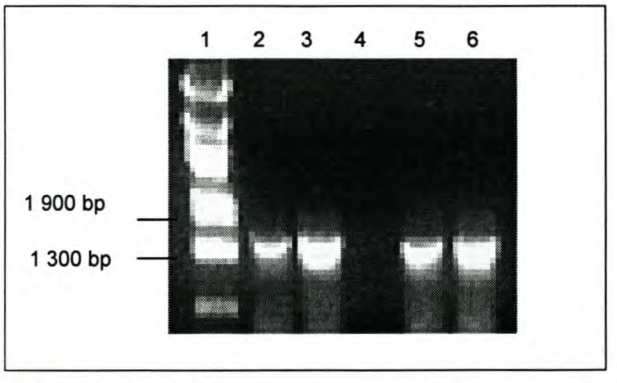


Figure 3.4. RT-PCR of the *ATF1* gene using the primers ATF1'F and ATF1'R. The marker is in lane 1, VIN13 in lane2, VIN13(pATF1s) in lane 3, VIN13(ΔATF1s) in lane 4 and VIN13(pATF1s, pJATF2oe) in lane 5 and 6

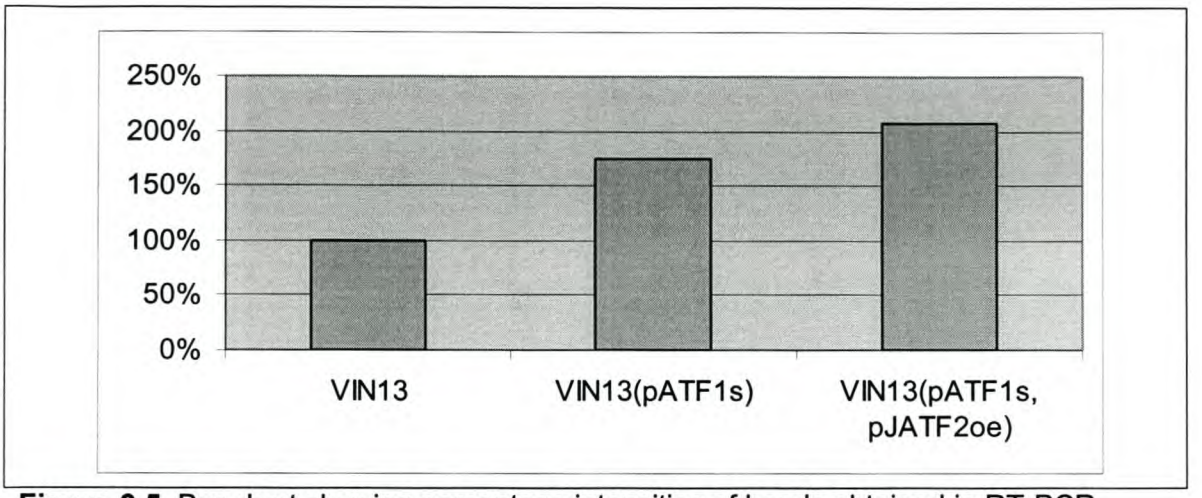


Figure 3.5. Bar chart showing percentage intensities of bands obtained in RT-PCR

3.3.3 Gas chromatographic analysis of wine and brandy distillates

GC analyses were carried out on the wine, base wine and brandy distillates. This was conducted in order to determine whether there was an increase or decrease in the levels of esters, higher alcohols and certain acids due to the overexpression or disruption of the *ATF1* and *ATF2* gene. Wine and brandy were also made with the strain VIN13(pATF1-s) from Lilly *et al.* (2000), in order to compare these results with the overexpression of the *ATF2* gene in VIN13 and the overexpression of both *ATF1* and *ATF2* in VIN13, as the conditions of the experiment were slightly different to those used by Lilly *et al.* (2000). The results of the GC analyses of the wine and distillates are shown in **Tables 3.3** and **3.4** respectively.

Wine made with VIN13(pJATF2oe) showed differences to the control strain. The total acid concentration was 20% less, although it produced 188% more isobutyric acid and 62% more propionic acid in comparison to the control strain. In general, the ester concentration did not differ from the control, except for diethyl succinate, of which 177% more was produced than by the control. Esters that showed a decrease were ethyl caprate (78% less), ethyl caprylate (61% less) and ethyl lactate, as no ethyl lactate was measured. Overall, the wine made from VIN13(pJATF2oe) showed a decrease in the total higher alcohols produced, producing 20% less *i*-butanol, 25% less isoamyl alcohol and 20% less *n*-butanol.

Wine made from VIN13(pATF1-s) differed significantly from the control, as expected. The wine made with this yeast showed a 62% decrease in the total amount of acids produced. This is largely due to the 70% decrease in acetic acid produced. Other acid formation was similar to that of the control, except for isobutyric acid, which was 41% more, and decanoic acid, which was 25% more than that produced by the control strain. However, as expected, there was a significant difference in the concentration of esters produced, with this yeast producing 327% more 2-phenylethyl acetate, 49% more diethyl succinate, 464% more ethyl acetate, 53% more ethyl caprate and 257% more isoamyl acetate. The total ester concentration was 377% more than that of the control strain.

Table 3.3. GC analyses for wine produced using the modified wine yeasts

COMPOUND (mg/L)	WINE YEAST STRAIN USED			
	VIN13	VIN13 (pATF1s, pJATF2oe)	VIN13 (pATF1-s)	VIN13 (pJATF2oe)
Acetic acid	191.35	48	56.85	149.826
Decanoic acid	4.25	3.6	5.75	2.79
Hexanoic acid	5.65	5.5	6.6	5.525
Isobutyric acid	0.28	0.45	0.395	0.805
n-Valeric acid	0.745	0.3	0.59	0.925
Octanoic acid	10.1	9.55	10.95	8.545
Propionic acid	1.13	0.865	0.97	1.83
2-Phenylethyl acetate	0.585	0.615	2.5	0.54
Diethyl succinate	0.51	0.82	0.76	1.415
Ethyl acetate	99.31	111.4	560.6	103.75
Ethyl butyrate	0	0.47	0	0.78
Ethyl caprate	4.7	3.65	7.2	1.03
Ethyl caproate	2.95	2.75	3.2	2.37
Ethyl caprylate	5.15	4.15	5.35	2.005
Ethyl lactate	1.1	1.35	1	0
Hexyl acetate	2.55	2.15	2.8	1.81
Isoamyl acetate	21.2	22.2	75.7	20.22
2-Phenylethyl alcohol	10.4	9.4	8.7	7.495
Hexanol	1.15	0.985	0.405	1.245
Isoamyl alcohol	109.95	102.4	56.45	82.68
Isobutanol	11.85	11.95	12.95	9.32
Methanol	15.8	16.8	19	0
n-Butanol	2	2.3	4.035	1.64
n–Propanol	72.45	40.25	55	78.54
TOTAL ESTERS	**138.055**	**149.555**	**659.11**	**133.92**
TOTAL HIGHER ALCOHOLS	**223.6**	**184.085**	**156.54**	**180.92**
TOTAL ACIDS	**213.505**	**68.265**	**82.105**	**170.246**

Wine made with VIN13(pATF1-s, pJATF2oe) differed from the control and the other two modified strains. It produced 68% less total acid than that of the control strain, mainly due to the 75% less acetic acid produced by this strain. Similarly to VIN13(pATF1-s) and VIN13(pJATF2oe), it produced 61% more isobutyric acid than the control strain. However, it differed from the other strains in that it produced 60% less *n*-valeric acid than that of the control. The total amount of esters produced by this strain was similar to that of the control, although some ester showed differences, such as diethyl succinate, of which 61% more was produced than by the control strain. The total higher alcohols produced by this strain were only 20% less than the control, with this strain showing 46% less *n*-propanol than the control strain.

GC analyses of the base wine produced using VIN13(pJATF2oe) and 70% distillate also showed differences. For the 70% distillate, the total acids produced were similar to that of the control, although this distillate showed 40% less acetic acid than the control strain. Other acid concentrations were generally higher than that of the control, with this distillate having 40% more decanoic acid, 47% more hexanoic acid and 40% more octanoic acid. As in the case of the wine, there was no increase in total ester concentration, except for an increase in 2-phenylethyl acetate (43%)

and diethyl succinate (60%). The ethyl acetate concentration decreased by 36%, ethyl caprate by 38%, ethyl caproate by 33%, ethyl caprylate by 43% and isoamyl acetate by 39%. The concentration of higher alcohols produced by this strain was not very different than that produced by the control strain.

Table 3.4. GC analyses of the base wine and 70% distillate

COMPONENT (mg/l)	COLOMBAR BASE WINE AFTER ALCOHOLIC FERMENTATION				70% DISTILLATE AFTER SECOND DISTILLATION			
	VIN13	VIN13 (pATF1-s, pJATF2oe)	VIN13 (pATF1-s)	VIN13 (pJATF2oe)	VIN13	VIN13 (pATF1-s, pJATF2oe)	VIN13 (pATF1-s)	VIN13 (pJATF2oe)
Acetic acid	231.725	61.72	52.575	224.22	25.695	15.1	7.52	15.385
Decanoic acid	4.495	5.68	7.725	4.135	34.24	34.47	37.32	47.87
Hexanoic acid	5.925	6.605	7.465	5.055	9.385	10.78	12.3	13.825
Isobutyric acid	0.742	2.66	1.425	0.76	0	0	0.74	1.01
n-Valeric acid	0.675	0.285	0.535	0.915	0	0	0	0
Octanoic acid	5.49	6.455	6.985	7.695	35.86	38.82	41.55	50.08
Propionic acid	14.175	3.255	1.55	1.165	0	0	0	0
2-Phenylethyl acetate	0.515	0.695	2.225	0.515	2.055	2.36	7.235	2.935
Diethyl succinate	0.87	0.89	0.95	0.98	1.08	1.22	1.57	1.725
Ethyl acetate	105.06	123.83	527.235	89.885	237.125	157.535	966.68	155.56
Ethyl butyrate	0.663	0.915	0.515		2.09	1.125	0	0
Ethyl caprate	3.715	4.945	7.315	2.12	44.47	24.68	37.69	18.52
Ethyl caproate	3.27	3.585	4.3	1.595	6.555	3.285	5.965	4.375
Ethyl caprylate	4.73	5.99	6.75	1.59	14.645	7.735	10.62	8.335
Ethyl lactate	0	0	0	0	2.47	2.985	2.325	2.21
Hexyl acetate	3.005	3.145	3.515	1.465	7.275	3.695	6.485	5.265
Isoamyl acetate	22.9405	28.21	77.31	15.025	73.22	42.335	185.39	44.305
2-Phenylethyl alcohol	6.975	6.855	5.505	7.09	4.645	4.905	3.875	6.21
Hexanol	1.205	1.155	0.455	1.19	7.41	6.45	3.905	6.48
Isoamyl alcohol	97.08	94.835	53.01	79.77	722.86	658.525	413.8995	599.875
i-Butanol	8.905	9.275	10.225	9.135	49.9	47.285	59.12	46.225
Methanol	20.19	17.205	0	0	97.005	97.805	103.03	92.605
n-Butanol	1.825	2.14	0.775	1.62	9.275	11.09	4.205	6.585
n-Propanol	62.32	37.09	50.525	93.525	334.175	188.5	275.065	386.47
TOTAL ESTERS	144.7685	172.205	630.115	113.175	390.985	246.955	1223.96	243.23
TOTAL HIGHER ALCOHOLS	198.5	168.555	120.495	192.33	1225.27	1014.56	863.0995	1144.45
TOTAL ACIDS	263.227	86.66	78.26	243.945	105.18	99.17	99.43	128.17

The 70% distillate made from the base wine using VIN13(pATF1-s) showed significant differences when compared to the control. The total amount of acids decreased, with this distillate having 71% less acetic acid than the distillate obtained from wine made with the control strain. Only hexanoic acid showed an increase of 31%. The ester concentration differed significantly from that of the control, as this distillate had 252% more 2-phenylethyl acetate, 45% more diethyl succinate, 308% more ethyl acetate and 153% more isoamyl acetate. In total, this distillate had 213% more total esters than the control. The concentration of the higher alcohols also showed differences, as this distillate had 47% less hexanol, 43% less isoamyl alcohol

and 55% less *n*-butanol than the control. In total, the higher alcohol concentration was 30% less than that of the control.

Differences were also seen in the 70% distillate made using VIN13(pATF1-s, pJATF2oe). The total acids produced by this strain were similar to that of the control, although a decrease in acetic acid (41%) was noted. This strain produced 37% less total esters, with a decrease observed for ethyl acetate (36%), ethyl butyrate (46%), ethyl caprate (45%), ethyl caproate (50%), ethyl caprylate (47%), hexyl acetate (49%) and isoamyl acetate (42%). An increase in ethyl lactate (21%) was observed, as well as small increases in 2-phenylethyl acetate (15%) and diethyl succinate (13%).

Statistical analyses conducted for the GC analyses and ANOVA results for the wine and brandy are given in Addendum C and Addendum D respectively. The statistical analysis showed that there was a statistical significance only for the following compounds in wine: acetic acid, decanoic acid, hexanoic acid, isobutyric acid, *n*-valeric acid, 2-phenylethyl acetate, ethyl acetate, ethyl caprate, ethyl caproate, ethyl caprylate, hexyl acetate, isoamyl acetate, hexanol, *i*-butanol and *n*-propanol. The statistical analysis also showed that the differences in the following compounds in the 70% distillate were statistically significant: isobutyric acid, 2-phenylethyl acetate, ethyl acetate, isoamyl acetate, hexanol, *i*-butanol, *n*-butanol and *n*-propanol. More wine fermentations will have to be conducted for further statistical analysis in order to statistically evaluate the wine and brandy more accurately.

3.3.4 Sensory and statistical evaluations

A panel of tasters rated the wine samples for the following characteristics: fruity, herbaceous, nutty, chemical, pungent and floral. All histograms obtained from the statistical analyses are given in Addendum A in Chapter 5.

When comparing the ratings given by the tasters for the fruity characteristic, it was found that VIN13(pATF1-s) and VIN13(pJATF2oe) received ratings of up to 5 and 4 respectively, implying them to be more fruity than the other two wine samples. VIN13(pATF1-s, pJATF2oe) received a very similar rating to the control for this characteristic. Slight differences were also observed for the characteristic of flowery, with all samples receiving slightly higher ratings than the control.

For the characteristic of chemical, VIN13(pATF1-s) received a high rating, indicating that this wine has a distinct chemical aroma in comparison to the control and the other two samples, which received ratings very similar to the control wine. For the pungent characteristic, VIN13(pATF1-s) was once again different to the other three samples and was rated as being more pungent. The ratings for the nutty and herbaceous characteristics were not very different to the control samples for all the wine made.

Although there were indications that the wines scored differently for various characteristics, only the characteristic of chemical showed statistical significance.

The ratings for the wine samples were analysed for their similarity to each other and a cluster diagram was drawn to illustrate how they related to each other (**Figure**

3.6). **Figure 3.6** shows that wine made with VIN13(pJATF2oe) and VIN13(pJATF1-s, pJATF2oe) and the control wine are clustered together, while VIN13(pATF1-s) clustered approximately twice the distance away from the rest. VIN13(pATF1-s, pJATF2oe) clustered the closest to the control wine.

The brandy made from the transformed yeasts was also rated for the following characteristics: fruity, flowery, sweet, chemical, oily and herbaceous. Ratings for all the characteristics varied, except for that of fruity, which did not vary much from the control wine. Histograms obtained from the tasting results are shown in Addendum B in Chapter 5.

For the characteristic of flowery, only VIN13 and VIN13(pATF1-s) got a rating of up to five, whereas VIN13(pJATF2oe) and VIN13(pATF1-s, pJATF2oe) were not rated high for this characteristic, both getting a rating of one from most of the panel. The distillates received similar ratings for the sweet characteristic. VIN13 and VIN13(pATF1-s) once again obtained a rating of up to five and VIN13(pJATF2oe) and VIN13(pATF1-s, pJATF2oe) rated low for this characteristic.

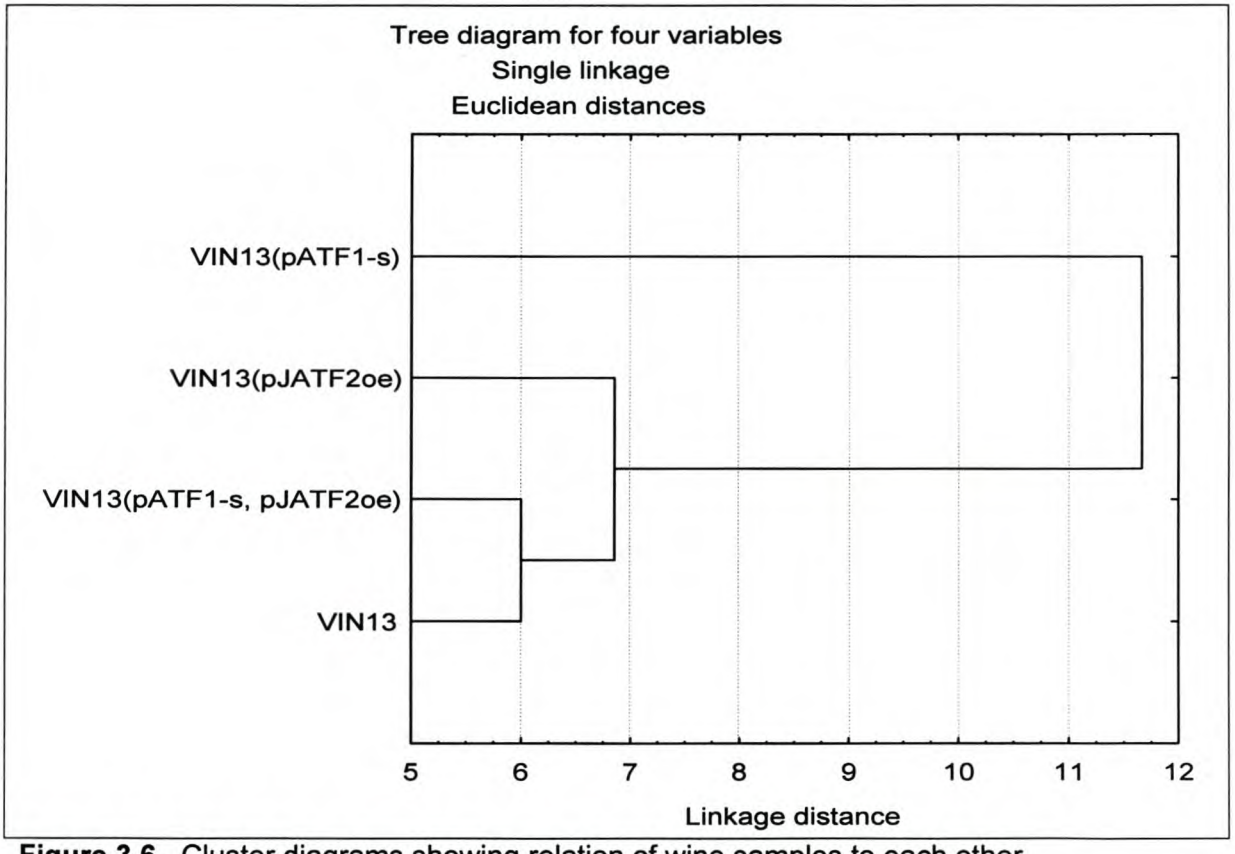


Figure 3.6. Cluster diagrams showing relation of wine samples to each other

Brandy made from VIN13(pATF1-s) was perceived as being the most chemical of all the brandies made, receiving a rating of up to five from the tasting panel. Brandy made with the other transformed yeasts was rated as having a chemical flavour, and received ratings similar to that of the control brandy.

For the characteristics of oily and herbaceous, brandy made from VIN13(pJATF2oe) was rated higher on both these characteristics in comparison to

the control. The tasting panel also rated VIN13(pATF1-s, pJATF2oe) as being more herbaceous than the control. The rating for this wine ranged from one to four ,whereas the ratings for the control were mostly one. Brandy made from VIN13(pATF1-s) did not get high ratings for either of these characteristics and received ratings of mostly one for this trait. These differences were noted in the tasting, although they were not statistically significant.

A cluster analysis in **Figure 3.7** shows the relationship between the brandies made. The cluster analysis was similar to that for the wine, with brandy made from VIN13(pATF1-s) clustering furthest from the control wine. However, the brandy differed from the wine in that VIN13(pJATF2oe) and VIN13(pATF1-s, pJATF2oe) clustered together and brandy made from VIN13 clustering on its own. The cluster analysis also shows that brandies made from VIN13(pJATF2oe) and VIN13(pATF1-s, pJATF2oe) are more closely related to the control brandy made from VIN13 than VIN13(pATF1-s). Cluster analysis also showed that the brandy samples were not as closely related as the wine samples, as clustering started at a linkage distance of 5 for the wine and at 8 for the brandy.

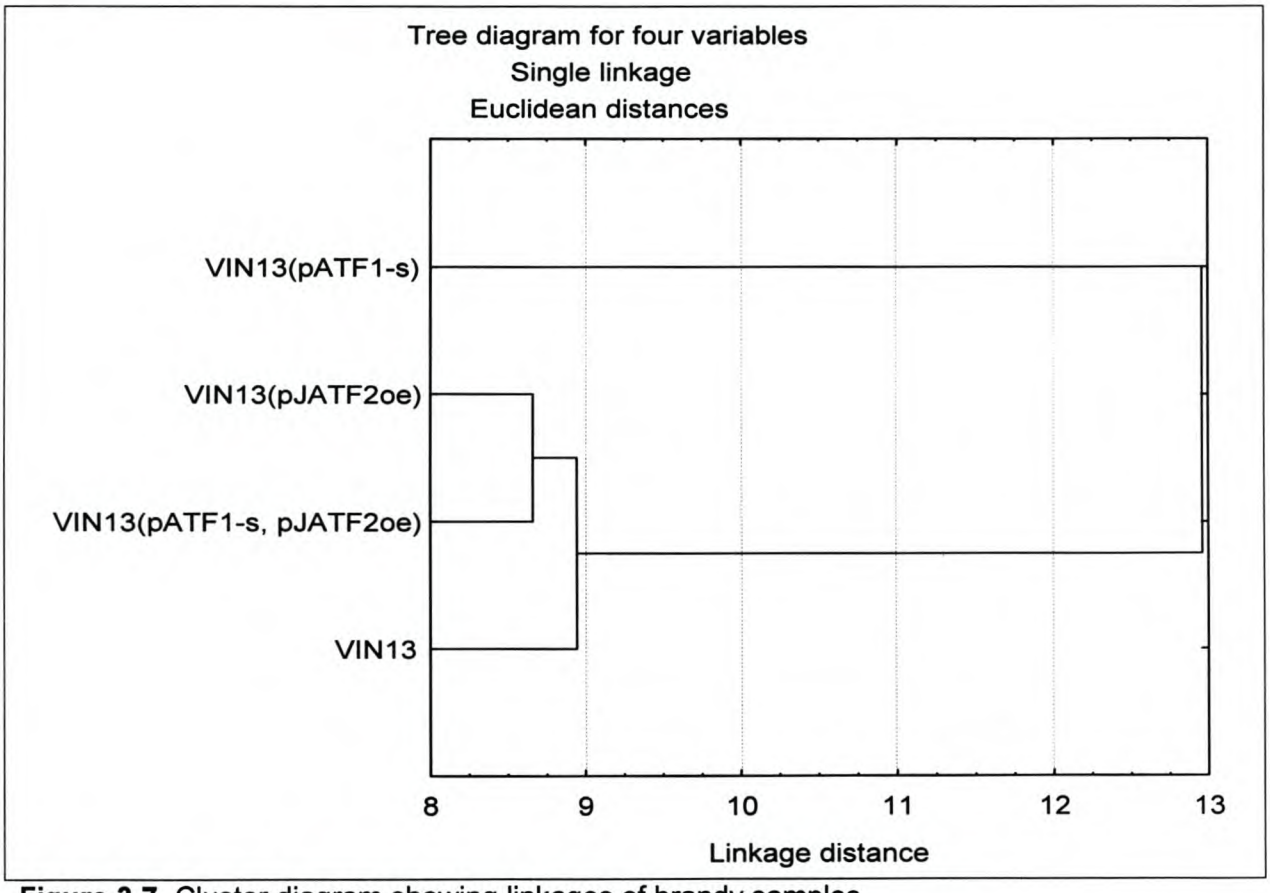


Figure 3.7. Cluster diagram showing linkages of brandy samples

The flavour characteristics describing the wine and brandy were also statistically analysed in order to obtain an idea of which characteristics correlated with each other. The factor analysis for the characteristics of the wine and brandy are shown in **Figures 3.8** and **3.9**. This analysis showed that the flavour characteristics of herbaceous and oily correlated with each other, while the flavour characteristics of

sweet, chemical, fruity and flowery correlated with each other and did not correlate with the characteristics of herbaceous and oily.

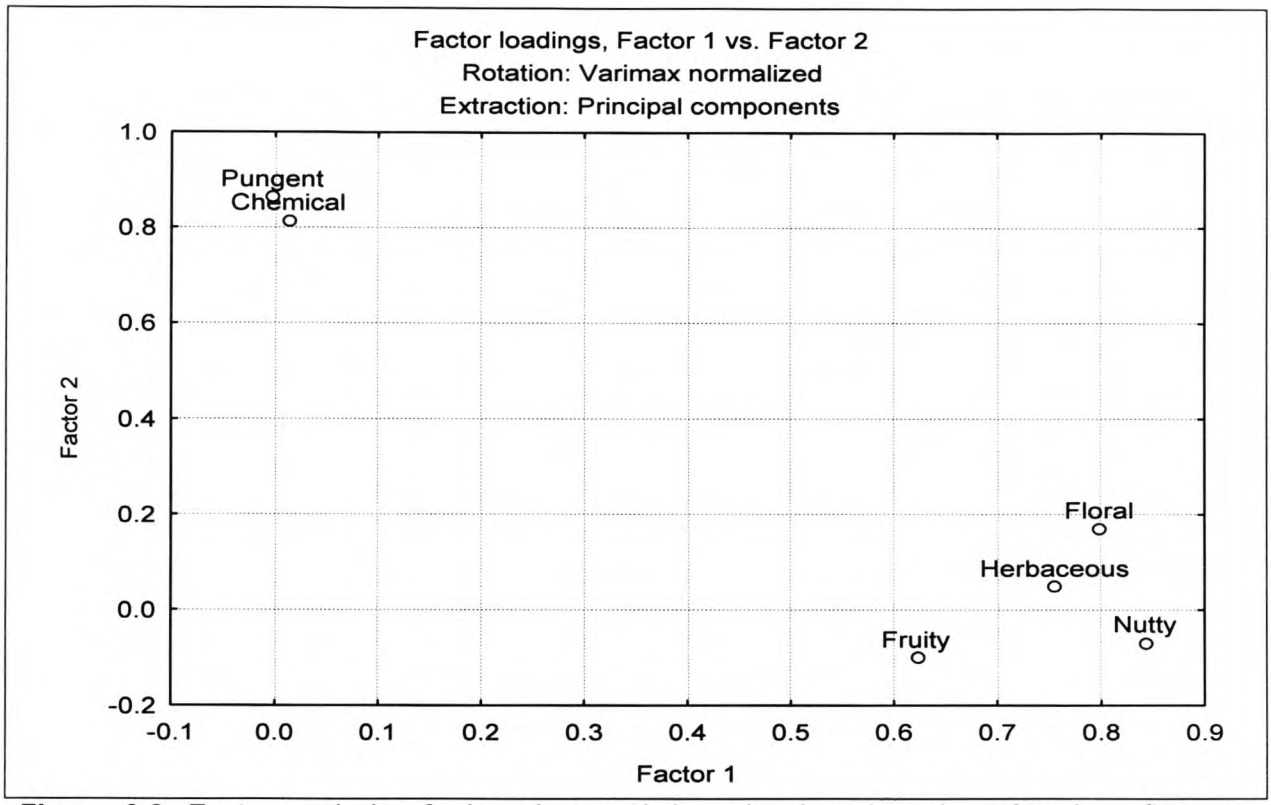


Figure 3.8. Factor analysis of wine characteristics, showing clustering of various flavour characteristics

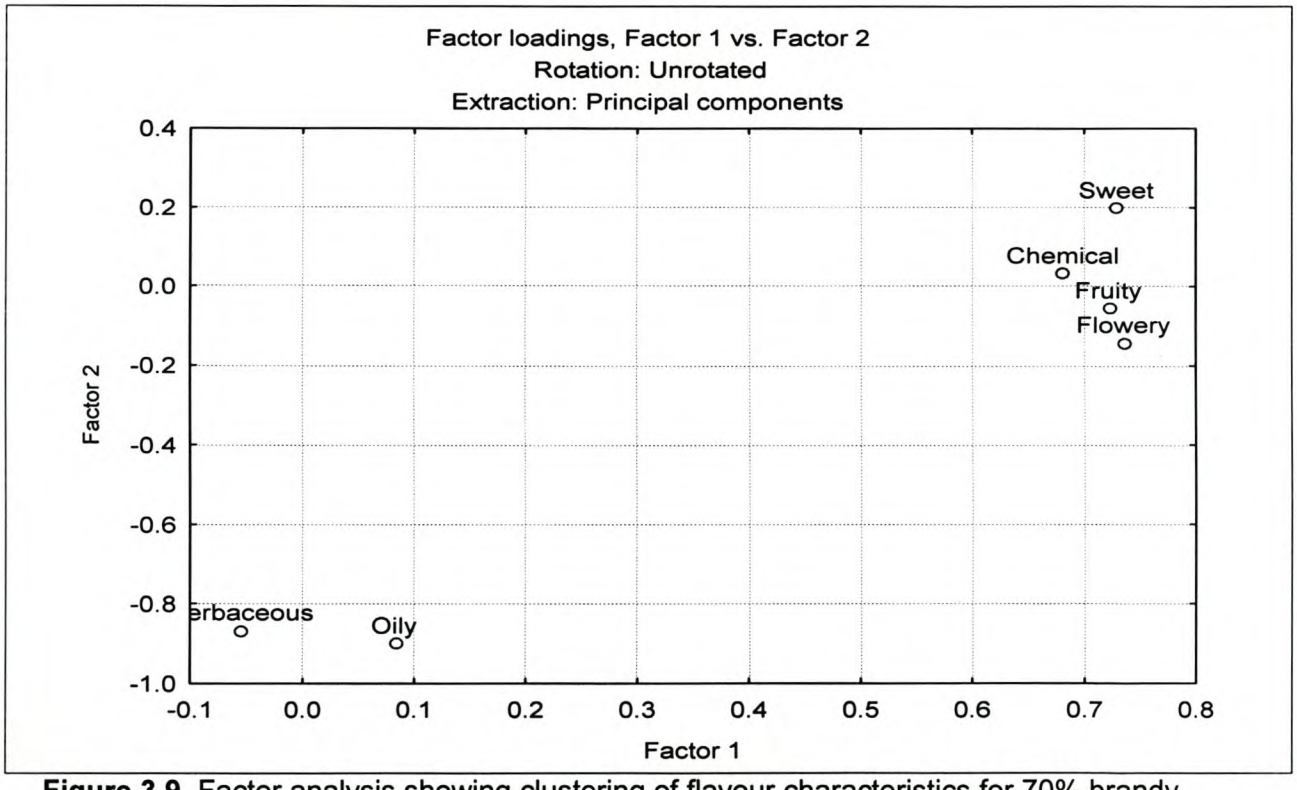


Figure 3.9. Factor analysis showing clustering of flavour characteristics for 70% brandy

For the factor analysis of the flavours describing brandy, the flavours chemical and pungent correlated with each other, while floral, herbaceous, fruity and nutty showed a correlation.

3.4 DISCUSSION

From the results obtained, it is clear that a change in the expression of a single gene can result in a difference in the aroma bouquet of the wine. This study examined whether there would be an increase in isoamyl acetate and ethyl acetate as a result of the overexpression of the acetyl transferase gene *ATF2*.

In order to achieve overexpression and have the *ATF2* gene expressed throughout alcoholic fermentation, it was placed under the constitutive promoter of the phosphoglycerate kinase gene (*PGK1*). This was achieved through the cloning of the *ATF2* gene into the plasmid pJ, which contained the *PGK1* promoter and terminator sequences. The *ATF2* overexpression cassette constructed in this study was then obtained through PCR and integrated into the genome of industrial wine yeast strain VIN13. A PCR-generated overexpression cassette was used, as it aided in the speed at which the overexpression and disruption cassettes could be obtained. This also allowed for easy selection of an integration site through the designing of specific primers. However, PCR can result in the incorporation of errors and, to eliminate this, the DNA polymerase Expand, which has proof-reading abilities, was used.

It was important for the gene to be integrated into the genome, as this would allow for growth under the non-selective conditions of alcoholic fermentation. Due to the use of an industrial wine yeast strain, dominant selective markers were essential and the *KanMX* gene was therefore used as a selective marker. The use of the sigma sequence was chosen for integration, as it would allow for the multiple integration of overexpression cassettes, as these sites are located throughout the yeast genome.

Because industrial wine yeast strains are polyploid, *loxP* sites, which can be used in the cre-recombinase system, were incorporated into the disruption and overexpression cassette. However, the recovery of the marker was unsuccessful and, due to this, wine yeast strains with only a single disruption of the *ATF1* and *ATF2* genes were obtained. In order to obtain double disruptions, it might be possible to design a different disruption cassette with the use of a different dominant selective marker, such as *SMR1*.

Although the initial idea for this study was concerned with the change in the levels in ethyl acetate and isoamyl acetate, GC analysis showed that there was no increase in these compounds when the *ATF2* gene was overexpressed in VIN13. The *ATF1* gene has been well researched and its role in the production of ethyl acetate and isoamyl acetate has been confirmed. However, the *ATF2* gene has not been studied as thoroughly and its function still remains unclear.

When examining the GC results, it can be seen that the overexpression of the *ATF2* gene results in a decrease in higher alcohols. The decrease in higher alcohols is often indicative of ester synthesis, although this was not the result in the overexpression of the *ATF2* gene and, even though there was a decrease in higher alcohols, no increase in total esters was observed. However, a significant increase in 2-phenyl ethyl acetate was observed. The decrease in total esters was unexpected, as the *ATF2* gene is an acetyl transferase that displays isoamyl alcohol acetyl transferase activity (Nagasawa *et al.*, 1998).

As expected, and in accordance with the results of Lilly *et al.* (2000), the *ATF1* gene resulted in high production of 2-phenylethyl acetate, ethyl acetate and isoamyl acetate. As a result of this, there was a decrease in 2-phenylethyl-alcohol, acetic acid and isoamyl alcohol.

The GC results obtained from the wine and brandy made with the yeast overexpressing both the *ATF1* gene and *ATF2* gene were surprising. The GC results generally tended to be more like that of the wine and brandy made with the yeast overexpressing the *ATF2* gene and not like that of the wine and brandy made with the yeast overexpressing the *ATF1* gene.

It was found that there was a general increase in isobutyric acid and diethyl succinate, and a decrease in acetic acid in wine, when either the *ATF1* or the *ATF2* gene was overexpressed. In the 70% distillates, an increase in hexanoic acid, diethyl succinate and 2-phenylethyl acetate, and a decrease in acetic acid was generally observed when either the ATF1 or the ATF2 genes were overexpressed.

The compound ethyl lactate is not produced by yeast and any increase in this ester could be the result of spontaneous malolatic fermentation, which may have started in some of the rebate wines.

Statistical analysis of the GC results was performed and the graphs obtained from an ANOVA analysis are shown in Addendums C and D. The large error bars seen in some of these figures are due to the values that were obtained for particular compounds being far apart. This is particularly so in the brandy samples. The reason for this could be that the distillation process was not accurate, as well as too few fermentation samples for each wine and brandy produced for a more accurate statistical analysis to be obtained. For the statistical analysis to be of more value, more fermentations should be conducted with the recombinant wine yeast strains.

Tasting results indicated clear differences in the wines and brandies made. For the wines tasted, differences observed for the flowery characteristic could be the result of the increased production of 2-phenylethyl acetate by all the recombinant yeasts. On the other hand the strong chemical characteristic for wine and brandy made with VIN13(pATF1-s) is due to the high level of ethyl acetate produced by this strain.

Factor analysis of the tasters showed that the tasters were different in their ratings and that their ratings did not always correlate for the various characteristics. The statistical analysis also indicated that the statistical significance was low for the

differences in the flavour characteristics. For better statistical analysis of the tasting, more tasters could be on the tasting panel, as this may improve the consistency of the results.

Grape must was not fermented with the recombinant yeasts made with only a single disruption and further studies could include these fermentations, along with fermentations using a wine yeast strain in which the *ATF1* and *ATF2* genes are completely disrupted. Fermentation using a wine yeast strain in which both the *ATF1* and *ATF2* genes are disrupted in a single wine yeast strain could also be carried out. This would provide more information on the role of the *ATF2* gene in the flavour bouquet of wine and a clearer picture of what this gene is responsible for during wine fermentations.

Further studies could also include barrel ageing of wine and brandy made with recombinant strains and the monitoring of ester levels throughout the ageing process. These studies would also be beneficial to winemakers and distillers, as they could see what happened to the fruity compounds in wine and brandy during wood maturation and thereby decide how long they want to age their wine or brandy.

Wine and brandy are complex products and research on aroma compounds may be able to provide the winemaker or distiller with more control over the aroma bouquet of the wine or brandy produced. This would allow the winemaker or distiller to make a product that would suit the likes of the consumer.

3.5 ACKNOWLEDGMENTS

I would like to thank Mr. J. Hignett for the plasmid pJ, Mr. G. Jakubetz for help with the Southern blot analysis of disrupted strains, Distell for the GC analysis of the wines and brandies that were made, as well as the National Research Foundation and Winetech for funding. I also would like to thank Prof. D. Ward for his initial help with the statistical data and Dr. M. Kidd for statistical analysis of the sensory data and GC data.

3.6 LITERATURE CITED

Carle GF, Olson MV. 1985. An electrophoretic karyotype for yeast. *Proc Natl Acad Sci USA* **82**:3756-3760.

Cauet G, Degryse E, Ledoux C, Spgnoli R, Achstetter T. 1999. Pregnenolone esterfication in *Saccharomyces cerevisiae*. *Eur J Biochem* **261**:317-324.

Engan S. 1974. Esters in Beer. *The Brew Dig* **49**:40-48.

Fujii T, Nagasawa N, Iwamatsu A, Bogaki T, Tamai Y, Hamachi M. 1994. Molecular cloning, sequence analysis and expression of the yeast alcohol acetyltransferase gene. *Appl Environ Micro* **60**:2786-2792.

Fujii T, Yoshimoto H, Tamai Y. 1996. Acetate ester production by *Saccharomyces cerevisiae* lacking the *ATF1* gene encoding the alcohol acetyltransferase. *J Ferm Bioeng* **81**:538-542.

Fujiwara D, Kobayashi O, Yoshimoto H, Harashima S, Tamai Y. 1999. Molecular mechanism of the multiple regulation of the *Saccharomyces cerevisiae ATF1* gene encoding alcohol acetyltransferase. *Yeast* **15**:1183-1197.

Gietz RD, Schiestl RH. 1995. Transforming yeast DNA. *Met mol cell biol* **5**:255-269.

Güldener U, Heck S, Fiedler T, Beinhauer J, Hegemann H. 1996. A new efficient gene disruption cassette for repeated used in budding yeast. *Nucleic Acid Res* **24**:2519-2524.

Lilly M, Lambrechts MG, Pretorius IS. 2000. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Appl Environ Micro* **66**:744-753.

Malcorps P, Cheval JM, Jamil S, Dufour JP. 1991. A new model for the regulation of ester synthesis of alcohol acetyltransferase in *Saccharomyces cerevisiae* during fermentation. *J Am Soc Brew Chem* **49**:47-53.

Mason AB, Dufour JP. 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* **16**:1287-1298.

Nagasawa N, Bogaki T, Iwamatsu A, Hamachi M, Kumagai C. 1998. Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (*ATF2*) from *Saccharomyces cerevisiae* Koyakai No. 7. *Biosci Biotechnol Biochem* **62**:1852-1857.

Nordström K. 1962. Formation of ethyl acetate in fermentation with brewer's yeast III. Participation of coenzyme A. *J Inst Brew* **68**:398-407.

Rosi I, Bertuccioli M. 1992. Influences of lipid addition on fatty acid composition of *Saccharomyces cerevisiae* and aroma characteristics of experimental wines. *J Inst Brew* **98**:305-314.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Van der Westhuizen TJ, Pretorius IS. 1992. The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. *Antonie Leeuwenhoek* **61**:249-257.

Yoshioka K, Hashimoto N. 1981. Ester formation by alcohol acetyltransferases from brewers' yeast. *Agric Biol Chem* **45**:2183-2190.

Yoshioka K, Hashimoto N. 1983. Cellular fatty acid and ester formation by brewers' yeast. *Agric Biol Chem* **47**:2287-2294.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 DISCUSSION

During alcoholic fermentation, the sugar in the grape must is converted by the yeast to ethanol, carbon dioxide and other byproducts (Pretorius, 2000). The yeast strain preferred for the alcoholic fermentation of grape must is *Saccharomyces cerevisiae*. This is not only due to its GRAS status, but also due to the favourable compounds that it produces during the alcoholic fermentation and because it is able to survive the selective pressures that occur during alcoholic fermentation (Pretorius, 2000). For these reasons, an industrial strain of *S. cerevisiae* was used for the genetic modifications in this study.

Flavour is considered the most distinctive characteristic of wine and brandy, describing not only the taste compounds, but also the aroma (Lambrechts and Pretorius, 2000). Important flavour products produced during alcoholic fermentation are esters, which impart fruity aromas to the final product (Engan, 1974). Acetate esters, such as isoamyl acetate and ethyl acetate, are produced during the fermentation of grape must and these contribute to banana-like and solvent-like flavours, respectively (Fujii *et al.*, 1997). Other esters that impart fruity aromas include ethyl caproate and ethyl caprylate, which have apple-like aromas, and 2-phenylethyl acetate, which has a fruity, flowery, honey-like aroma (Pretorius, 2000). Acetate esters are produced through the action of acetyltransferases (AATases). Genes showing AATase activity in *S. cerevisiae*, namely *ATF1* and *ATF2*, have been identified, cloned and sequenced (Fujii *et al.*, 1994; Nagasawa *et al.*, 1998).

The *ATF1* gene is involved as a catalyst in the production of short-chain and medium-chain aliphatic esters from isoamyl alcohol or ethanol and acetyl-CoA (Malcorps and Dufour, 1992). Studies on the overexpression of this strain in an industrial wine yeast have shown that there is a significant increase in isoamyl acetate and ethyl acetate when this gene is overexpressed (Lilly *et al.*, 2000). However, the exact role of the *ATF2* gene is unclear (Mason and Dufour, 2000), although it shows isoamyl alcohol activity and is therefore thought to play a role in the production of isoamyl acetate (Nagasawa *et al.*, 1998).

By overexpressing or disrupting the *ATF1* and *ATF2* genes in wine yeast, the aroma profile of the wine or brandy can be modified to meet the likes of the consumer. By modifying single genes through overexpression or disruption, various wine yeasts may be obtained, which can produce wine or brandy with different aroma profiles, each one suitable for a different market.

In this study, the *ATF2* gene was overexpressed in the industrial yeast strain VIN13 in order to assess whether it would change the ester profile of wine or brandy. The *ATF2* gene was placed under the constitutive promoter of the phosphoglycerate kinase gene ($PGK1_p$) so that expression occurred throughout fermentation. The

overexpression cassette was amplified through PCR and integrated into the genome of the industrial wine yeast strains VIN13 and VIN13(p*ATF1*-s), in which the *ATF1* gene already was overexpressed. The integration of the overexpression cassette into VIN13 and VIN13(pATF1-s) was confirmed by Southern blot hybridisation. RT-PCR results also confirmed that the ATF1 gene was overexpressed in VIN13(pATF1s) and VIN13(pATF1-s, PJATF2oe). Unfortunately, attempts at RT-PCR for the *ATF2* gene were unsuccessful, even for the RT-PCR of the *ATF2* gene from untransformed VIN13. Various changes were made for optimisation, but none were successful.

The results showed that there was no significant increase in either ethyl acetate or isoamyl acetate when the *ATF2* gene was overexpressed. The result for ethyl acetate was expected, as the *ATF2* gene has not been implicated in ethyl acetate production (Mason and Dufour, 2000). However, it was surprising that there was no increase in isoamyl acetate. The results also showed a decrease in higher alcohols, which could mean that this gene produces other esters, but no measurement took place for these particular esters. GC analysis also showed a decrease in the amount of acetic acid produced by VIN13(pJATF2oe) in comparison to VIN13. This is favourable, as acetic acid contributes largely to volatile acidity, which is an unwanted characteristic, and has been associated with stuck fermentations. A wine yeast that produces less acetic acid may therefore be very useful in the commercial production of wine.

The results obtained for the wine yeast strain with both the *ATF1* and *ATF2* genes overexpressed were also interesting. This yeast did not produce high levels of ethyl acetate or isoamyl acetate, but, as in the case of the overexpression of the *ATF2* gene, showed decreases in higher alcohols, suggesting esters may be formed that were not measured. A decrease in the levels of ethyl acetate in comparison to the wine or brandy produced using VIN13(pATF1-s) was a positive result. Wine and brandy made using VIN13(pATF1-s) was described as potent and chemical due to high ethyl acetate and isoamyl acetate concentrations, therefore the decrease due to the overexpression of *ATF1* and *ATF2* is a positive result.

Tasting results also showed that there were differences among the wines and brandies produced with the modified strains. The tasting analysis showed low statistical significance, except for the characteristic of chemical. Although the statistical significance was low, the slight changes encourage further research on the modification of ester-producing genes in wine yeasts in order to manipulate the aroma profile.

4.2 CONCLUDING REMARKS AND FUTURE PROSPECTS

It is important for the winemaker or distiller to choose not only a suitable yeast for the production of the wine or brandy, but also to be selective in the choice of wood used for maturation. As discussed in chapter two, maturation in wood is important for the

quality of the wine and brandy, but it is also a sensitive process and must be monitored in order to extract the desired amounts of the many components found in the wood. It is also important for the winemaker or distiller to choose what type of toasting level is required for the maturation, as this factor results in significant changes in wood structure and therefore significant changes in extractives from the wood.

Many studies have shown the importance of esters in the aroma bouquet of wine and brandy. In this study, it was shown how the aroma profile of wine or brandy can change with a change in the expression of ester-producing genes. As previous research has shown (Mason and Dufour, 2001), the exact role of *ATF2* remains unclear. In this project, no significant increase in ethyl acetate or isoamyl acetate was observed when the *ATF2* gene was overexpressed in VIN13, therefore the function of the *ATF2* gene still remains unknown. However, there were other changes in the aroma bouquet and future studies will be needed to clarify the exact role of this gene.

Further studies on the regulation of the *ATF1* and *ATF2* genes will be needed for an understanding of what happens during the fermentation process when both these genes are overexpressed. In this study, it was observed that the ester production in a yeast overexpressing both the *ATF1* and *ATF2* genes was not as high as in a yeast overexpressing only the *ATF1* gene. Regulation studies may clarify what happens in the metabolic pathways to cause this loss of high ester production.

Future studies on the aroma differences between a control strain and a double disruption of the *ATF1* or *ATF2* gene would be most interesting and would continue to provide more information on the exact role of these yeasts in the production of esters in wine and brandy. Further studies may also include the manipulation of gene expression through the use of various promoters. This could result in the winemaker choosing when in the fermentation process he/she would like the esters to be produced, thus giving him or her more control over the final product.

Winemaking still remains a blend of art and science. However, as knowledge on the biology of human perception of flavour preferences increase, so wines will become more targeted to the genetic differences of the consumer (Bisson *et al.*, 2002). Through the use of modern techniques winemakers and distillers can make a product that is more to the olfactory profile of the consumer.

"The biochemistry of fermentation not only beautifully illuminates and clarifies the ancient art of making wine, but also explains and makes possible a calculated control of many of the "mysteries" which baffled the artisan winemaker. Aesthetics, however, is still a part of (and should not be displaced from) wine production and wine appreciation, but mystique should not remain if knowledge can be substituted" (Amerine and Singleton, 1977).

4.3 LITERATURE CITED

Amerine MA, Singleton VL. 1977. Alcoholic fermentation. In *Wine, an introduction*. University of California Press, Ltd: London; 74

Bisson LF, Waterhouse AL, Ebeler SE, Walker MA, Lapsley JT. 2002. The present and future of the international wine industry. *Nature* **418:**696-699.

Engan S. 1974. Esters in beer. *The Brew Dig* **49:**40-48.

Fujii T, Nagasawa N, Iwamatsu A, Bogaki T, Tamai Y, Hamachi M. 1994. Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. *Appl Environ Microbiol* **60:**2786-2792.

Fujii T, Kobayashi O, Yoshimoto H, Furukawa S, Tamai Y. 1997. Effect of aeration and unsaturated fatty acids on expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase gene. *Appl Environ Microbiol* **63:**910-915.

Malcorps P, Dufour JP. 1992. Short-chain and medium-chain aliphatic-ester synthesis in *Saccharomyces cerevisiae*. *Eur J Biochem* **210:**1015-1022.

Mason AB, Dufour JP. 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* **16:**1287-1298.

Nagasawa N, Bogaki T, Iwamatsu A, Hamachi M, Kumagai C. 1998. Cloning and nucleotide sequence of the alcohol acetyltransferase gene (*ATF2*) *from Saccharomyces cerevisiae* Kyokai No. 7. *Biosci Biotechnol Biochem* **62:**1852-1857.

Lambrechts MG, Pretorius IS. 2000. Yeast and its importance to wine aroma – a review. *S Afr J Enol Vitic* **21:**97-129.

Lilly M, Lambrechts MG, Pretorius IS. 2000. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Appl Environ Microbiol* **66:**744-753.

Pretorius IS. 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16:**675-729.

CHAPTER 5

ADDENDUMS

ADDENDUM A

HISTOGRAMS OBTAINED FROM WINE TASTING

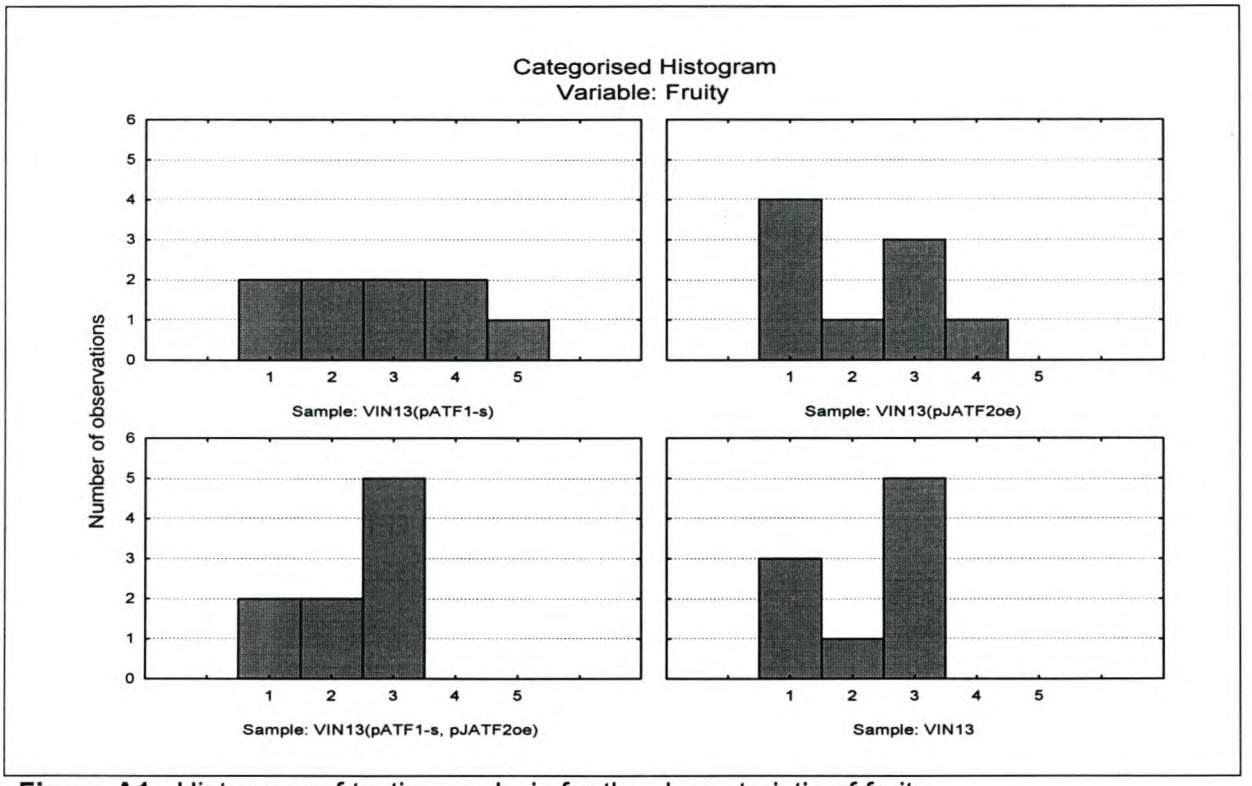


Figure A1. Histogram of tasting analysis for the characteristic of fruity

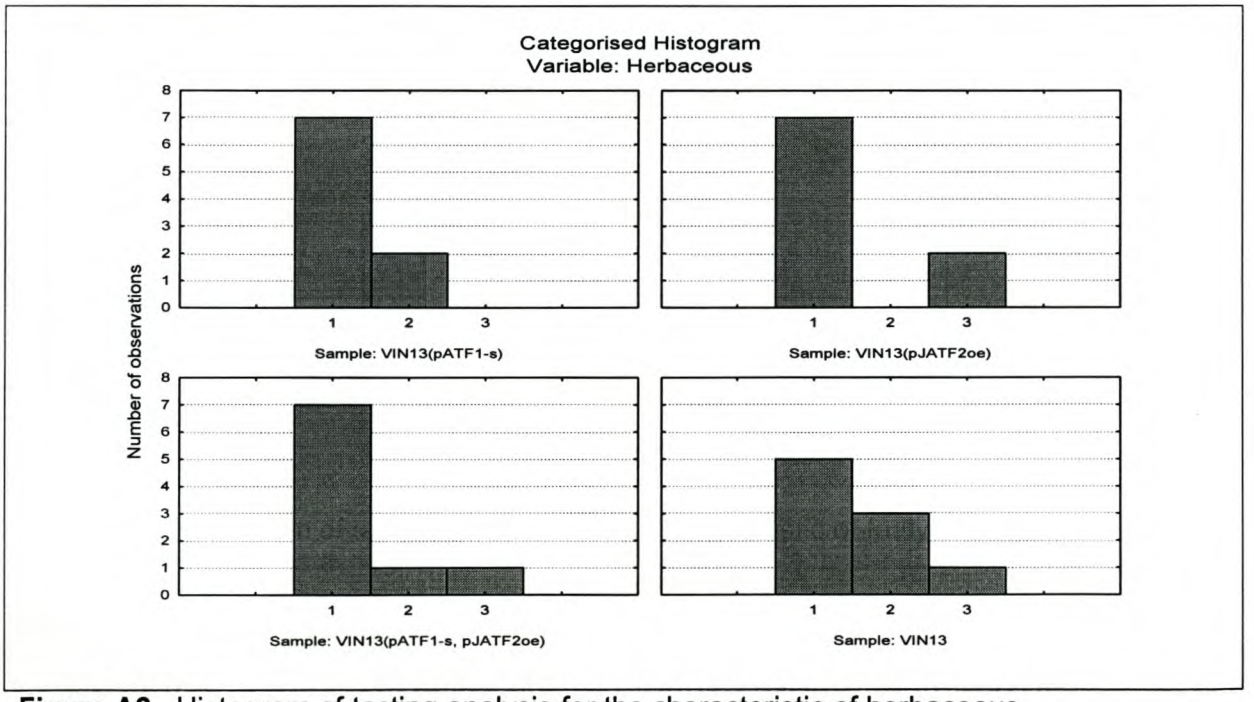


Figure A2. Histogram of tasting analysis for the characteristic of herbaceous

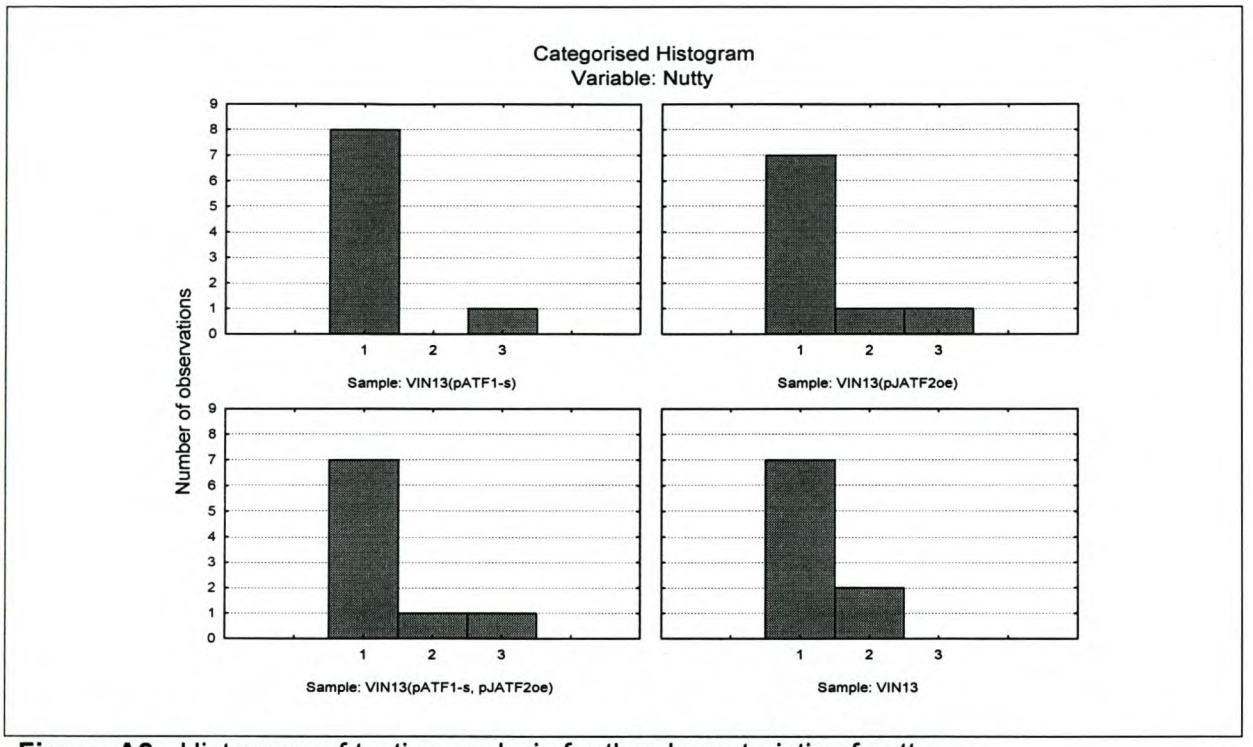


Figure A3. Histogram of tasting analysis for the characteristic of nutty

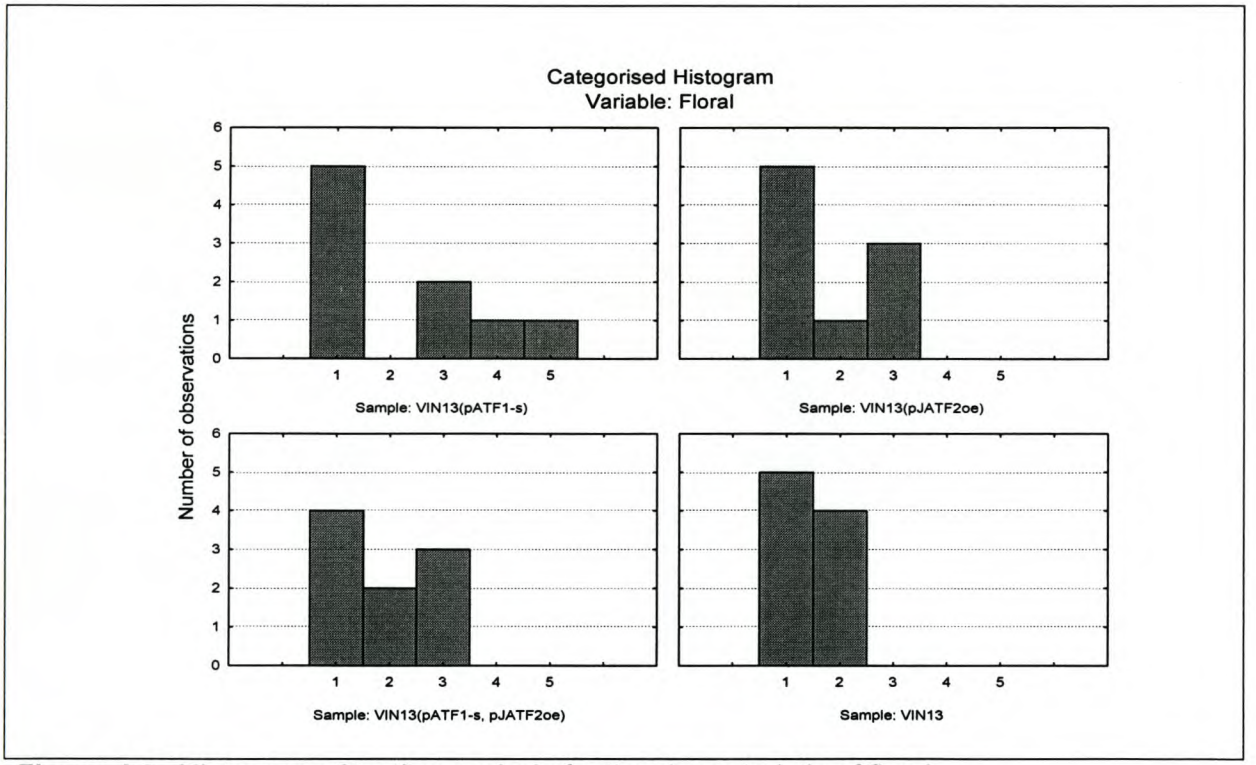


Figure A4. Histogram of tasting analysis for the characteristic of floral

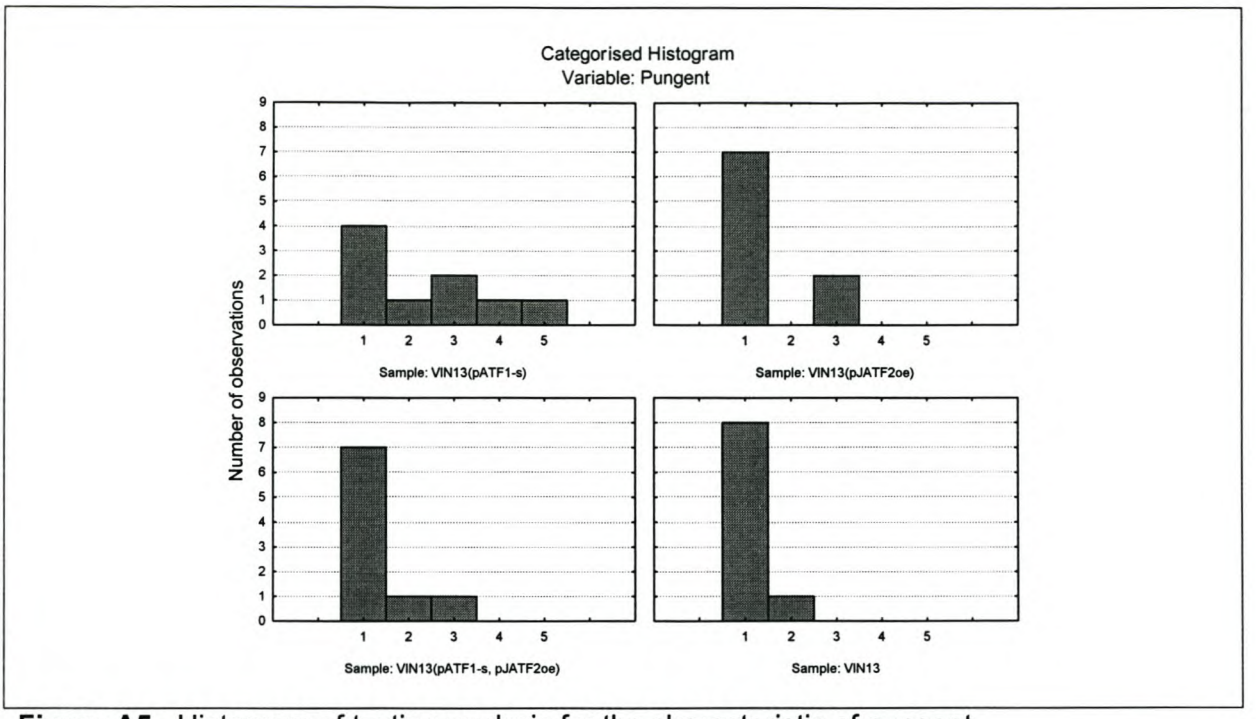


Figure A5. Histogram of tasting analysis for the characteristic of pungent

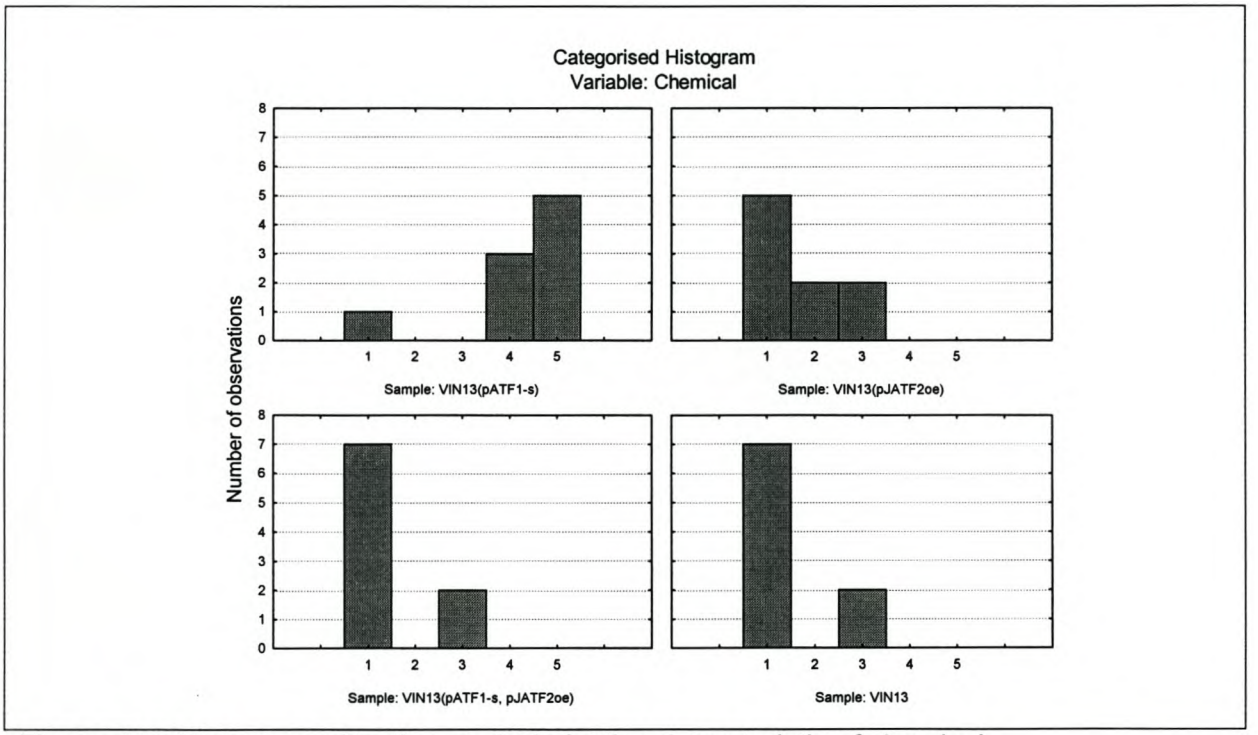


Figure A6. Histogram of tasting analysis for the characteristic of chemical

ADDENDUM B

HISTOGRAMS OBTAINED FROM BRANDY TASTING

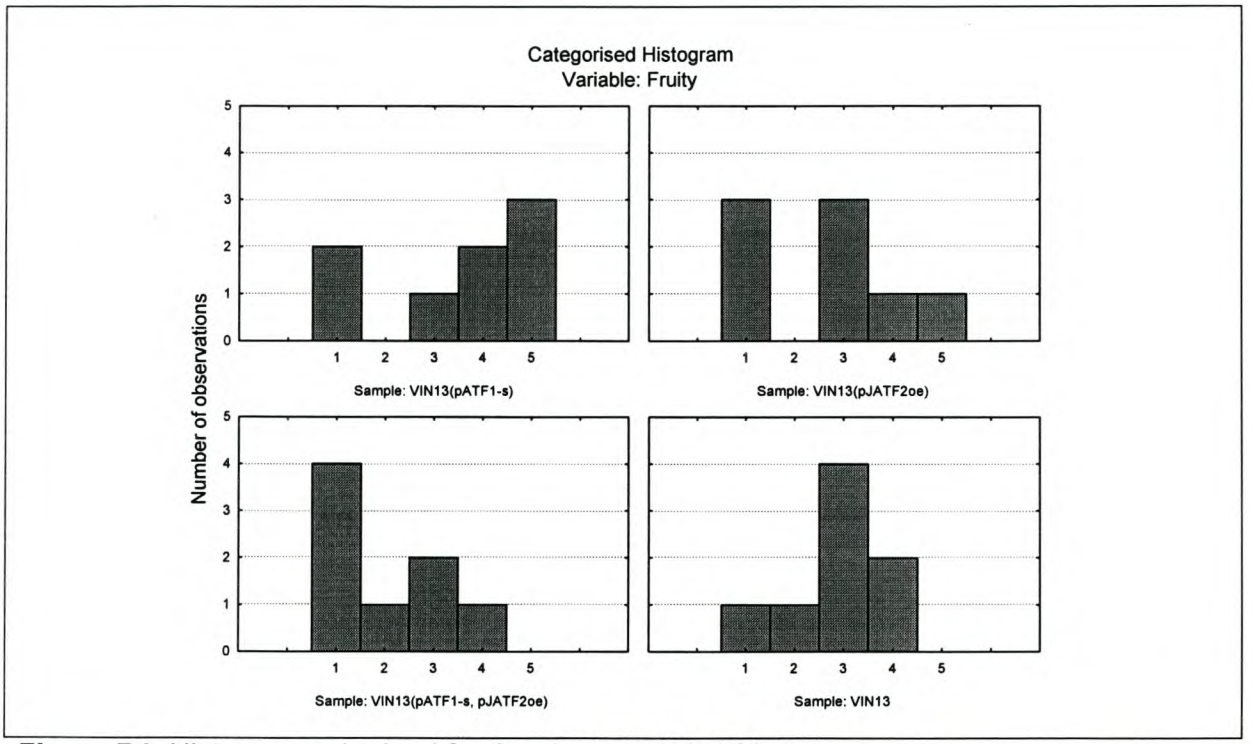


Figure B1. Histograms obtained for the characteristic of fruity

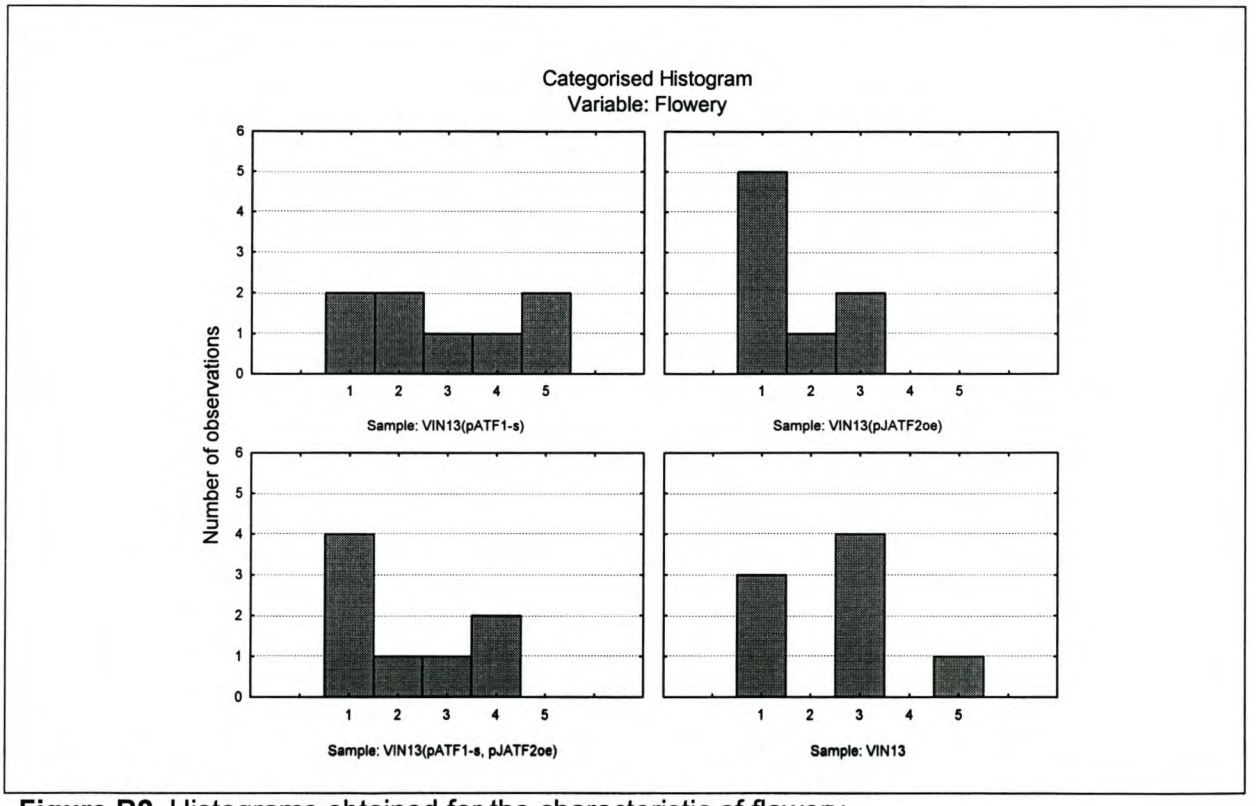


Figure B2. Histograms obtained for the characteristic of flowery

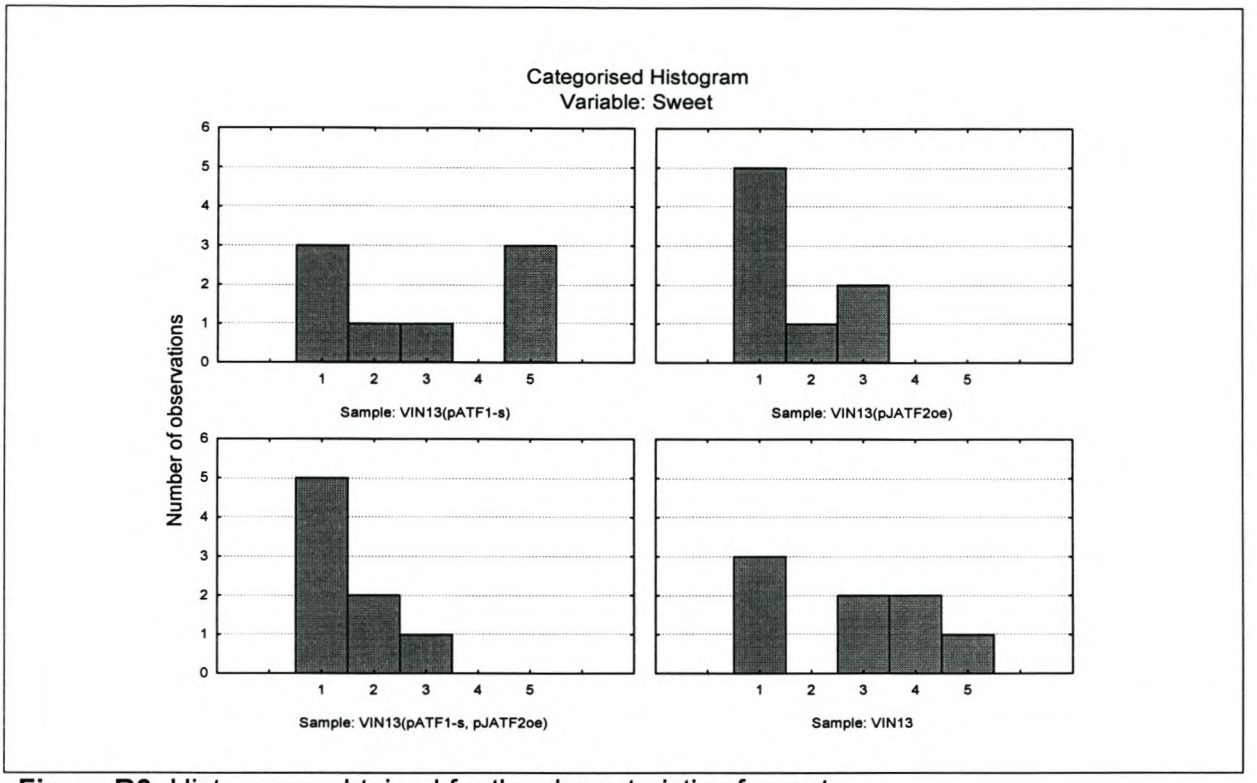


Figure B3. Histograms obtained for the characteristic of sweet

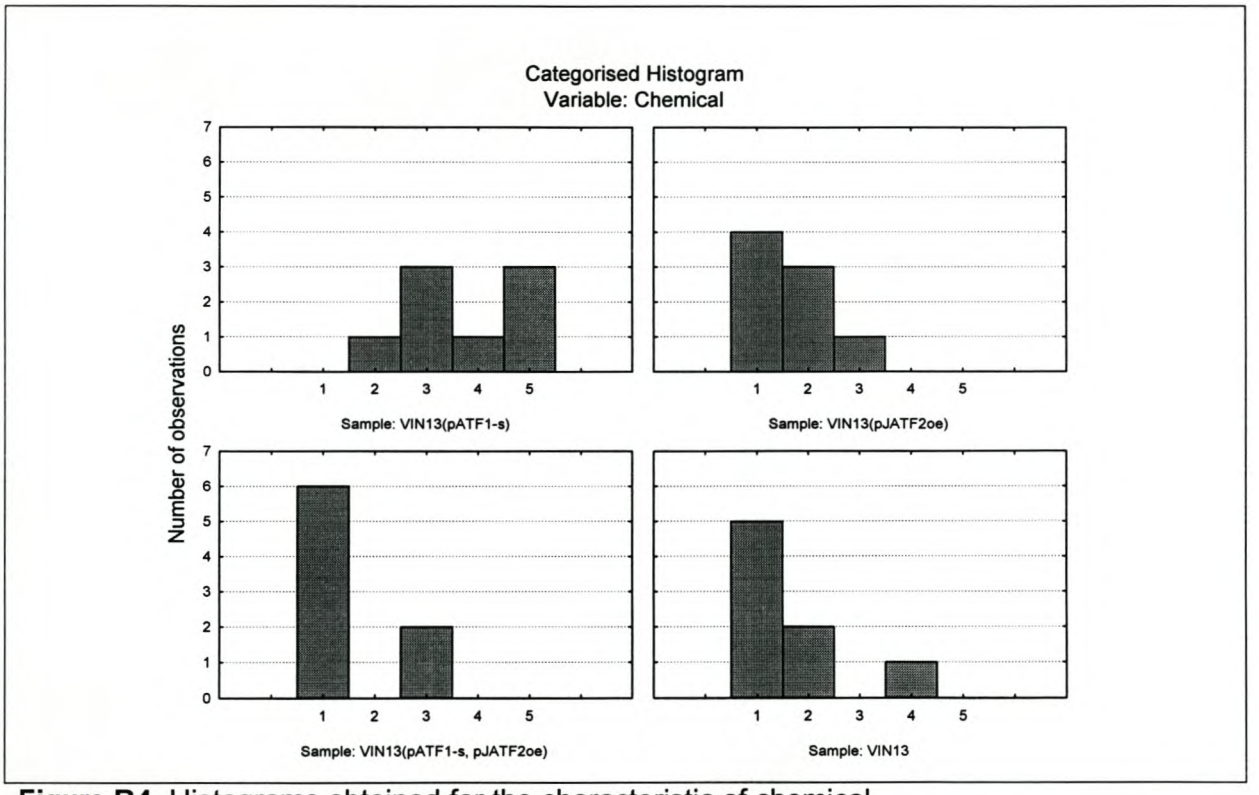


Figure B4. Histograms obtained for the characteristic of chemical

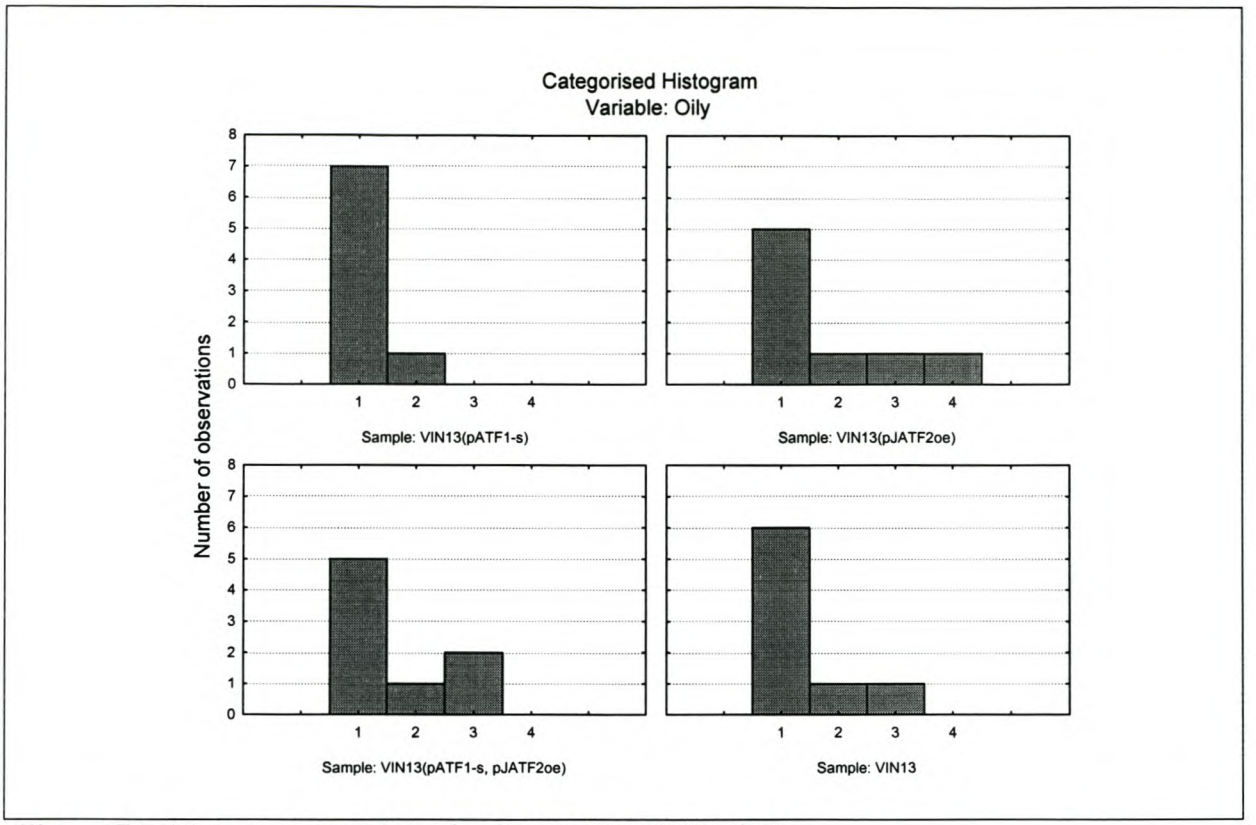


Figure B5. Histograms obtained for the characteristic of oily

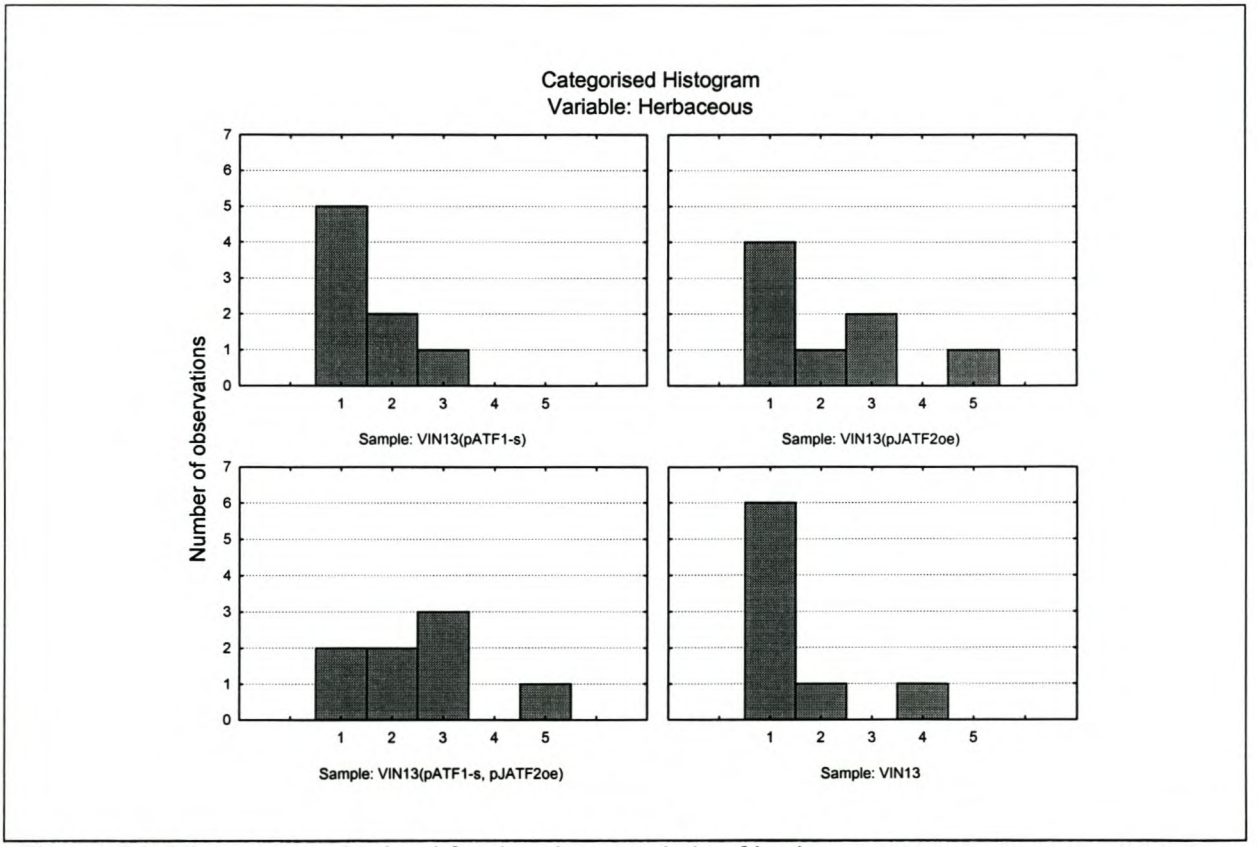


Figure B6. Histograms obtained for the characteristic of herbaceous

ADDENDUM C

ANOVA RESULTS OF COMPONENTS IDENTIFIED IN GC ANALYSIS OF WINE

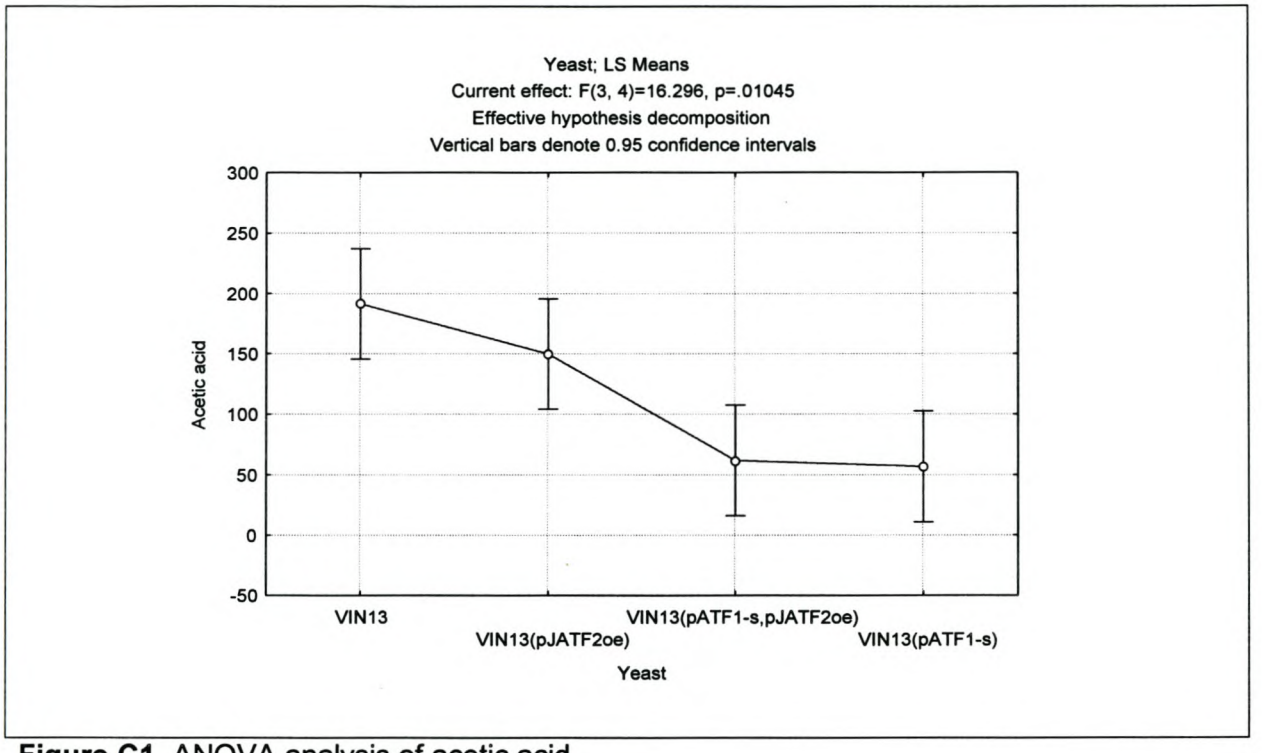


Figure C1. ANOVA analysis of acetic acid

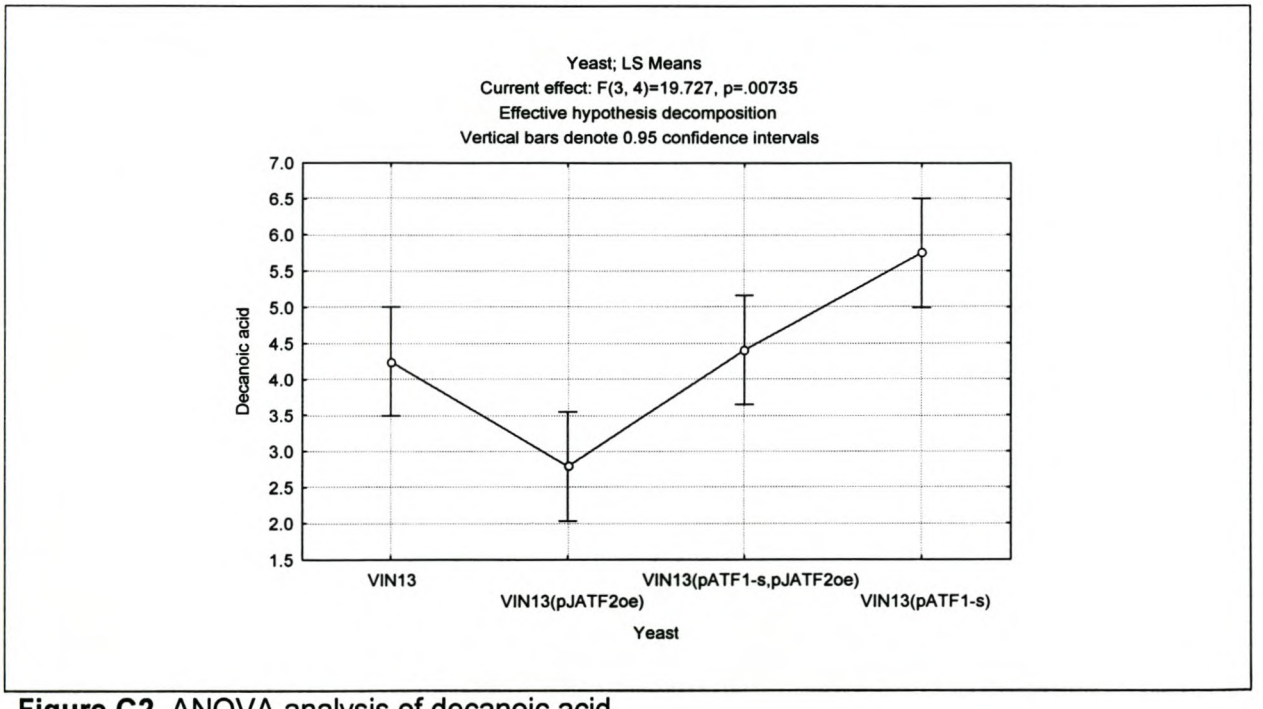


Figure C2. ANOVA analysis of decanoic acid

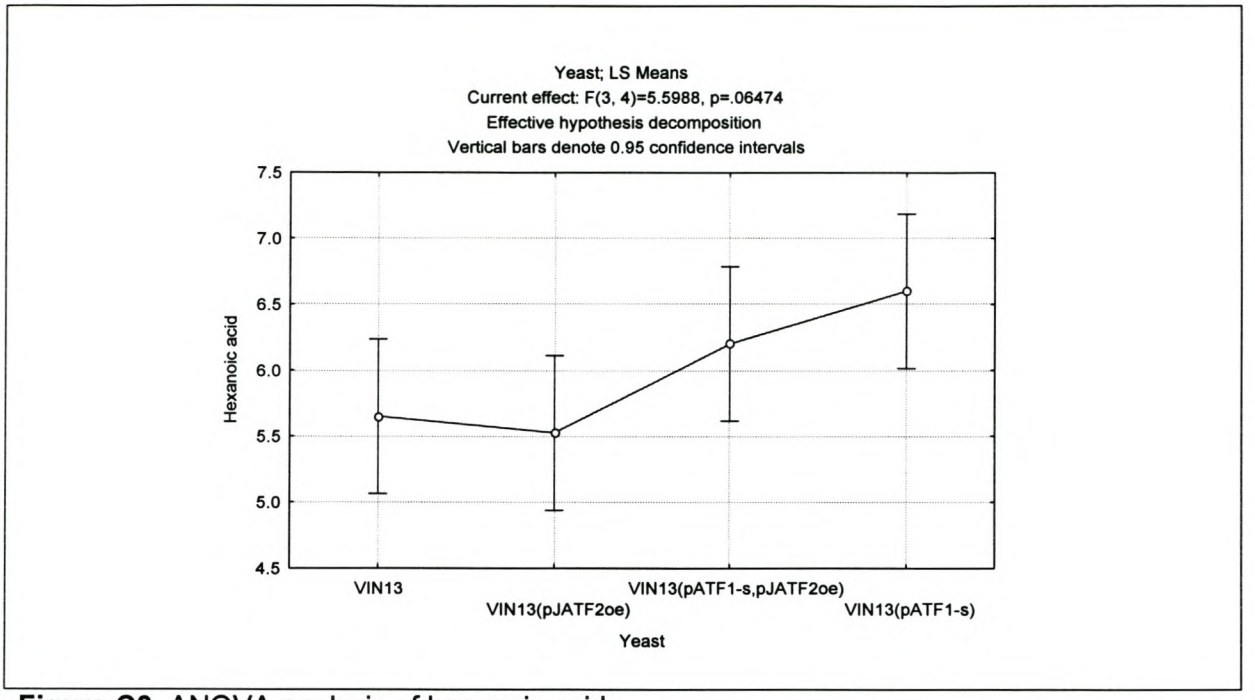


Figure C3. ANOVA analysis of hexanoic acid

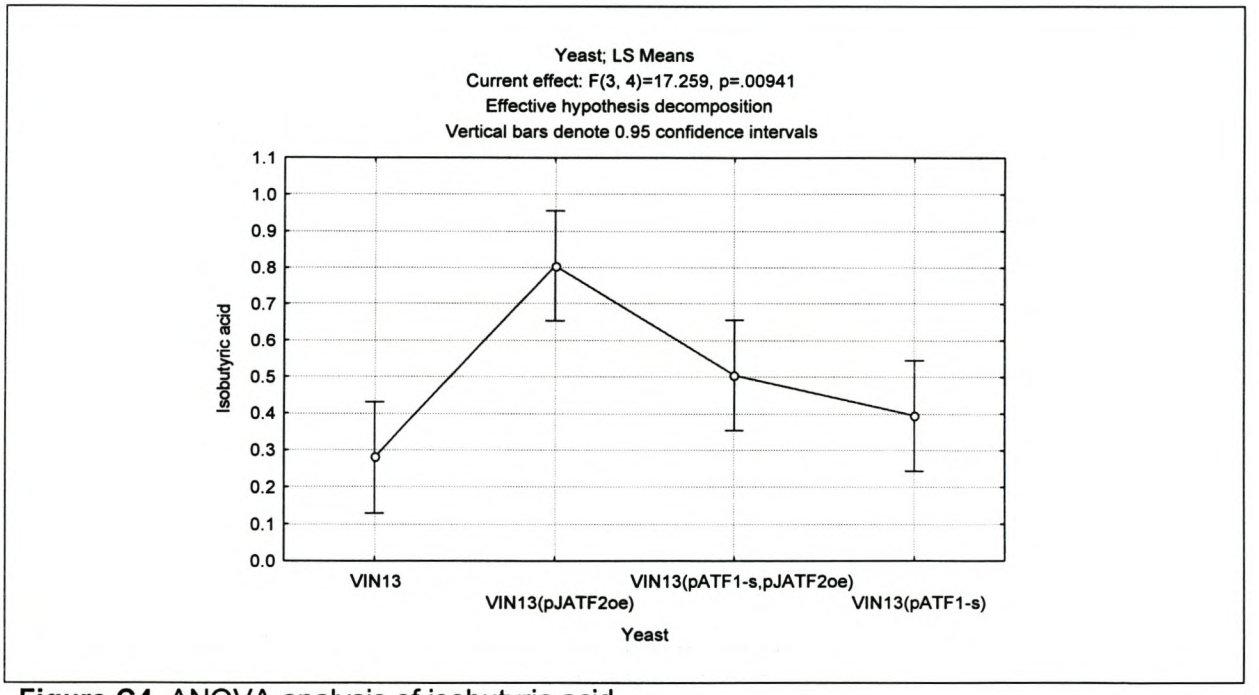


Figure C4. ANOVA analysis of isobutyric acid

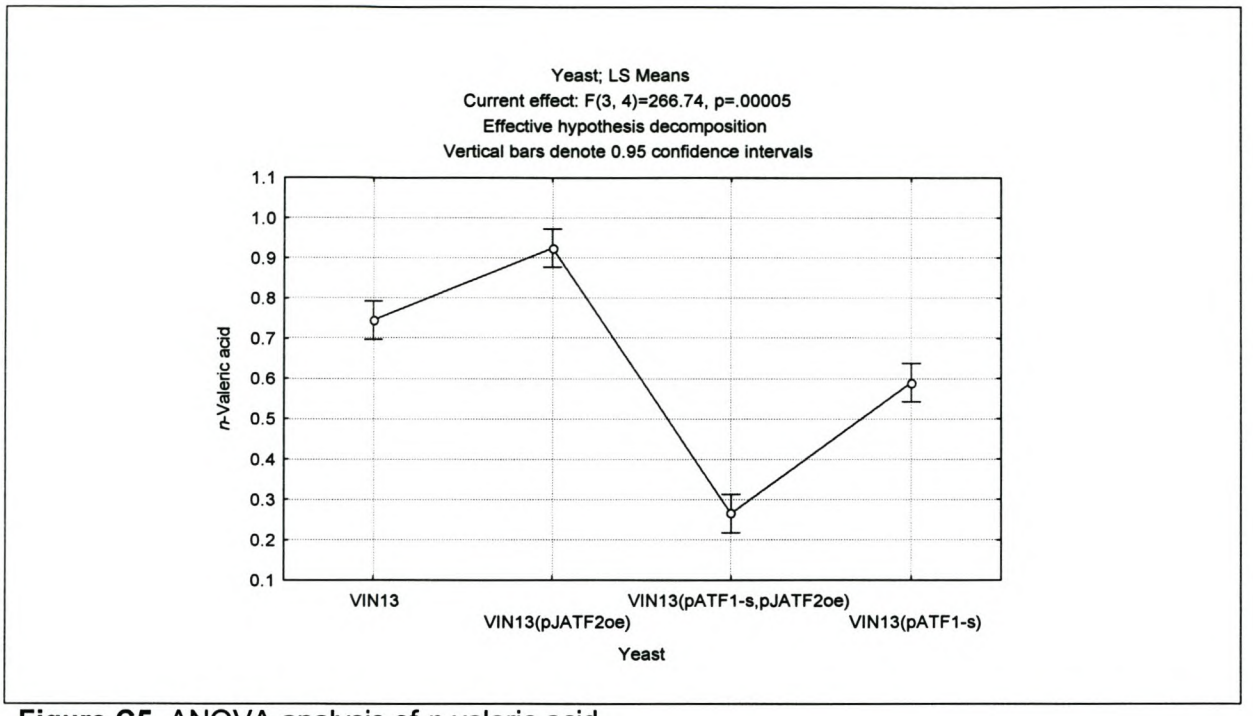


Figure C5. ANOVA analysis of *n*-valeric acid

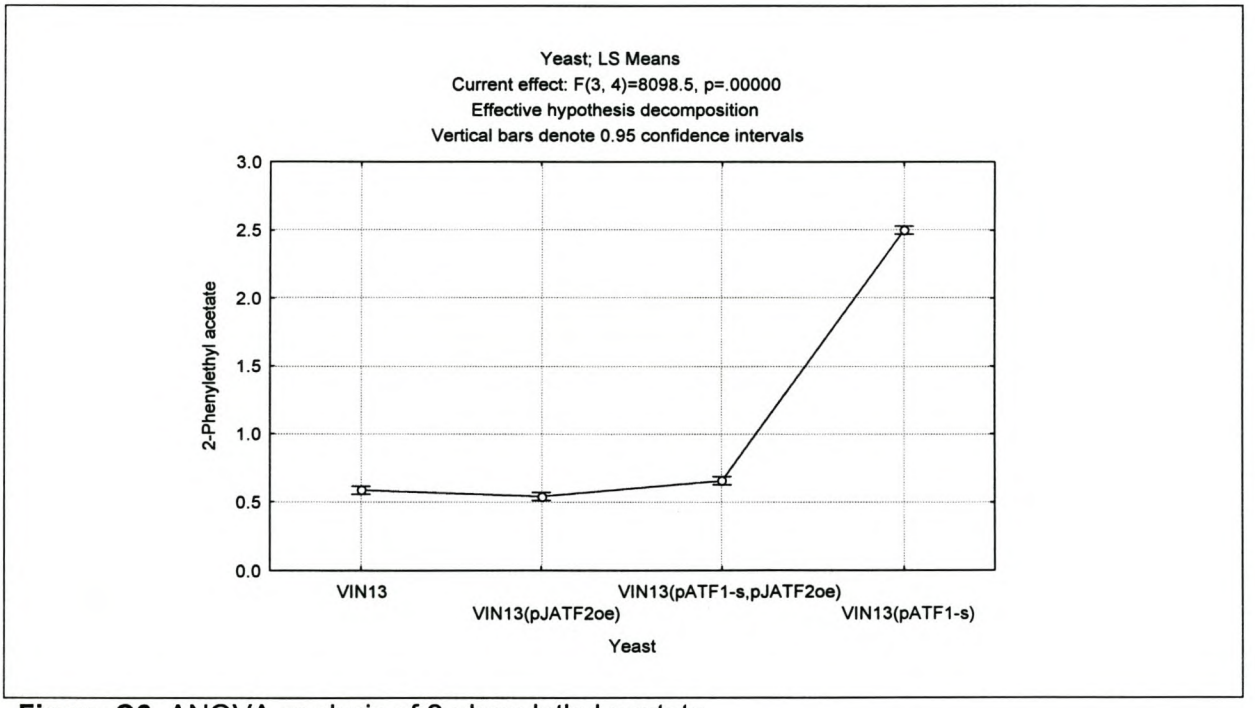


Figure C6. ANOVA analysis of 2-phenylethyl acetate

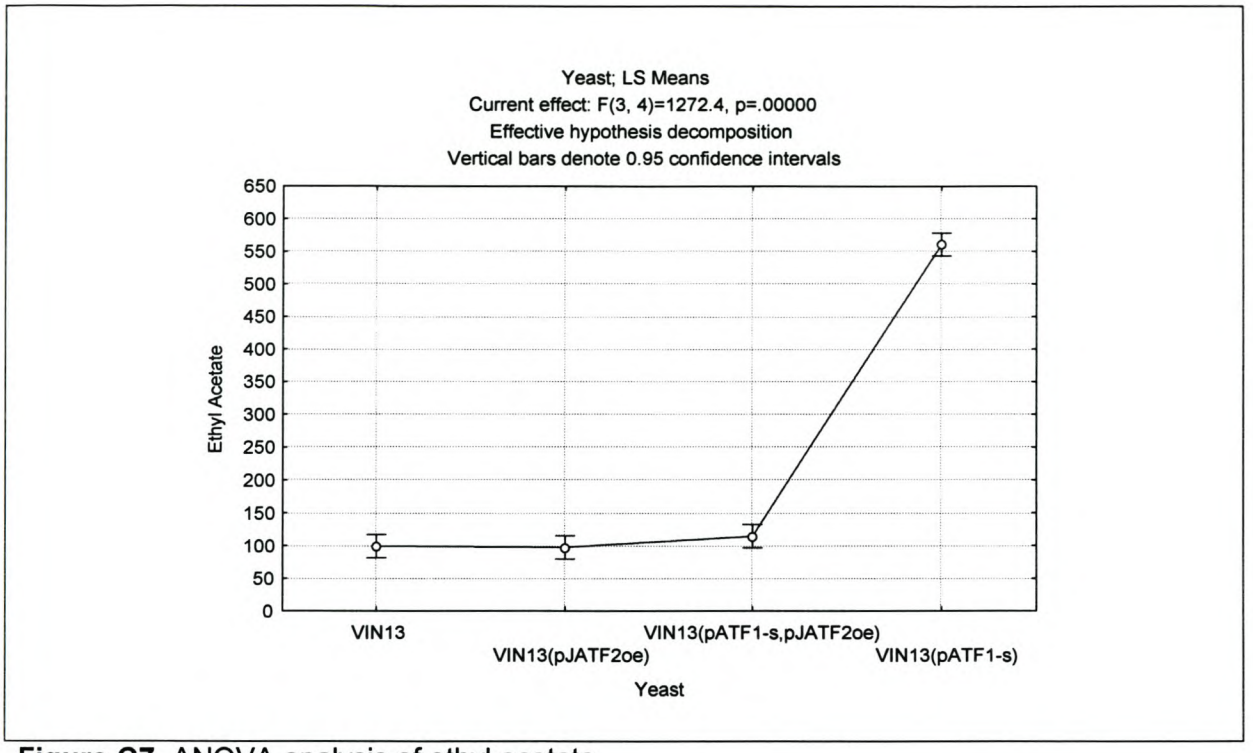


Figure C7. ANOVA analysis of ethyl acetate

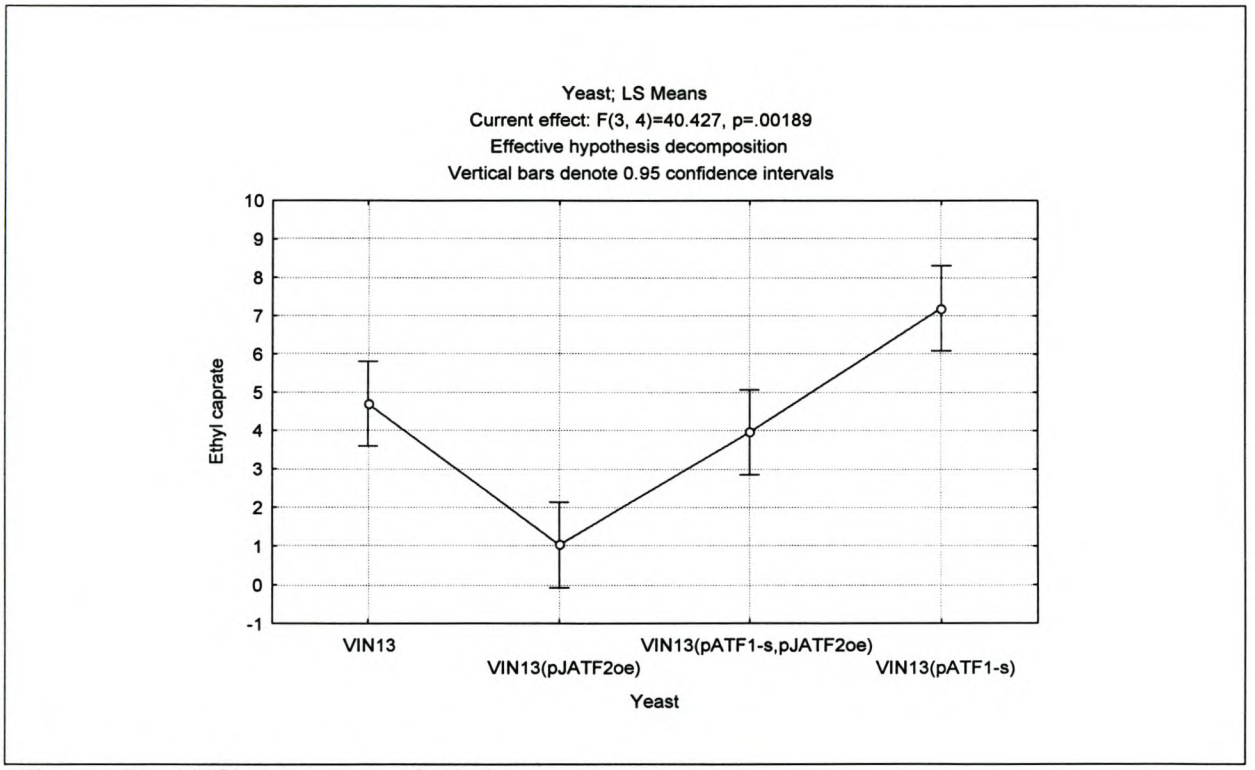


Figure C8. ANOVA analysis of ethyl caprate

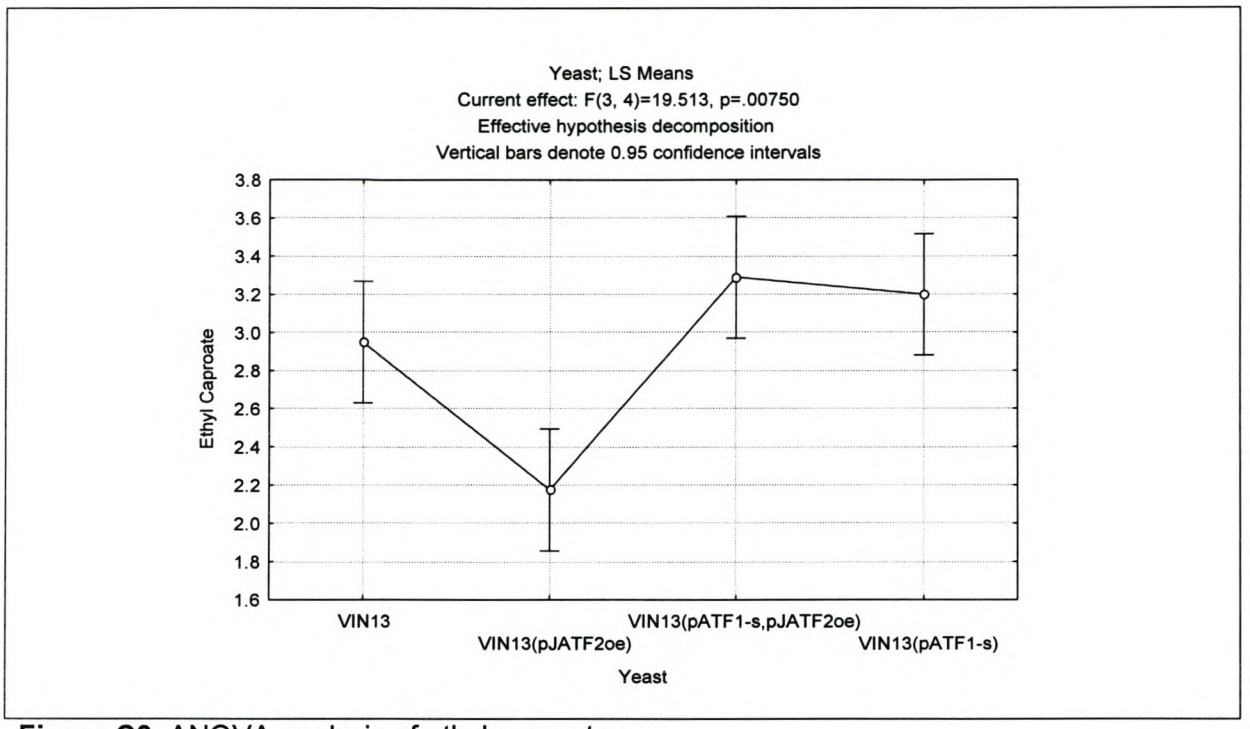


Figure C9. ANOVA analysis of ethyl caproate

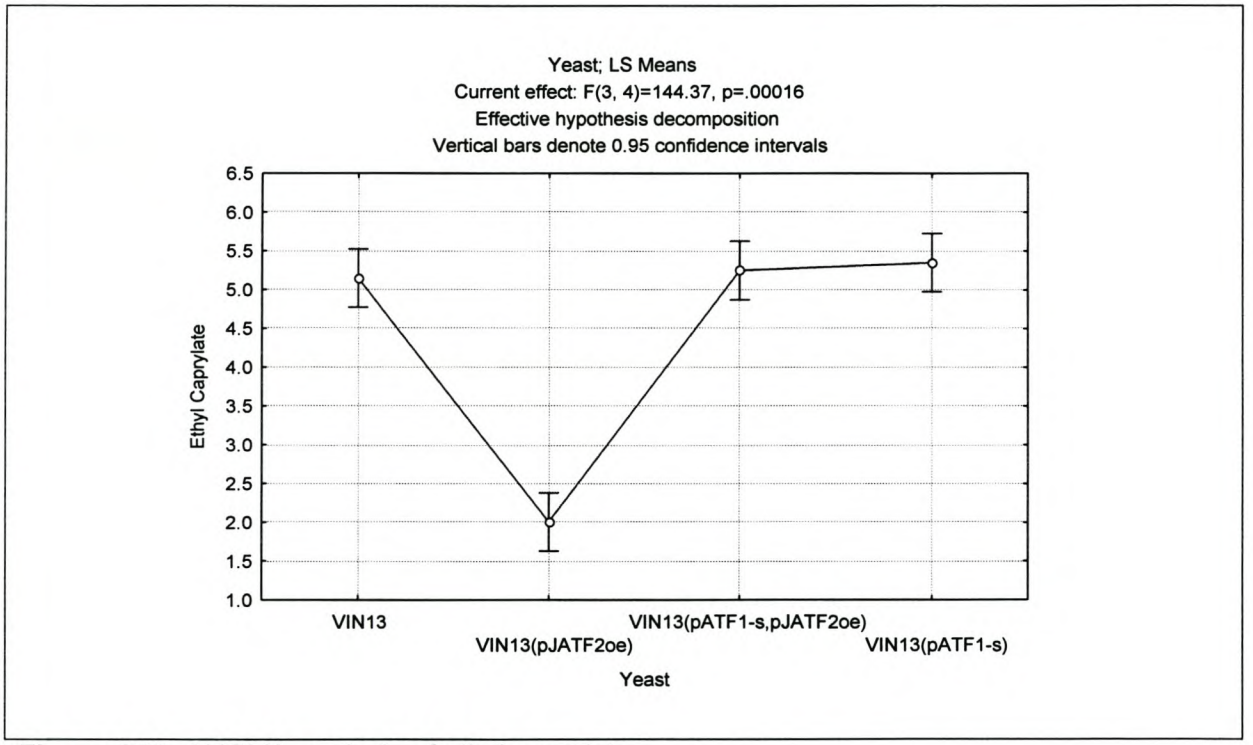


Figure C10. ANOVA analysis of ethyl caprylate

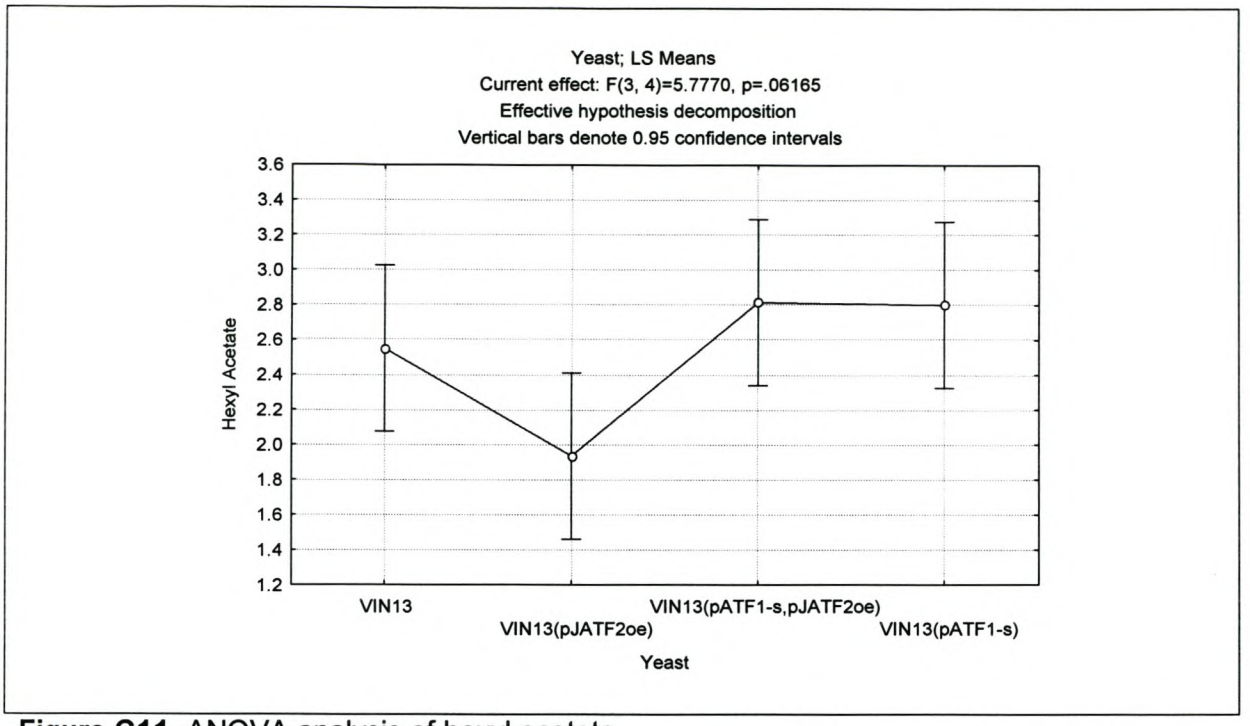


Figure C11. ANOVA analysis of hexyl acetate

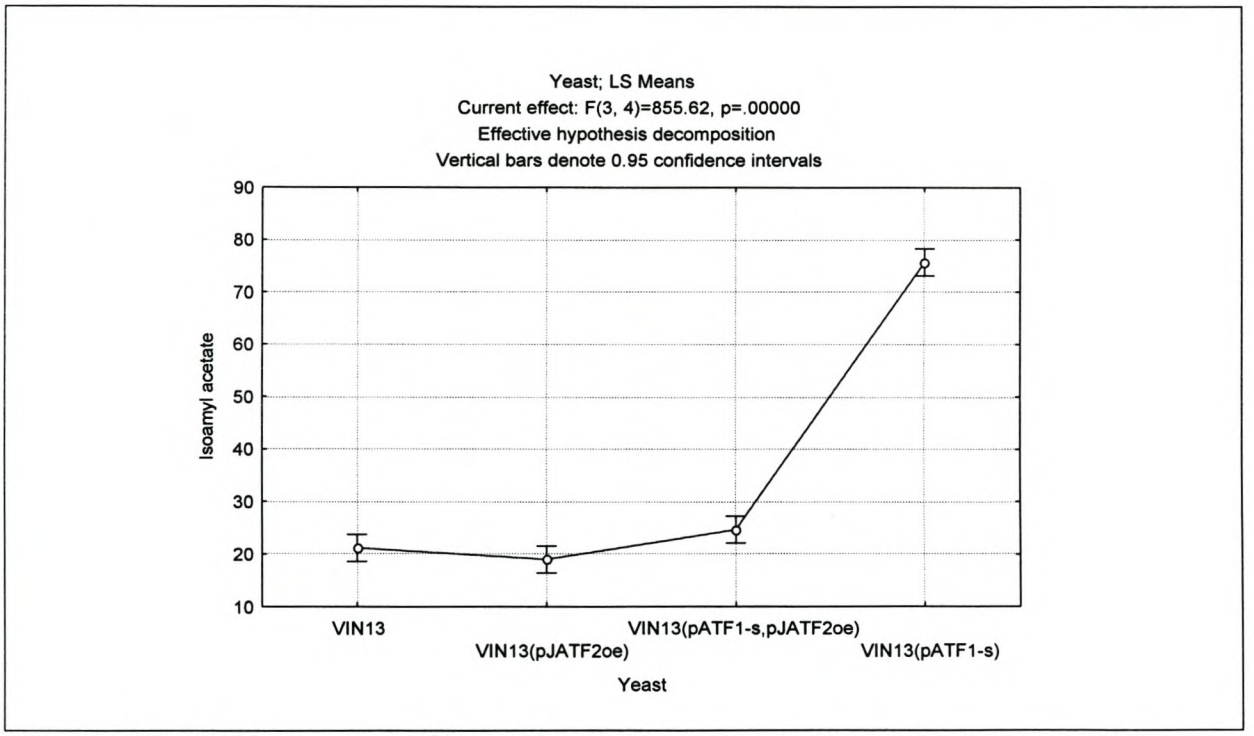


Figure C12. ANOVA analysis of isoamyl acetate

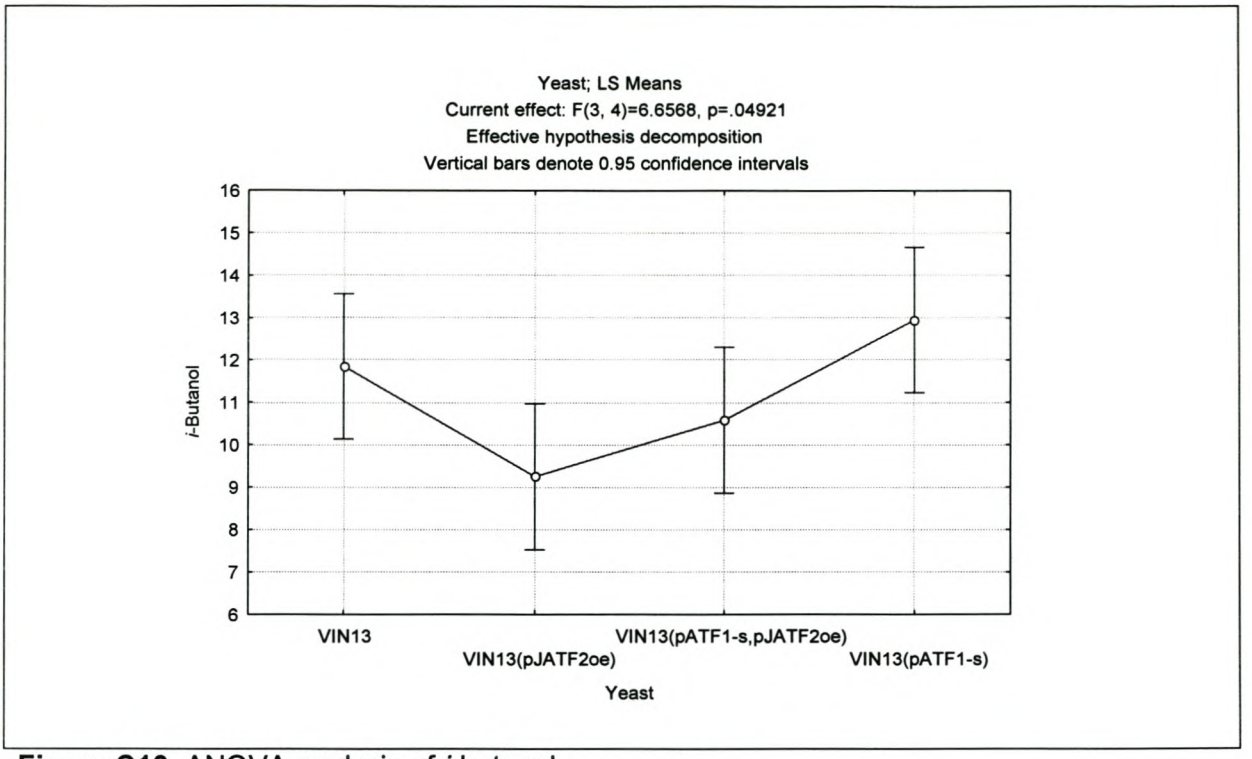


Figure C13. ANOVA analysis of *i*-butanol

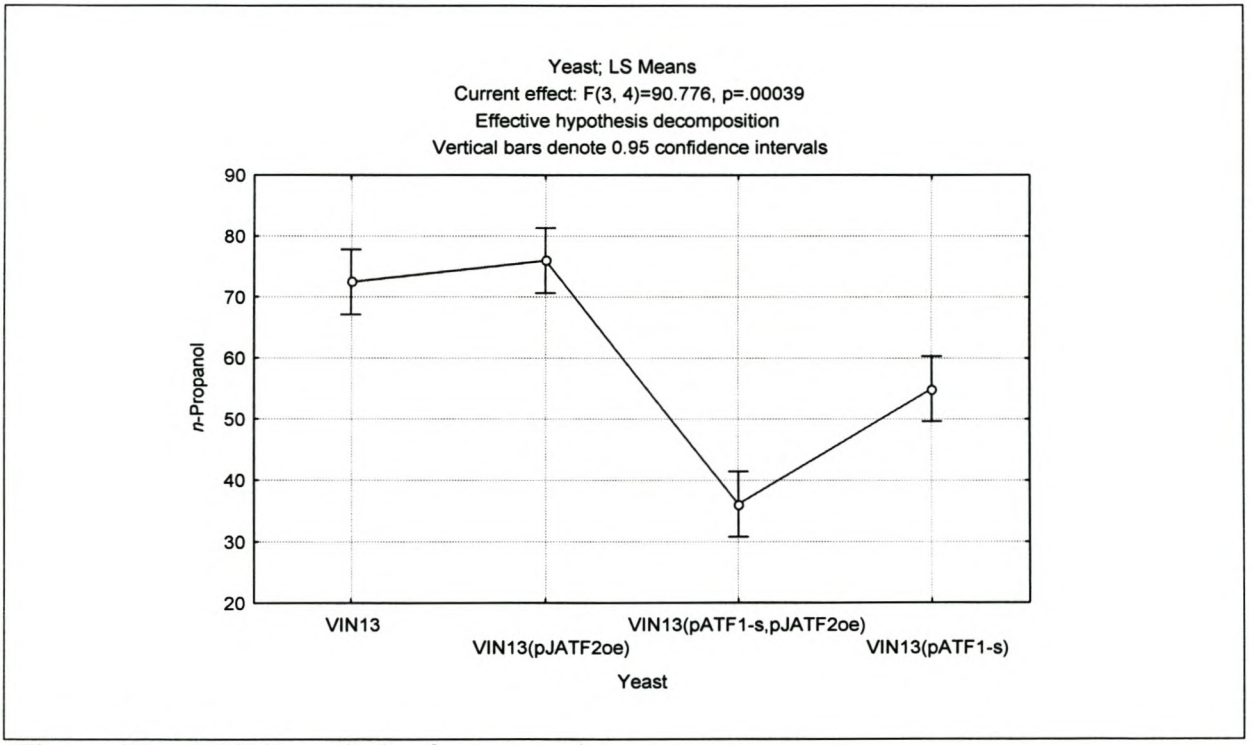


Figure C14. ANOVA analysis of *n*-propanol

ADDENDUM D

ANOVA RESULTS OF COMPONENTS IDENTIFIED IN GC ANALYSIS OF BRANDY

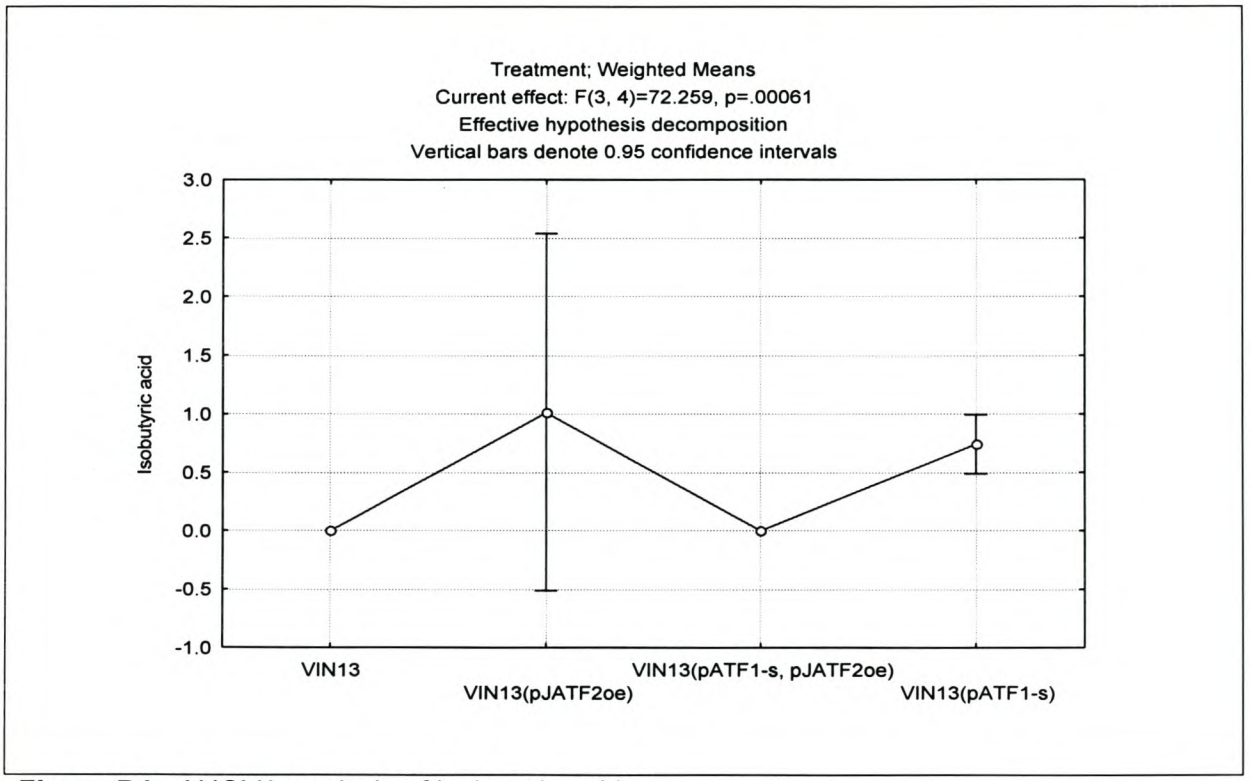


Figure D1. ANOVA analysis of isobutyric acid

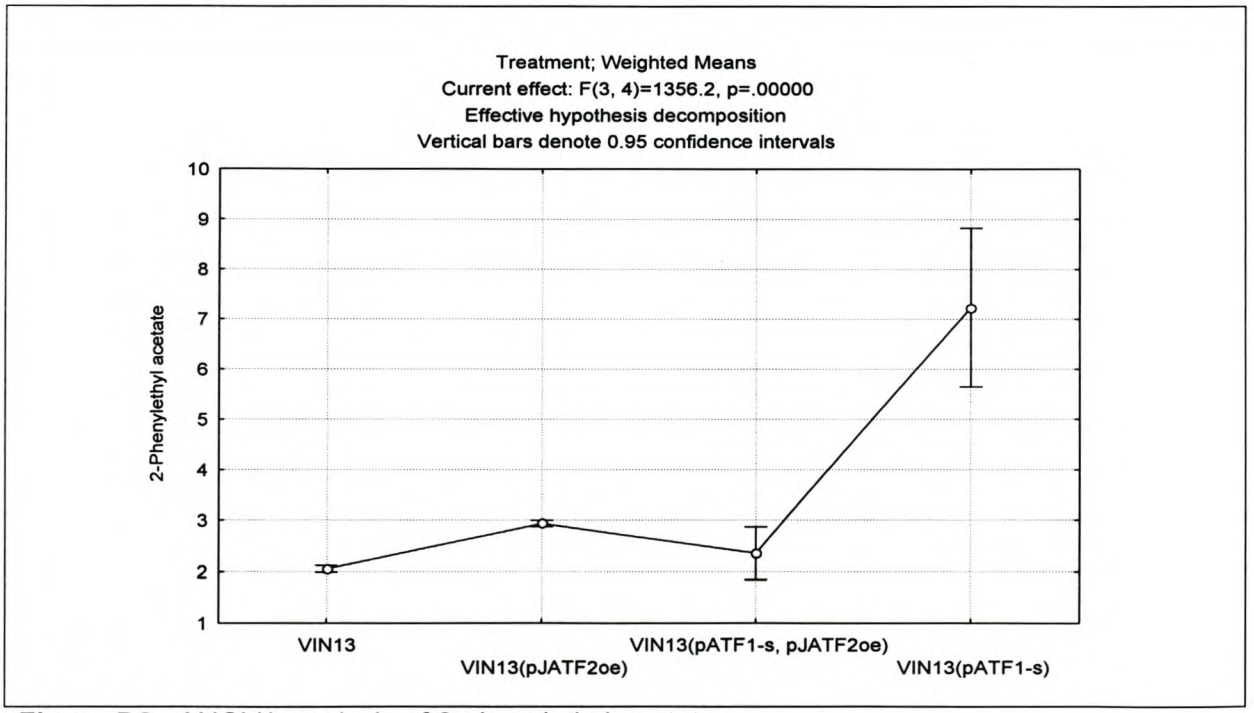


Figure D2. ANOVA analysis of 2-phenylethyl acetate

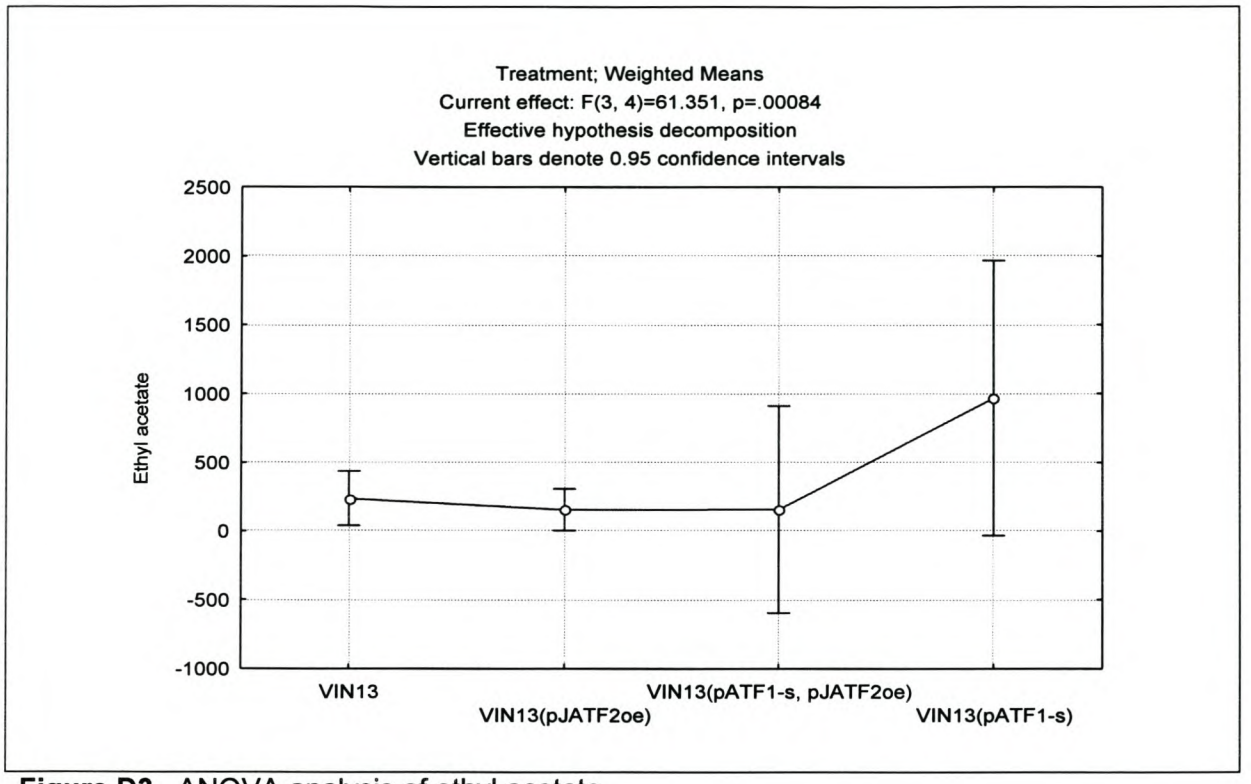


Figure D3. ANOVA analysis of ethyl acetate

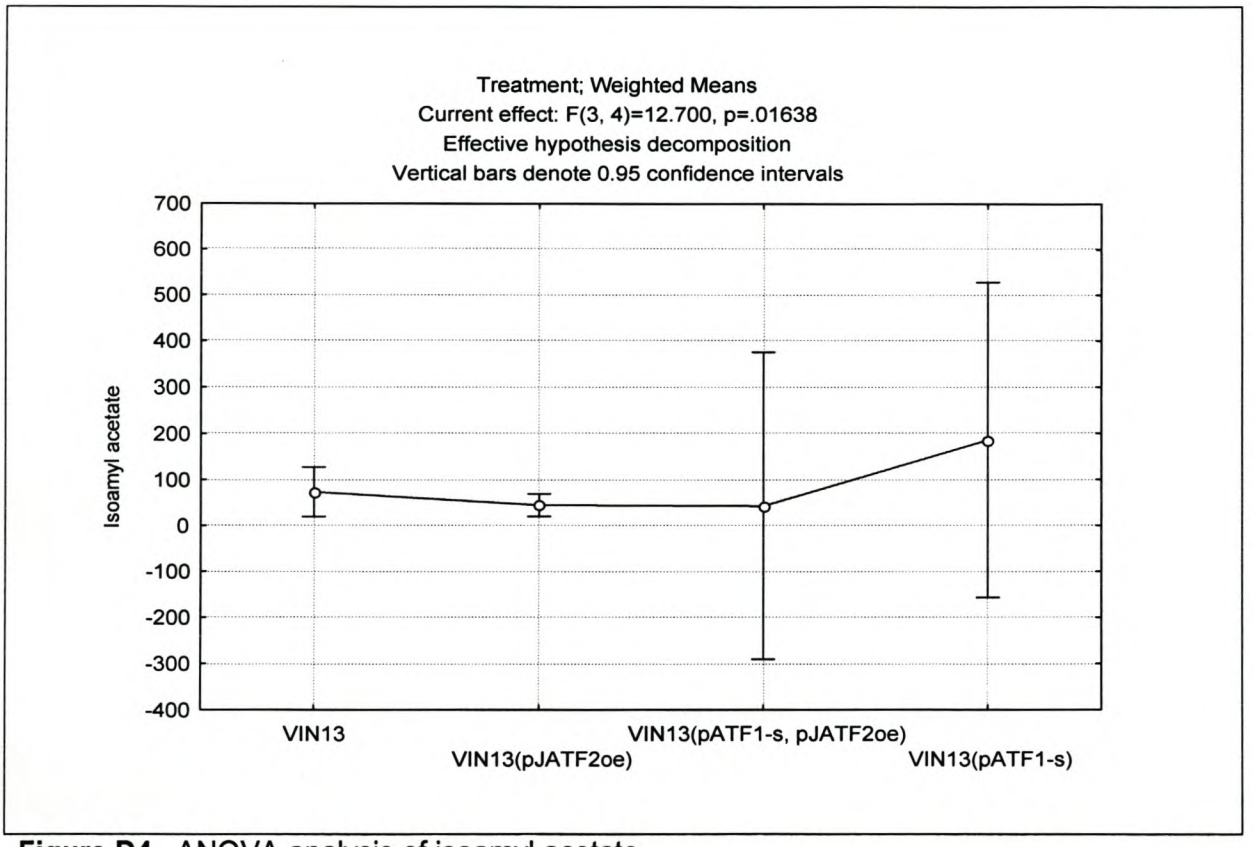


Figure D4. ANOVA analysis of isoamyl acetate

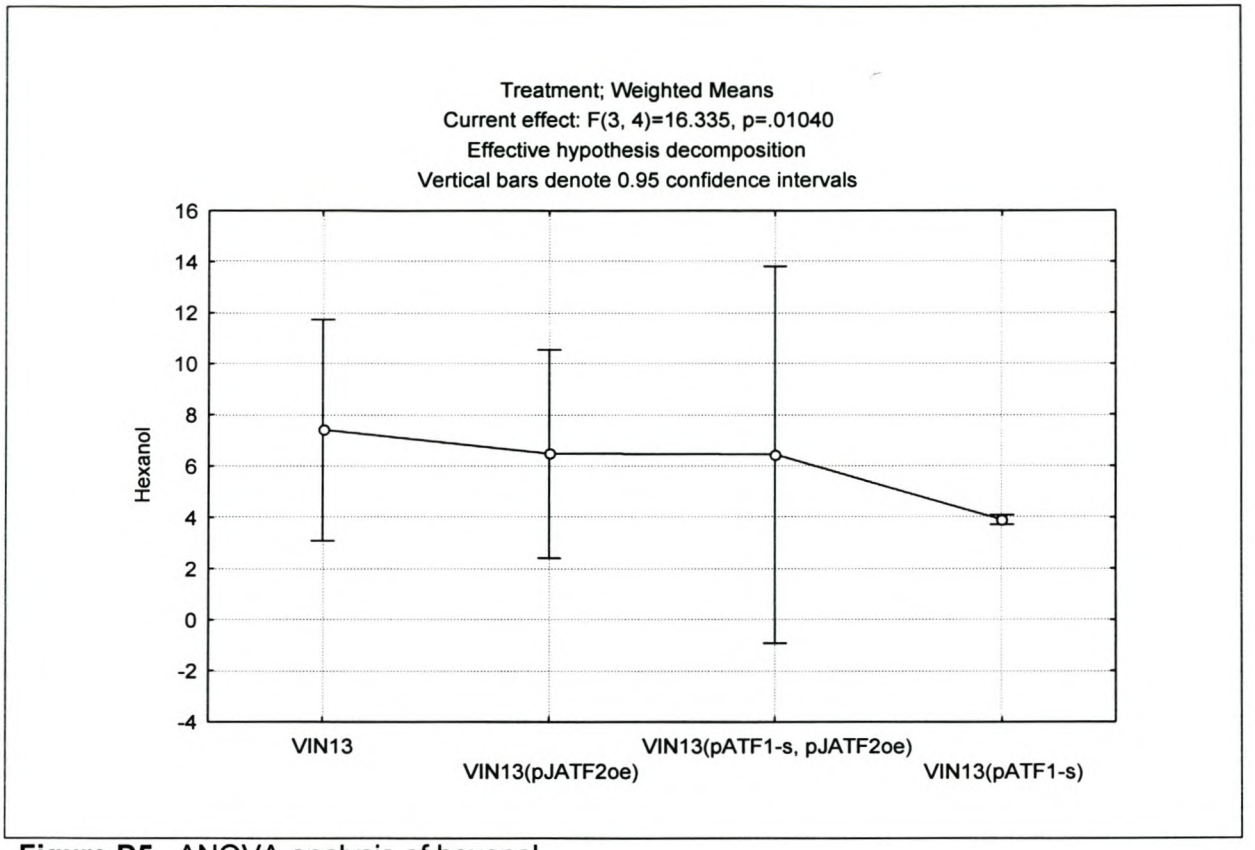


Figure D5. ANOVA analysis of hexanol

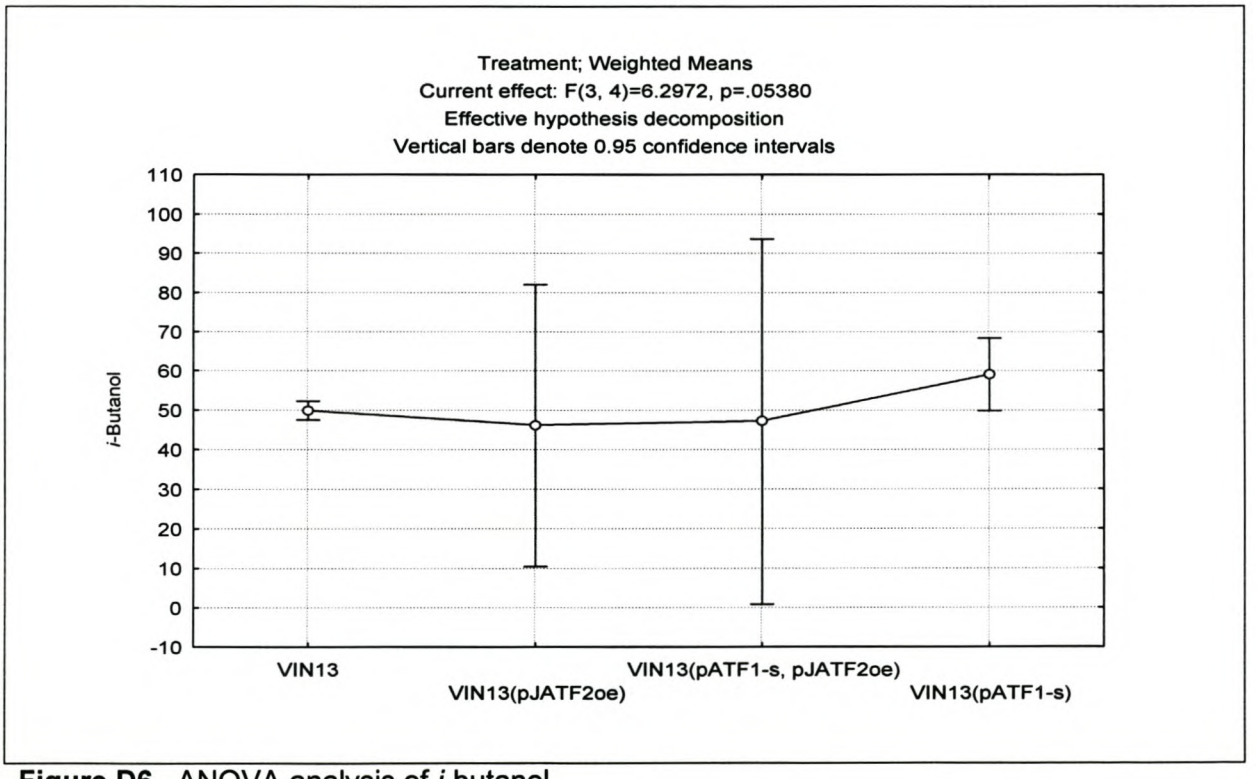


Figure D6. ANOVA analysis of *i*-butanol

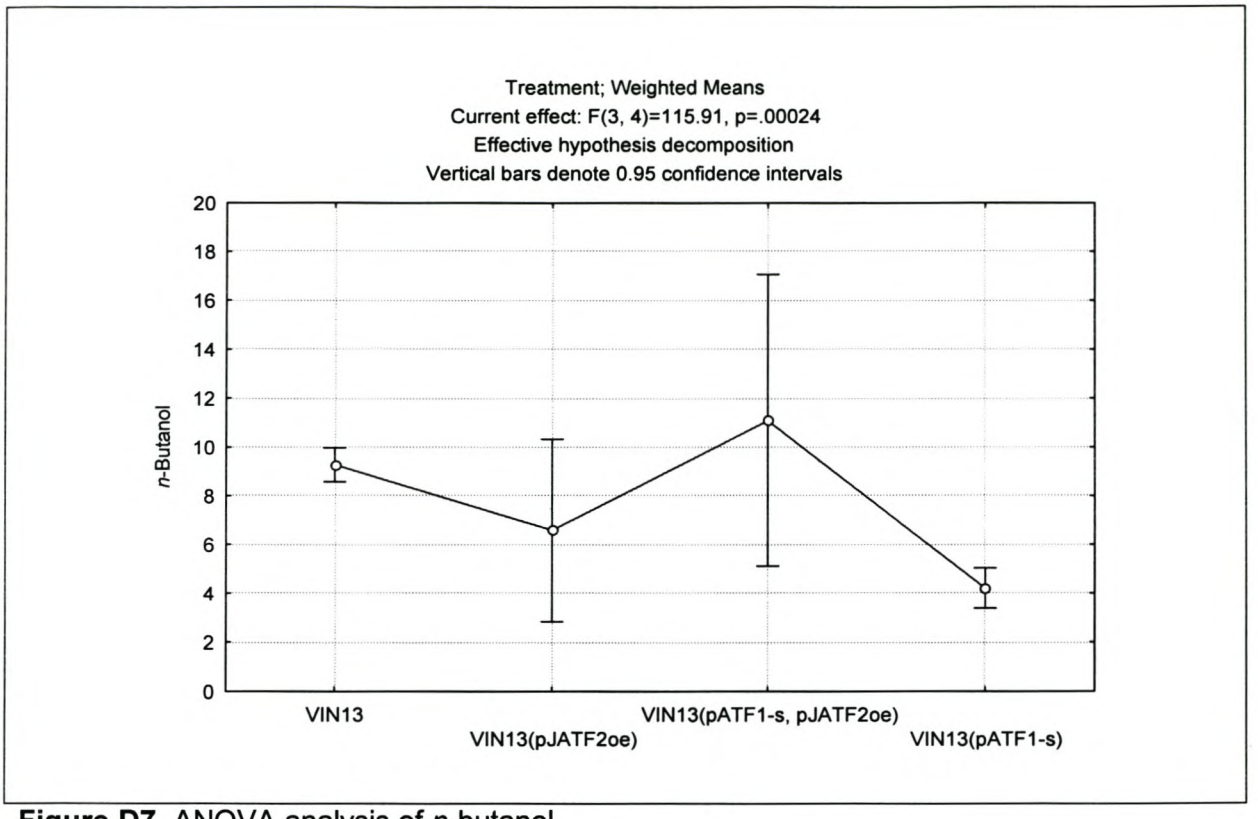


Figure D7. ANOVA analysis of *n*-butanol

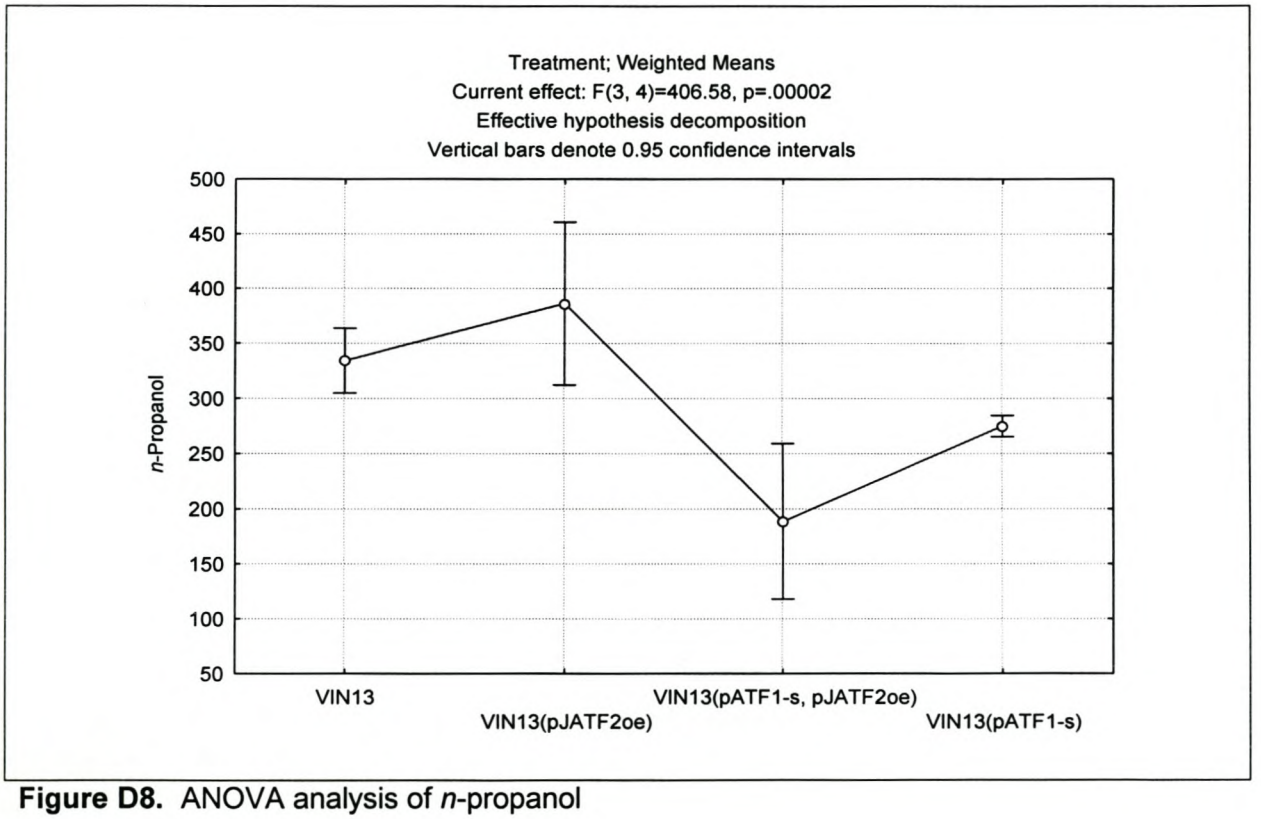


Figure D8. ANOVA analysis of *n*-propanol