

Optimization of fermentation processes for the production of indigenous fruit wines (Marula)

by

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

March 2001

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DECLARATION

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

Ms M. Fundira

SUMMARY

The importance of indigenous fruit wines is not well researched and documented. There is a need to develop and exploit these valuable food resources through improved production practices, storage, preservation and utilization technologies. The marula fruit is beneficial in many ways, it can be used for making juice, jam, beer or can be eaten as a whole fruit. The highly nutritive nature of the fruit, its distinctive tropical flavor, its wild occurrence and demand by the local and international communities for the by-products of the fruit necessitated efforts to optimize the technological processes for the production of the possible by-products. This study focuses on the fermentation technology of the marula fruit.

The effect of enzymes prior to the fermentation process and post-fermentation was evaluated. For pre-fermentation processes we focused on the ability of commercial enzymes to increase juice yield, improve the clarification and filterability. For pre- and post-fermentation applications, aroma release was considered. The results indicated a significant increase in the yield depending on the enzyme used. An increase of at least 2% was recorded and a maximum of 12% yield increase was observed. The enzymes also had a phenomenal effect on the release of bound monoterpenes and hence enhancing the flavor of the juice. The panel of judges confirmed the results from the gas chromatography analyses by noting an increase in flavor intensity in the enzyme treated juice.

The possibility of selecting a yeast strain that performs best during the fermentation of marula pulp was also looked at. This study aimed at selecting a strain that produces wine and distillate with the typical marula flavor complex. We showed the effect of the different yeast strains, in the wines and distillates, on the principal volatile compounds. We then correlated the performance of the different strains as perceived by the panel to the various volatile compounds. The effect of fermentation temperature on the performance of the different yeast strains was also considered. Fermenting the marula pulp at different temperatures resulted in the production of wines and distillates with different volatile profiles for the different yeast strains. The wines and distillates fermented at a low temperature of 15°C were preferred to the wines and distillates fermented at 30°C. However, not all strains performed well at 15°C, strains like NT116 performed better at 30°C. The different commercial strains produced wines and distillates with significantly different flavor profiles. These differences in the flavor profiles were reflected in the sensory evaluation where, depending on the interaction of the volatile compounds some wines and distillates were preferred to others. The effect of the different commercial enzymes and yeast strains should thereof be further evaluated and optimized on a larger scale. This would greatly help prevent variation in quality of the fermented by-products of the marula fruit.

OPSOMMING

Die belang van inheemse vrugte wyne is nie goed nagevors en gedokumenteer nie. Daar is 'n behoefte om hierdie waardevolle voedselbronne te ontwikkel en te benut, deur verbeterde produksiepraktyke, storing, preserving en benuttingstechnologieë. Die maroelavrug is veelsydig op baie wyses, deurdat dit gebruik word vir die maak van sap, konfyt, bier, of as heel vrug geëet kan word. Die vrug is hoog in voedingswaarde, het 'n kenmerkende tropiese geur, kom wild voor, en is in aanvraag by plaaslike en internasionale gemeenskappe vir die by-produkte van die vrug. Dit maak dit essensieel om die tegnologiese prosesse vir die produksie van hierdie moontlike by-produkte te optimaliseer. Hierdie studie fokus op die fermentasie-tegnologie van die maroelavrug.

Die effek van ensieme voor en na die fermentasie-proses is geëvalueer. Vir prosesse wat voor fermentasie plaasvind, het ons gefokus op die vermoë van kommersiële ensieme om sapopbrengs te verhoog, asook om verheldering en filtrering te verbeter. Vir beide voor- en na-fermentasie toepassings is die vrystelling van aroma gemonitor. Die resultate dui op 'n betekenisvolle verhoging in die sapopbrengs, afhangende van die ensiem wat gebruik is. 'n Verhoging van ten minste 2% is opgeteken, en 'n maksimum van 12% opbrengsverhoging is waargeneem. Die ensieme het ook 'n geweldige effek op die vrystelling van gebonde monoterpene gehad, en dus die verhoging in die geur van die sap. Die proefpaneel het die resultate bevestig van die gaschromatografie-analises, deur 'n verhoging in die geurintensiteit in die ensiem-behandelde sap te bemerk.

Daar is ook gekyk na die moontlikheid om 'n gisras te selekteer wat die beste presteer tydens die fermentasie van maroela-pulp. Hierdie studie het die doelstelling gehad om 'n gisras te selekteer wat wyn en distillaat produseer met 'n tipiese maroela-geurkompleks. Ons het die effek van verskillende gisrasse aangedui in die wyne en distillate, op grond van van vlugtige komponente. Ons het dan die prestasie van die verskillende rasse, soos waargeneem deur die paneel, gekorrelleer met die verskeie vlugtige komponente. Die effek van fermentasie-temperatuur op die werkverrigting van die verskillende gisrasse is ook in ag geneem. Fermentasie van die maroela-pulp by verskillende temperature het gelei tot die produksie van wyne en distillate met verskillende vlugtige profiele vir die verskillende gisrasse. Die wyne en distillate wat by 'n laer temperatuur van 15°C gefermenteer is, is verkies bo die wyne en distillate wat by 30°C gefermenteer is. Alle rasse het egter nie baie goed presteer by 15°C nie, soos byvoorbeeld NT116 wat beter presteer het by 30°C. Die verskillende kommersiële rasse het wyne en distillate geproduseer met betekenisvol verskillende geurprofile. Hierdie verskille in geurprofile is gereflekteer in die sensoriese evaluering waar, afhangende van die interaksie van die vlugtige komponente, sommige wyne en distillate bo ander verkies is. Die effek van die verskillende kommersiële ensieme en gisrasse moet

verkieslik verder op groter skaal geëvalueer en geoptimiseer word. Dit sal veral help om variasie in kwaliteit van die gefermenteerde by-produkte van die maroelavrug te voorkom.

This thesis is dedicated to my mother for her unfailing love and support

BIOGRAPHICAL SKETCH

Margaret Fundira was born on the 1st of March 1972. She obtained her Ordinary Level Certificate at Goromonzi high School. From there she proceeded to St David's Girls High School where she did her Advanced Level. She obtained her Bachelor of Applied Sciences (Hons) dual degree in Biology and Biochemistry in 1995 at the National University of Science and Technology (Zimbabwe). In 1996 she enrolled at the Université de Bordeaux where she did Oenologie and did practical work on the French wineries. In 1999 she enrolled for the masters degree in Microbiology at the University of Stellenbosch.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions for their contributions to the successful completion of this study.

Dr P. Van Rensburg, Prof. IS Pretorius and Mr M. Blom, my supervisors for their guidance and critical reading of this manuscript.

The Technical Research Department and the Brandy and Spirits Department of Distillers Corporation for their part in research and in the sensory analysis of my wines and distillates.

The ARC Infruitec-Nietvoorbij for allowing me to use their laboratory distillation equipment.

The Scientific and Industrial Research and Development Center: for financial support.

Caroline Steger for answering my questions, giving me direction on my study and her critical input to my work.

My friends, Joseph, Kudakwashe and Candide: for their support.

The Almighty for this opportunity.

PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and chapters 3 and 4 will be submitted for publication. The writing style for the Journal of Food Science was used for all the chapters.

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GENERAL INTRODUCTION AND PROJECT AIMS

Chapter 2

LITERATURE REVIEW

Alcoholic fermentation of fruit wines and the technological processes related to their production.

Chapter 3

RESEARCH RESULTS I

Comparison of commercial enzymes on the processing of marula pulp, wines and spirits.

Chapter 4

RESEARCH RESULTS II

Effect of different commercial yeast strains on the flavor profile of the marula fruit wines and distillates.

Chapter 5

GENERAL DISCUSSION AND CONCLUSIONS

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1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 PRODUCTION OF FRUIT WINES

The production of high quality wines requires carefully optimized parameters including the growing environment for a given fruit and adapted wine technology. Amongst other characteristics of wines, flavor and aroma are a good measure of the quality of wine. The concentration of wine aroma compounds can be influenced by various factors, including the environment (climate, soil), grape variety, the degree of ripeness, fermentation conditions (pH, temperature, yeast flora), wine production (oenological methods, treatment substances) and aging (bottle maturation) of the wine (Rapp 1998).

A major controversy exists about the relevance of the selection of wine yeast strains with respect to their influence on the sensory characteristics of the wine produced (Kunkee and Vilas 1994). Differences in wine yeast strains in their fermentation behaviors have been noted since the earliest selection of pure cultures, over a century ago (Kunkee 1984). However, the question of the dependency of the sensory characteristics of the resulting wine upon the specific yeast strain used for the fermentation does not seem yet to have been satisfactorily answered (Kunkee and Vilas 1994). Even though the resolution of this conflict is of crucial importance to the wine producer, whether using pure yeast starters or indigenous yeasts, definitive information is scanty. Of course, inappropriate winemaking procedures certainly cause unacceptable flavor effects, which may be yeast strain related. For example, vitamin or nitrogen deficiencies may bring about production of displeasing amounts of hydrogen sulfite or acetic acid, dependent upon yeast strain (Rankine 1963; Bisson and Kunkee 1991). Sluggish fermentations may result in an over oxidized product or one with residual sugar, both detected organoleptically, as a result of yeast strain (Kunkee 1991).

In the wine making process enzymes play a significant role; in fact, more than ten different enzymes are involved during the alcoholic fermentation in the glycolysis cycle of yeast. These enzymes are responsible for the yeast's metabolic processes. Without these enzymes one cannot transform juice into wine. These enzymes can be either beneficial or detrimental to wine quality, hence the need for the enologist to know the precise nature and the behavior of these enzymes. Optimal conditions need to be created for the desirable enzymes to function and at the same time inhibit those that are detrimental to the wine quality (Jean-Claude Villetaz 1993).

It is also important to note that besides yeast, bacteria contribute to the aroma formation of an alcoholic beverage. For example Arroyo (1983) showed that *Clostridium saccharobutyricum*, affects fermentation in rum. Its presence in the fermentation medium was found to accelerate fermentation. Arroyo (1983) also showed that the best rum yield and aroma were related to the bacteria/yeast ratio of 1:5.

Maturation is also an important technological aspect to the production of a good quality alcoholic beverage, affecting the aroma composition. New compounds are formed and others consumed in chemical reactions during maturation. In general maturation makes wines and alcoholic spirits more acceptable (Lehtonen and Jounela-Eriksson 1983).

As part of the optimization process one cannot do away with sensory analysis of the product. In the past three decades it has become common practice to supplement traditional sensory and chemical analysis of wines and spirits with additional gas chromatographic (GC) analysis. This allows for both qualitative and quantitative analysis of a large range of volatile compounds that may be present in wines and spirits. GC analysis also helps to identify relationships that may exist between particular compounds or groups of compounds and their effects on the flavor and aroma of a beverage.

1.2 PROJECT AIMS

Various aspects of African foods and beverages have been studied and the results published. Emphasis has been on the microorganisms used and the nutritional value of the products after fermentation. The preparation of these products is still uncontrolled, hence the variations in the quality and stability of these products.

Marula (*Sclerocarya birrea* sub. *Caffra*) is a typical fruit of Southern Africa. It has an exotic flavor and high nutritive value, with a vitamin C content three times higher than orange juice. One of the most popular traditional alcoholic beverages is marula beer, also known as marula wine, and its production is still at grass roots level. The scientific approach to the production of marula by-products is still at an early stage.

This study aims to optimize the alcoholic fermentation of the marula fruit, for the improvement and standardization of the quality of its fermented by products. The work focuses on the determination of the influence of the fermentation temperature, and the selection of pectolytic enzymes and commercial yeast strains which can enhance the typical flavor complex of the fruit in the fermented product.

The specific aims of the study were the following:

- (i) comparing the effect of 10 different commercial yeast strains on the flavor and quality of the marula wines and distillates ;
- (ii) fermentation of the marula pulp at 15°C and 30°C to determine the influence of fermentation temperature on the wines and on the distillates;
- (iii) testing of commercial enzymes for post-fermentation aroma release;
- (iv) testing of commercial enzymes for the improvement of filtration and juice extraction;

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CHAPTER 2

LITERATURE REVIEW

Alcoholic fermentation of fruit wines and the technological processes related to their production

LITERATURE REVIEW

2.1. INDIGENOUS FRUIT WINES

2.1.1 Fruit wines

Upon fermentation, fruits, berries and grapes yield wines. The techniques used for fruit wine production closely resemble those for the production of white and red grape wines. Differences however arise from two facts:

- (i) it is more difficult to extract the juice or hydrolyze the sugar and other soluble materials from the pulp of some fruits than it is from grapes.
- (ii) the juices obtained from most of the fruits are lower in sugar content and higher in acids than grapes.

The use of specialized equipment to thoroughly chop or disintegrate the fruit or berries, followed by the use of presses that extract the juice from the finely divided pulp solves the first problem. The second problem is solved by the addition of water to dilute the excess acid and of sugar to correct this deficiency.

(Amerine 1980).

Most frequently used non-grape wine sources include apples, pears, plums, cherries, currants, oranges and various types of berries.

2.1.2 Pear wine

In Europe, special varieties of pears with high tannin content are used for making "perry", a fermented pear beverage. The pears are grated and pressed, after which the sugar and acidity levels are adjusted to suit the type of wine to be made. About 100 ppm of sulfur dioxide (SO₂) and pure wine yeast are added and the juice is left to ferment. The wine is raked, aged with oak chips, clarified and filtered. Pectic enzymes are used to enhance the filtration; the dry wine can then be sweetened, pasteurized or fortified as with other fruit wines (Amerine 1980).

2.1.3 Cherry wine

Sour cherries are recommended in preference to sweet cherries for making wine, as the acidity of the latter is too low. A blend of the two or a blend of currant and a table variety of cherries may be used, ordinarily to make a dessert wine rather than a table wine. Sugar is added to get the required alcohol content, and to sweeten the wine before bottling. In these wines, the use of a small amount of potassium metabisulfite is advisable before fermentation; the use of pectic enzymes to hasten and improve clarification is also desirable (Amerine 1980).

2.1.4 Wine from oranges

Oranges are the base for a fortified, sweet, dessert orange wine, dark amber in color. Orange wines darken rapidly and develop a harsh, stale taste unless a fairly high level of sulfur dioxide is maintained. To avoid a stale flavor, the fruit must not be overripe. Only the juice without the peel is extracted, to avoid excessive essential oil from the peel that slows fermentation. Approximately 150 ppm of sulfur dioxide or an equivalent amount of bisulfite or metabisulfite is added to the fresh juice. In order to facilitate clarification approximately 0.1% pectic enzyme is added. After fermentation, 200 mg/L of SO₂ is added to the wine, which is then filtered, pasteurized and bottled (Amerine 1980).

2.1.5 Grapefruit wine

Grapefruit may be used in the production of table wine, dessert wine and cordials. The wines are typically somewhat bitter, but if excessively so, a calculated amount of potassium carbonate may be added. The mixture is heated to 71.1°C to hasten the reaction and to make the calcium citrate less soluble, followed by hot filtering and cooling. Ion-exchange treatment may also be used to reduce acidity (Amerine 1980).

2.1.6 Wines of tropical fruits

Many subtropical and tropical fruits have low acidity and are difficult to make into attractive, stable wines. Since the flavors are 'strange' to consumers in temperate regions, export markets are difficult to establish (Amerine 1980).

2.2 THE MARULA FRUIT

2.2.1 Distribution

The marula, also known as the cider tree (*Sclerocarya birrea* sub. *caffra*), belongs to the family *Anacardiaceae* which includes 73 genera and 600 species, of which the mango, cashew nut and pistacio nut are well known examples. The tree is well adapted to deep sandy soil in warm, dry areas with an annual rainfall of 250-800 mm (Pretorius and others 1985). The marula fruit is one of the most commonly utilized wild fruits of southern Africa. The marula is widely distributed throughout the African continent. In southern Africa, only the subspecies *caffra* is found, and it occurs over practically all the subtropical regions (van Wyk and Gericke 2000). The marula is found in the Northern and Eastern parts of South Africa, Botswana, Mozambique, Swaziland, Zimbabwe and northwards into tropical Africa at altitudes up to 1000 m above sea level (Gous and others 1988). In its wild state the marula provides quality timber, edible leaves, medicinal bark (for heartburn, diarrhea, diabetes, fever, malaria) (van Wyk and Gericke 2000) and edible fruit.

2.2.2 Description

2.2.2.1 Physical characteristics

The marula is one of the most commonly utilized wild fruits of Southern Africa. The fruit, (**Fig. 2.1**) is a round oval drupe with a diameter of 30-40 mm and has a thick leathery soft exocarp that encloses a white juicy mucilaginous flesh (Gous and others 1988). The flesh adheres tightly to the single stone and has a tart, sweet refreshing taste and a slightly turpentine-like aroma (Palmer and Pitman 1972). The fruit has been classified by the Pedi, to whom the marula is part of the diet, into three types: sweet and palatable; sour and unpopular, and undesirable due to objectionable odor (Schäefer and McGill 1986).



Figure 2.1: Marula fruit, whole fruit and sectioned fruit (Nerd and Mizrahi 1993).

The marula fruit has a unique flavor and high nutritive value, and is rich in vitamins. Each fruit contains an extremely hard seed covered by fibrous matter (Shone 1979). Mature fruit drop, while still green, and ripens to a yellow color on the ground, usually during January and February. Harvesting is simply picking up the fallen fruit (Weinert and others 1990). The contribution of the various parts of the fruit to its total weight is shown in **Table 2.1**. The wide variation in fruit weight can be influenced by climatic and geographic conditions, even by genetic make up (Weinert and others 1990).

Table 2.1: Approximate weight (g) distribution of ripe marula fruit (Weinert and others 1990)

	Source	
	Quin (1959)	Shone (1979)
Fruit (average)	17.9	30
Peel	7.4	9
Stone	9.4	-
Stone shell	-	3
Nut	-	1
Edible flesh	1.1	12
Inedible flesh	-	5

The viscosity of the marula puree is generally high, hence it can be expected that the handling and processing of the purees will be more difficult than other products.

2.2.2.2 Chemical characteristics

The marula fruit has, on average, a moisture content of approximately 85%, with the moisture of the skin slightly less than that of the flesh (see **Table 2.2**). The fruit contains low amounts of fat and protein and it can contain more than 2% crude fiber. Sucrose and smaller quantities of glucose and fructose add up to a 7-14% sugar content. Ascorbic acid and citric acid are the most abundant organic acids; the presence of malic and tartaric acid has also been reported (Gous and others 1988). The mineral composition of the fruit seems to vary with its geographical origin. Most notable are the relatively high concentrations of potassium, calcium and magnesium (Grivetti 1982).

At 2 mg/mL the vitamin C content of the fruit is four times that of ordinary orange juice (Van Wyk 1974) and has been recognized as a valuable antiscorbutic agent. In areas where the trees occur, a reduction in the occurrence of scurvy has been noted during the harvest period (Shone 1979).

The general amino acid pattern of the fruit has been noted to be consistent with differences occurring due to climatic conditions. Asparagine, glutamine and arginine are the most abundant amino acids. Glutamic acid, alanine and serine are present in intermediate amounts and the other amino acids are occasionally present at low concentrations (Gous and others 1988).

Table 2.2: Composition of marula fruit from different regions in Southern Africa

	Zimbabwe ¹	Botswana ²	Sibasa ³	SWA-Namibia ⁴		SWA-Namibia ⁵	
	Fruit	Fruit	Fruit	Flesh	Skin	Flesh	Skin
	g/100 g						
Moisture	87	91.7	86.4	85.2	80.7	85.5	78.8
Protein		0.5	0.3	0.5	0.8	0.6	0.6
Fat		0.1	0.1	0.4	0.6	0.4	0.4
Ash		0.2	0.4	0.9	1.0	1.3	1.0
Crude fiber		0.5	0.8	1.2	2.4	2.0	2.6
Carbohydrate		7.0 ⁶		12.0	14.4		
Fructose	2.3 ⁷			0.97 ^a			
Glucose				0.75 ^a			
Sucrose	5.9			5.95 ^a			
	mg/100 g						
Malic acid				0.22 ^b			
Tartaric acid				0.75 ^b			
Citric acid				11.6 ^b			

⁷ % invert sugar

^a juice

^b fruit

2.2.2.3 Volatile compounds

Very little study has been done on the volatile components of the marula aroma. Ballschmieter and Torline (1973) found that the aroma was mainly contained in the skin and investigated different methods for its isolation, such as vacuum stripping, carbon absorption of volatiles after vacuum stripping and extraction of the skin with either freon 11 or freon 12. Experiments showed that steam distillation of the skins caused a "cooked aroma", whereas extraction of the skins with methylene chloride resulted in a loss of "freshness". The aroma obtained by vacuum stripping of the skins was thus investigated: 12 major components (all sesquiterpene hydrocarbons) were found by capillary gas chromatographic separation in the methylene chloride extract of the aqueous condensate obtained through vacuum stripping.

More than 100 components were identified in the aroma fraction obtained after carbon absorption, but the overall note was sour and unbalanced. The conclusion of the authors was that extraction of the skins with freon 12 gave the most authentic marula flavor. Thirty-nine components, identified in the freon 12 extraction, are shown in

Table 2.3.

Pretorius and others (1985) investigated the volatile aroma of marula juice obtained by liquid-liquid extraction or headspace analysis followed by GC and GC-MS identification. They found that 38 components (from α -ylangene to benzenemethanol) in

Table 2.3 accounted for over 95% of the volatiles of the extracts. Like the results obtained from the extracts, the major aroma compounds in the headspace of the juices were sesquiterpene hydrocarbons and benzyl alcohol.

Table 2.3: Volatile aroma compounds identified in Marula skins and juice

Skins ¹	Juice ²
n-pentane	ethylacetate
n-hexane	ethyl-3-methylbutanoate
benzene	3-methyl-1-butanol
2-octene	pentan-1-ol
1,5-hexadiene	styrene
diethylbenzene	3-hydroxybutan-2-one
	3-methylbutyl-3-methylbutanoate
methanol	hexan-1-ol
n-pentanol	(E+Z)-3-hexen-1-ol
3-methoxy-2-butanol	an ethylester
2-methyl-1-pentanol	trans-linalool oxide furanoid
2-ethoxypropanol	furfural; alkylbenzene
2-ethyl-3hexen-1-ol	α -cubebene
	δ -elemene
acetone	α -ylangene
	α -copaene
acetaldehyde	β -bourbonene

glycolaldehyde	an ethyl ester
crotonaldehyde	β -cubebene
n-pentanal	benzaldehyde
3-methylbutanal	linalool
2-hexenal	α -bergamotene
n-hexanal	β -caryophyllene
n-heptanal	a sesquiterpene hydrocarbon
n-octanal	tetrahydro-2-H-pyran-2-one
2-ethylbutanal	aromadendrene
	γ -elemene
ethylformate	(Z)- β -farnesene
ethylpropanoate	3-methylbutanoic acid
ethylisobutyrate	α -humulene
ethylvalerate	a sesquiterpene hydrocarbon
ethylbutanoate	(E)- β -farnesene
ethylisovalerate	γ -amorphene
ethylcaproate	a sesquiterpene hydrocarbon
benzylacetate	γ -muurolene
	three sesquiterpene hydrocarbons
glycolic acid	α -muurolene
oxalic acid	(Z,Z)- α -farnesene
2-methylpropanoic acid	a α -farnesene isomer; two
2-methylbutanoic acid	sesquiterpene hydrocarbons
	(E,E)- α -farnesene
ethyl-2-propenylether	δ -cadinene
ethylisopropenylether	γ -cadinene
ethylamine	a sesquiterpene hydrocarbon
acetamide	ethylnicotinoate
	geraniol
	a sesquiterpene hydrocarbon
	benzenemethanol
	benzene-ethanol
	calcorene
	2,5-furandialdehyde
	a sesquiterpene hydrocarbon
	dodecan-1-ol
	pentadecan-2-one
	heptadecan-2-one
	nonadecan-2-one
	5-(hydroxymethyl)-2- furancarboxaldehyde

¹Ballschmieter and Torline (1973); ²Pretorius and others, (1985).

No characteristic aroma impact compound could be identified, indicating that a variety of compounds contribute to the typical aroma. Despite having common aroma compounds, the similarity in flavor of the marula and the grapefruit were attributed to nonvolatiles causing a bitter taste. On the other hand, the resemblance observed

between the aromas of the marula fruit and the pineapple cannot be due only to the few common volatile components (e.g. ethyl acetate, benzaldehyde, linalool) (Weinert and others 1990).

2.2.2.4 Traditional utilization

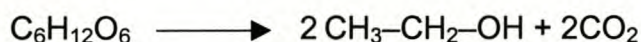
Ripe marula fruit can be consumed by sucking the juice or chewing the mucilaginous flesh after removal of the skin. A popular fermented alcoholic beverage is prepared from ripe fruit. The ripe fruits are washed before fermentation (Madovi 1981). In some cases the skin is removed and the juice fermented together with the flesh still on the seed. In other cases the skin is only cut and the whole fruit allowed to ferment. The yeasts occurring naturally on the fruit ferment the fruit spontaneously (Weinert and others 1990, Gadaga and others 1999). The stones and skin of the fruit are then filtered to produce a wine which, with slight improvements in technology could result in a lovely sparkling wine with a rich flavor (Madovi 1981). This beverage is commonly known as marula beer, but might more appropriately be called a 'marula wine' (Shone 1979). The wine or beer has twice the amount of ascorbic acid as orange juice and thus has excellent antiscorbutic activity. The nuts inside the seed of the fruit are regarded as a delicacy and are used to supplement diet during winter. They are mixed with vegetables or meat, may be pounded and formed into a cake, or used in biscuits (Shone 1979). Marula pulp is also used to make jelly (Palmer and Pitman 1972).

2.2.2.5 Marula liqueur

At the present moment limited amounts of juice are processed for the manufacture of the liqueur, "Amarula". Ripe fruit is gathered, the kernels are removed in a destoner and the flesh crushed from the skin. The marula flesh is then fermented under conditions similar to winemaking. After fermentation the marula wine is distilled in copper pot-stills. The young liqueur is then matured in small casks of oak for approximately 2 years and enriched with pure marula extract obtained through a special process that captures the unique flavors of the marula in a concentrated form. The spirit is then blended with fresh cream until a smooth consistency is reached. The creaming process is of very high standard, resulting in a product that is completely stable, rich and soft. The final product has an alcohol content of 17% by volume (Amarula website 2000).

2.3 ALCOHOLIC FERMENTATION

The essential part of the wine flavor is formed during alcoholic fermentation. Alcoholic fermentation is the conversion of the principal sugars, glucose and fructose to ethanol and carbon dioxide. It is conducted by yeasts and is expressed by the Gay-Lussac equation as:



In the case of wine, the primary yeast carrying out fermentation belongs to the genus *Saccharomyces*. Pure alcoholic fermentation is very rare due to the fact that all the sugar molecules do not follow the Gay-Lussac equation. A certain proportion is degraded by the glyceropyruvic fermentation through the Neuberg equation with the formation of glycerol.



Pyruvic acid is also formed and is decarboxylated into acetaldehyde, which is the origin of diverse secondary products that form during anaerobic respiration. During alcoholic fermentation, the pyruvic acid that appears at the end of glycolysis is decarboxylated and acetaldehyde is reduced into alcohol by the NADH_2 that formed during the oxidation of glyceraldehyde-3-phosphate. The two reactions are coupled to give an oxidation-reduction mechanism.

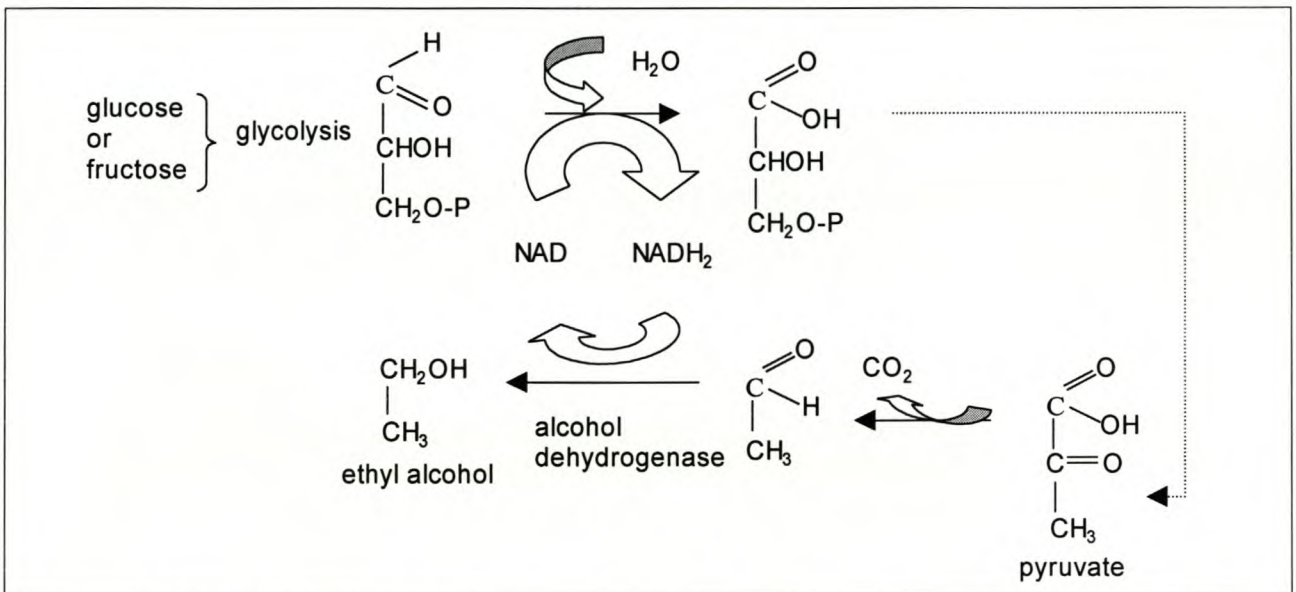


Figure 2.2: Alcoholic fermentation.

The complete chemical summary of the fermentation affected by the yeast can therefore be expressed as;



Fermentation increases the chemical and flavor complexity of wine by assisting the extraction of compounds from solids present in must, modifying the must-derived compounds and producing yeast metabolites (Henschke and Jiranek 1992).

2.3.1 Yeast

The three essential microbiological characteristics of vinification are; must rich in sugar, a large natural inoculum of microorganisms (10^6 cell/mL) and an anaerobic fermentation.

The three principal phases in the yeast growth cycle during fermentation are:

- (i) a limited phase of multiplication that lasts for 2 to 5 days and carries the cell population to a level around 10^7 to 10^8 cells/mL;
- (ii) a stationary phase where the viable cell level remains approximately constant;
- (iii) a decline phase in which the viable cell population progressively reduces to a level of about 10^5 cells/mL. This phase may last several weeks.

These phases represent a classical microbiological growth cycle, but with wine fermentation, several variations may be noted:

- (i) the duration of the growth cycle is particularly long;
- (ii) the total growth is limited and corresponds to about four or five generations;
- (iii) the cessation of growth does not arise from the exhaustion of sugar in the must; a deproportion exists between the principal phases with the decline phase lasting three or four times longer than the phase of multiplication (Ribereau-Gayon 1985).

2.3.2 Factors affecting yeast growth during fermentation

2.3.2.1 Clarification of juice

The clarification of must before fermentation is known to affect the quality of white wines. Fermentation of clear must has been shown to form more higher alcohols, fatty acids and corresponding esters. On the other hand, the must suspended solids can impart heavy and disagreeable vegetal odors, hence the importance of must racking.

Clarification essentially modifies must fermentability. Clear must is known to ferment with more difficulty than cloudy must, though the elimination of yeast is not the only reason for this fermentation difficulty. The procedures used to clarify juices, especially for white wine fermentations, influence the population of indigenous yeasts in the juice and their subsequent contribution to the fermentation. The phenomenon of fermentation is affected by clarification. Clarification can remove indigenous yeasts and therefore decrease their contribution to fermentation, or it can encourage the selective growth of some species, thereby enhancing their contribution to the fermentation. Clarification also eliminates nutritive elements released by must sediment and a possible support effect which would permit a greater yeast activity, possibly by fixation of toxic compounds (short chain fatty acids) (Ribereau-Gayon and others 2000).

In consequence, the level of must clarification should be controlled for each type of white wine making by measuring must cloudiness or turbidity, expressed in NTU. For vineyards in the Bordeaux region, a turbidity of less than 60 NTU can lead to serious

fermentation difficulties. Above 200 NTU, the risk of olfactory deviations due to presence of must lees is certain (Ribereau-Gayon and others 2000).

The clarification procedures include cold settling, enzyme treatment, centrifugation and filtration. Centrifugation and filtration are likely to remove yeast cells, thereby decreasing or even eliminating any contribution from indigenous yeasts. In contrast cold settling presents opportunities for the growth of indigenous yeasts, especially those species that grow well at low temperatures (Fleet and Heard 1993).

2.3.2.2 Sulfur dioxide (SO₂)

Sulfur dioxide is added to juice for controlling oxidation reactions and restricting the growth of indigenous microflora during winemaking (Kunkee 1984). SO₂ has the following principal properties:

- (i) **Antiseptic:** It inhibits the development of microorganisms. SO₂ has a greater activity on bacteria than on yeast. During storage SO₂ hinders the development of all microorganisms (yeast, lactic acid bacteria, acetic acid bacteria). In this way, haze formation by yeast, the refermentation of sweet wines, the development of mycodermic yeast (flo yeast) and various bacterial spoilages are avoided;
- (ii) **Antioxidation:** In the presence of catalysts, SO₂ binds with dissolved oxygen, thus protecting wines from chemical oxidations, of phenolic compounds and certain elements of aroma. SO₂ prevents madeirisation, and contributes to the establishment of a sufficiently low oxidation-reduction potential, favoring wine aroma and taste development during storage and aging;
- (iii) **Antioxidant:** SO₂ inhibits the functioning of oxidation enzymes (tyrosine and laccase), and helps to avoid oxidasic casse in white and red wines made from rotten grapes;
- (iv) **Binding ethanal and other similar products.** By doing so SO₂ protects wine aromas and eliminates flat character (Ribereau-Gayon and others 2000).

In this way SO₂ affects the metabolic activity of the participating yeasts and therefore the chemical composition of the wine. Wines fermented in the presence of SO₂ have been shown to have higher concentrations of acetaldehyde, for example than those fermented in its absence (Herraiz and others 1989).

An increase of off-flavors in bottled wines over the years have been seen to be noticeably a result of thiols (odor threshold < 2 µg/L) like methanethiol (MeSH) and ethanethiol (EtSH) in connection with significant amounts of other S-substances with higher boiling points (Rauhut and others 1999). As an antimicrobial agent, SO₂ can be expected to have the following effects on the kinetics of yeast growth during fermentation:

- (i) increasing the lag phase and delay the onset of fermentation;
- (ii) decreasing the growth rate and increase the time to complete fermentation;
- (iii) accelerating the decline or death phase;

- (iv) selective effects upon the species or strains that grow and contribute to the fermentation.

The extent of these influences will range from negligible to significant, depending upon the concentration of added SO_2 , the composition of the juice which affects the proportion of active SO_2 , and the SO_2 tolerance of the species or strains of yeasts present in the juice (Fleet and Heard 1993). **Figure 2.3** summarizes the formation of off-flavors during winemaking and storage.

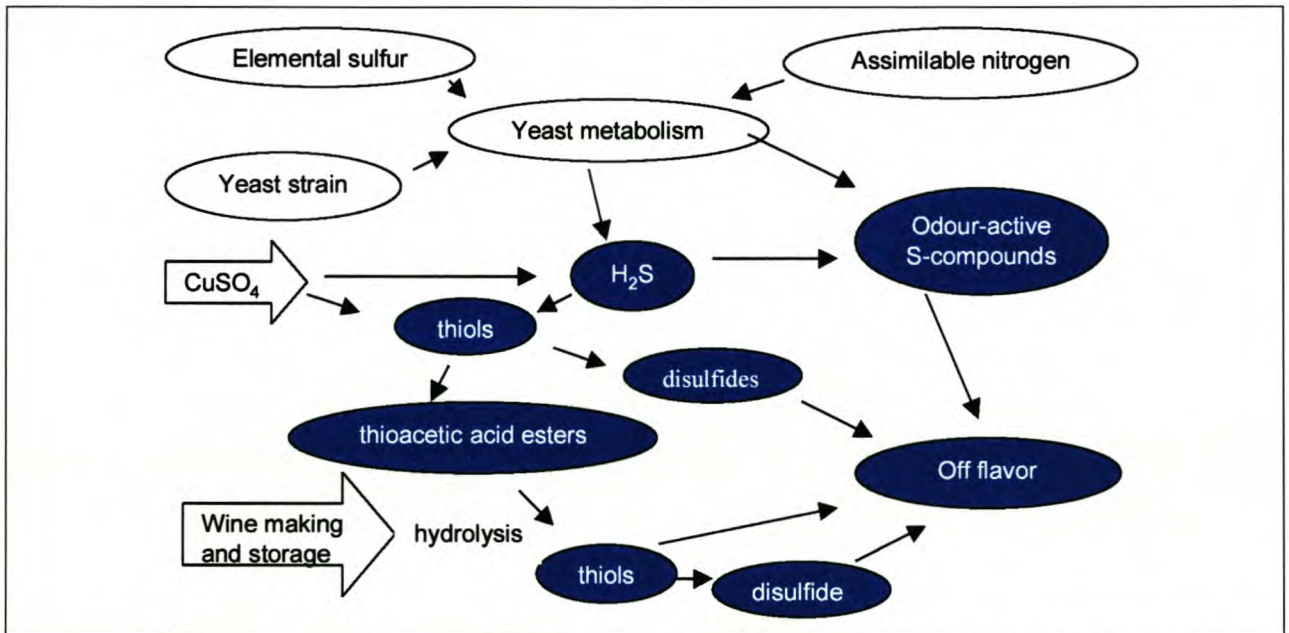


Figure 2.3: Formation of off-flavors during winemaking and storage (Rauhut and others 1999).

2.3.2.3 Temperature

The temperature at which the alcoholic fermentation is conducted affects:

- (i) The rate of yeast growth and, consequently the duration of fermentation;
- (ii) The extent to which different yeast species contribute to the fermentation;
- (iii) The biochemical reactions of the yeasts which ultimately determine the chemical composition and sensory quality of the wine.

The rate of yeast growth and alcoholic fermentation increases as the temperature increases, with maximum rates generally occurring at temperatures between 20°C and 25°C (Amerine 1980). Red wines are generally fermented at 20°C-30°C, while white wines are often fermented in the range 10°C-20°C (Fleet and Heard 1992). In recent years there has been a preference by some winemakers to ferment white wines at lower temperatures to enhance the production and retention of flavor volatiles (Killian and Ough 1979; Kunkel 1984). Hence the selection and use of strains of *S. cerevisiae* which exhibit good growth rates at low temperatures is crucial. Due to the fact that fermentation produces heat, a good cooling system is often required. Cooling control is particularly important if there is a possibility of the temperature increasing above 30°C,

where sensitivity of *S. cerevisiae* to ethanol is enhanced as is the risk of a stuck fermentation (Van Uden 1989).

Shaft and Margalith (1983) have shown that temperature also affects the ecology of wine fermentation. A wide range of studies have shown that increased ethanol tolerance of the non-*Saccharomyces* yeasts at low temperatures appears to be a major factor accounting for their stronger, if not dominant, contribution to low temperature fermentations.

Temperature also affects the biochemical activities of yeasts and their production of secondary metabolites (Bisson 1984).

2.3.2.4 Juice composition

The chemical and physical composition of the juice affects the rate and completeness of fermentation as well as the concentration of many aroma and flavor constituents in the wine. Important variables likely to affect yeast growth are sugar concentration, nitrogenous substrates, presence of adequate vitamins, concentration of dissolved oxygen and concentration of insoluble solids. Other factors include fungicide residues from the fruits and any yeast inhibitory or stimulatory substances produced by the growth of fungi or bacteria on the grapes prior to harvest.

Juice composition varies according to variety, soil condition, use of fertilizers and maturity of the berries at harvest. In addition, processing operations such as pressing, clarification, ion exchange, desulfiting and blanketing with nitrogen or carbon dioxide can also affect composition.

The initial concentration of sugar in grape juice selectively influences the species and strains of yeast responsible for the fermentation. Such influences have been shown to be amplified at lower fermentation temperatures (15°C) (Monk and Cowley 1984). Pure culture studies with *S. cerevisiae* show that the rate and completeness of fermentation decreases as the initial concentration of the sugar in the juice increases above 200 g/l. Such high concentrations affect yeast growth by increasing the lag phase, decreasing growth rate and decreasing the maximum population of cells produced (Lafon-Lafourcade 1983; Monk and Cowley 1984; Nishino and others 1985).

Free amino acids and ammonium ions are the principal nitrogen sources used by yeast for growth during alcoholic fermentation (Fleet 1992). The nitrogen demand by yeast increases with increasing sugar concentration in the juice and can vary significantly (from 460-780 mg/L of free amino nitrogen) with the *S. cerevisiae* strain (Jiranek and others 1990). Thus to ensure that nitrogen availability is not a factor that limits yeast growth, supplementation of juices with diammonium phosphate is common practice. The rate of growth and juice fermentation by *S. cerevisiae* decreases as the pH is decreased from 3.5 to 3.0 (Ough 1966a; 1966b).

Juices containing residual fungicides may be prone to altered alcoholic fermentation depending upon the concentration and type of fungicide present. Such residues could

delay the onset of yeast growth, decrease the rate and yield of growth and accelerate death of the yeast cells after growth has ceased. This results in slower and incomplete fermentation (Beuchat 1973).

2.3.2.5 Inoculation with selected yeast

The use of selected yeast cultures as starters for wine fermentation has led to the production of more consistent wines. There is a wide range of commercial selected yeasts. From an ecological point of view it is expected that the inoculated strain of *S. cerevisiae* will suppress and outgrow indigenous non-*Saccharomyces* species as well as indigenous strains of *S. cerevisiae*, and dominate the fermentation. However Heard and Fleet (1985) have shown that growth of *Kloeckera apiculata* and *Candida* species is not suppressed in fermentations inoculated with commercial strains of *S. cerevisiae*. From these and other findings, it can be concluded that inoculation of the grape juice with high initial populations of *S. cerevisiae* will not necessarily prevent the growth of indigenous non-*Saccharomyces* yeasts, and that these species may also contribute to the overall fermentation. Loiseau (1987) concluded that the dominance of the inoculated strain was not always assured and depended on specific vinification conditions. Martinez and others (1989) using production of hydrogen sulfide as a marker to differentiate between strains of *S. cerevisiae*, demonstrated that the inoculated strain tended to dominate, but the indigenous strains remained in significant numbers throughout the fermentation. Significant factors that will influence the outcome will be the population of indigenous yeasts in the juice and the extent to which they have adapted to grow in that juice.

2.3.2.6 Stimulation of the alcoholic fermentation, notion of survival factors

The addition of yeast to the must increases the cellular concentration and accelerates the speed of the fermentation at the start. But the principal reason for adding yeast lies in the possibility of increasing the total quantity of sugar fermented by using a selected yeast strain that tolerates a high concentration of ethanol.

New perspectives have been introduced by the notion of survival factors, which derive from the specific role of steroids in vinification (Ribereau-Gayon 1985). The steroids have a "survival factor" action when they act on aerobically cultivated yeasts, fermenting under anaerobic conditions, in grape must of high sugar concentration. In this case, the substances have no effect on the cellular multiplication but they maintain viability and fermentation activity in the resting cells (Larue and others 1979). Hence they do not function as factors of growth but contribute to yeast survival. Steroids may also have an inhibiting effect. The interest in these survival factors rests in their action on the population responsible for the degradation of the final grams of sugar, and they may serve to prevent premature cessation of fermentation (Ribereau-Gayon 1985). Certain growth conditions during fermentation affect the survival factor action of steroids:

high temperature, low pH, vitamin deficiency, low amount of inoculum, insufficient aeration and excessive sugar concentration (Larue 1979).

2.3.3 Effect of yeast on the quality of wine

The transformation of grape juice to wine is essentially a microbial process (Boulton and others 1996), and yeasts, among other microorganisms, play a significant role in this process. They conduct the alcoholic fermentation, they can spoil wines during conservation in the cellar and after packaging, and they affect wine quality through autolysis (Fleet 1993). The primary wine yeast is *S. cerevisiae* originating in both the grape and the winery environment. Indigenous or wild yeasts such as species of *Kloeckera*, *Hanseniaspora* and *Candida*, play a role in determining the flavor and overall character of wine. Non-*Saccharomyces* species and naturally occurring strains of *S. cerevisiae* can contribute to the flavor and aroma of wines by the production of higher alcohols, esters, aldehydes and through acid metabolism (Fleet 1997). *Kloeckera apiculata*, *Candida anomala* and *Candida stellata* have been reported to produce undesirably high concentrations of volatiles such as acetic acid and ethyl acetate while producing only low concentrations of ethanol (maximum 6.5% for *K. apiculata*) (Fleet and Heard 1993). Despite the possible negative impact of certain species, some may enhance the flavor and aroma properties of wines during fermentation. Hence there is a growing interest in exploiting the potential of these wild yeasts in the production of more complex-flavored and novel wine products (Heard 1999). The most noticeable aroma components are produced by yeast during fermentation (Schreier 1977), and the nature of the final aroma depends primarily on the kind of yeast used, and on the fermentation conditions (Nykänen 1985). In distilled beverages, the technique of distillation and the maturing process of the beverage (Suomalainen 1970), further affect the aroma composition.

Volatile organic acids, aldehydes, alcohols and esters are important components of fermented beverages that are formed or modified by yeast fermentation (Soles and others 1981). The yeast plasma membrane regulates the movement of compounds into the yeast cell and of yeast metabolites from the cell into the medium (Suomalainen and Lehtonen 1978). The lipophilic nature, the molecular size and the degree of branching of compounds determine the ease of penetration. Short C-chain compounds pass into the yeast more rapidly than long chain compounds. It has been found that branching of the carbon chain retards the penetration. (Lehtonen and Jounela-Eriksson 1983).

Lehtonen and Jounela-Eriksson (1983) reported that a particular yeast strain produces the same aroma compounds irrespective of the juice used. In their study white currant juice was used to produce Finnish berry wine and grapes to produce Spanish sherry.

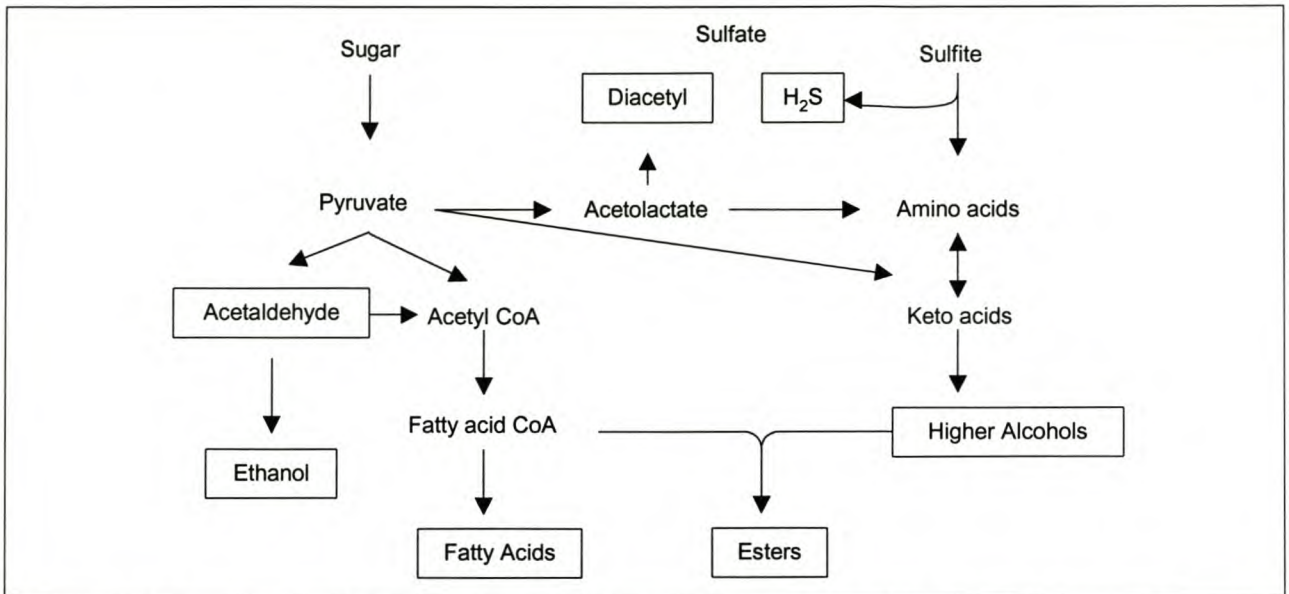


Figure 2.4: Derivation of flavor compounds from sugar, amino acids and sulfur metabolism by yeast (Henschke and Jiranek 1992).

For convenience, the yeast derived flavor compounds in the winemaking process can be placed into three groups: the major volatile products of fermentation; the trace “fermentation bouquet” compounds; and the undesirable or “negative” aroma compounds. The main groups of compounds that form the “fermentation bouquet”, are the organic acids, higher alcohols and esters and to a lesser extent, aldehydes (Rapp and Versini 1991). The most deleterious “negative” aroma compounds are the reduced sulfur compounds, hydrogen sulfide, organic sulfides and thiols (mercaptans) (Suomaläinen and Lehtonen 1978; Henschke and Jiranek 1991). In excess concentrations, the fermentation bouquet compounds, acetaldehyde, acetic acid, ethyl acetate, higher alcohols and diacetyl may also be regarded as undesirable (Henschke and Jiranek 1992).

Lubbers and others (1994) studied the influence of yeast cell walls on wine aroma using a model wine. They reported that yeast cell walls lead to a decrease of volatility of all the aroma compounds, depending on the physico-chemical nature of the volatile compound. This suggests that great quantities of aroma compounds, especially the most hydrophobic, could be lost if musts are processed with large amounts of yeast walls. The quantity of yeast walls required for treatments is therefore important.

2.3.4 Fermentation by-products

Apart from ethanol and glycerol, as well as diols and higher alcohols, numerous other wine constituents are formed by yeast metabolism (especially acids, esters, aldehydes, ketones and S-compounds). Ethyl esters of straight chain fatty acids and acetates of higher alcohols are the dominant esters in wine and they are formed during the alcoholic fermentation. These compounds can contribute to the evaluation of optimal wine

technology, but are however not suitable to the evaluation of a varietal characterization (Rapp 1998).

2.3.4.1 Esters

A great variety of volatile metabolic by-products are formed in yeast cells during fermentation. Quantitatively, the largest group is comprised of esters. Volatile esters have been described as contributing mostly floral or fruity characters to wine (Soles and others 1982). Reported concentrations are generally near sensory threshold levels, hence esters also play an important role (both positive and negative) in wine quality (Zoecklein and others 1995). The basic odor of a wine has been attributed to the four esters, (ethyl acetate, isoamyl acetate, ethyl caproate and caprylate), and to isobutyl alcohol, isoamyl alcohol and acetaldehyde. The other compounds are thought to modify the basic odor (Rapp and Mandery 1986).

According to the mechanism in **Fig. 2.5**, acyl-coenzyme-A functions as the key compound in the biosynthesis of esters. During fermentation, yeast produces acyl-CoA in cells either through the activation of fatty acid or through the oxidative decarboxylation of keto acid. These mechanisms differ in their capacity to utilize ATP, with the former requiring ATP and the latter not. Furthermore, in ester as well as fatty acid synthesis, the lengthening of the carbon chain of the acid moiety occurs such that malonyl-CoA bonds with acyl-CoA in the enzyme complex, bringing two more carbon atoms into the chain of the acid. The final step is the cleavage of the enzyme from the complex. In the presence of alcohol the reaction produces an ester, whereas when water is present the result is a free fatty acid. The metabolic pathway in yeast cells starting from acetyl-CoA therefore leads to esters whose corresponding acid moiety has an even number of carbon atoms. In fact, esters having an even carbon number regularly appear as main components in ester fractions of wines and distilled alcoholic beverages (Nykänen 1985).

The major factors affecting ester content of wine are medium composition and fermentation procedure. Variations in media include carbon source and concentration (Nykänen and others 1977; Ough and others 1968), pH of the medium (Nordström 1982), micronutrient availability (Haikeli & Lie 1971) and unsaturated fatty acid/sterol levels (Anderson and Kirsorp 1974; Soufleros and Bertrand 1979; Nordström and Carlson 1965). Important fermentation procedure factors include fermentation temperature (Killian and Sough 1979), carbon dioxide concentration, oxygenation of the media (Nordström and Carlson 1964) and yeast strain (Soles and others 1982; Rapp and Mandery 1986).

The short chain fatty acids, (acetic, propionic, butanoic, and lactic acid) are by-products of fermentation. Acetic acid is quantitatively the most important, ranging from less than 0.2 to more than 2 g/L volatile acidity (acetic acid is the major component of volatile acidity) with a mean of 0.54 g/L in Australian table wine (Englinton 1992). Depending on the style of wine, acetic acid becomes objectionable near its flavor

threshold of 0.7 to 1.1 g/L (Corison and others 1979). The yeast strain and extracellular lipids are the main factors affecting the acetic acid concentration in wine.

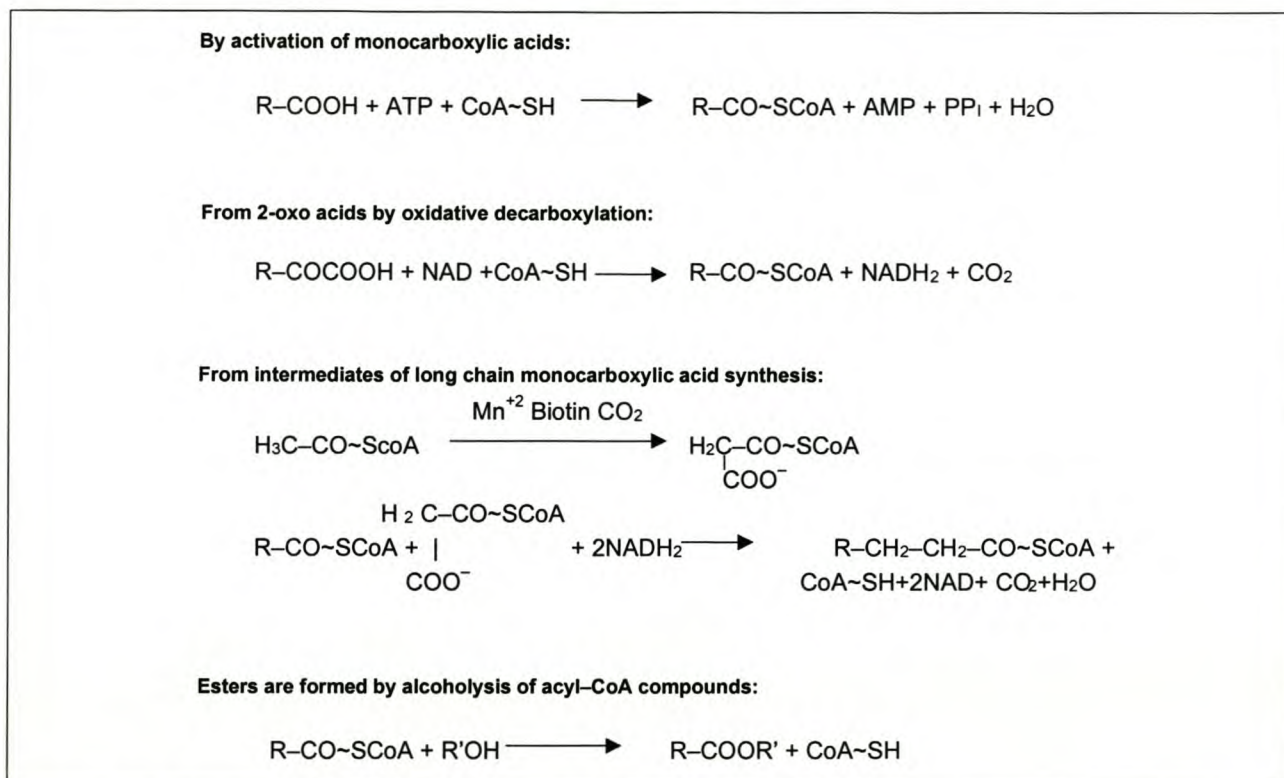


Figure 2.5: Scheme for the fatty acid ester synthesis (Nykänen 1985).

The longer straight-chain fatty acids (C8 to C12) are intermediates in the formation of long chain acids (C16 to C18), which are structural components of cell membranes. Concentration of the shorter chain acids increases while that of the longer chain acids decreases during fermentation (Henschke and Jiranek 1992). Two major groups of esters are formed during fermentation: the ethyl esters of straight-chain fatty acids and acetates of the higher alcohols. Esters of the first group include the ethyl esters of hexanoic, octanoic and decanoic acids. The fatty acid component of this group originates during formation or degradation of long-chained membrane fatty acids. This group of esters plays an important role in sensory properties of distillates. Esters of the second group include ethyl-, isobutyl-, isoamyl-, 2-phenethyl-, and hexyl-acetate. Ethyl acetate is generally present in wines in the highest concentration. Ethyl lactate (arising from malolactic fermentation) may also be present in substantial amounts (Zoecklein and others 1995). Some of the fatty acid CoA derivatives, which react with malonyl-CoA during chain elongation, take part in ester formation. Reaction with an alcohol forms the fatty acid ester (Henschke and Jiranek 1992). Ester formation is related to the availability of the alcohol and yeast demand for fatty acid synthesis (Nordström 1964; Nykänen 1985).

2.3.4.2 Higher alcohols

Higher alcohols form an important group of by-products of alcoholic fermentation since, along with their ester derivatives, they are determinants of the flavor and aroma of fermented products (Nordström and Carlson 1964). Higher alcohols occur in varying amounts in alcoholic beverages and they can be detected by their strong, pungent smell and taste (Nykänen 1986). Higher alcohols can be defined as those possessing more than two carbon atoms with a higher molecular weight and boiling point than ethanol (Lambrechts 2000). Higher alcohols are quantitatively the largest group of aroma compounds in alcoholic beverages and are secondary products of alcoholic fermentation (Amerine and others 1980). It has been demonstrated that both the catabolic process starting from amino acids and anabolic process from sugars (Fig. 2.6) are involved in the formation of higher alcohols via 2-keto acids, even when amino acids are present in excess.

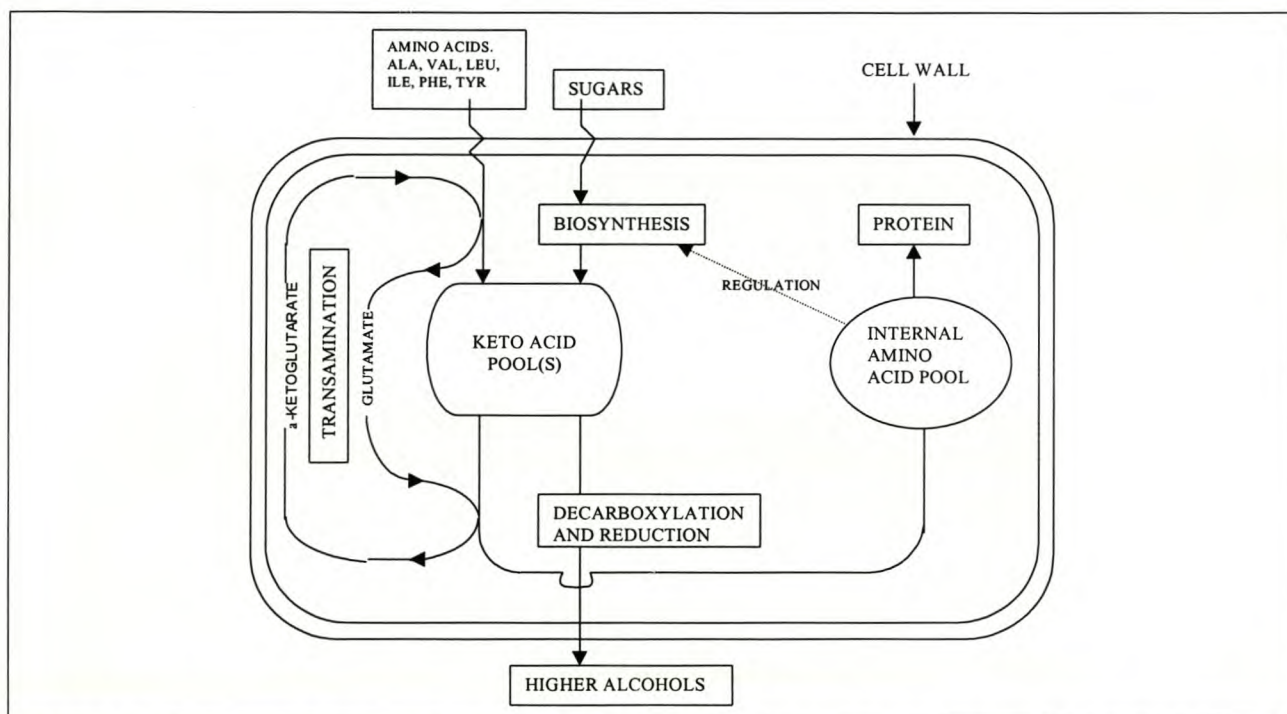


Figure 2.6: Schematic presentation of the formation of principal higher alcohols (Nykänen 1984).

The most frequently encountered fusel alcohols are isoamyl- (3-methyl-1-butanol), “active amyl” (2-methyl-1-butanol), isobutyl (2-methyl-1-propanol), and n-propyl alcohols (Henschke and Jiranek 1992). At low concentrations (less than 300 mg/L) they contribute to a wine’s aromatic complexity. On the other hand, at high levels, they mask the wine’s aromatic finesse due to penetrating odors (Ribereau-Gayon and others 2000). This group of alcohols may present problems in distillation when they concentrate in the ‘tails’ fractions of distilled spirits. In cases of ‘stills’ without fractionation capabilities

or of malfunction in column still operation, excessive amounts of these alcohols may appear in the product. Depending on the product (brandy or neutral spirits), excessive amounts of these compounds may exert a negative influence in the sensory characteristics of the distillate (Zoecklein and others 1995).

It has been reported that at lower concentrations below 300 mg/L (unless the must contains a high level of suspended solids), they usually contribute to the desirable complexity of the wine. Quantitatively, isoamyl alcohol generally accounts for more than 50% of all fusel oil fractions in distilled beverages (Muller and others 1993).

The distillation procedure influences the flavor composition of distillates. As a part of flavor compounds is removed during distillation, not only do the total amounts of the compounds in the distillate change, but their mutual relationships change as well. Thus the flavor compound ratios of the distillate may be quite different from those in wines. The relative proportions of these higher alcohols are relatively similar in whisky and brandy, as they are in wines (Nykänen 1985).

2.3.4.3 Terpenols

Monoterpenes play an important role in the grape and wine flavor of some aromatic cultivars (Seung 1996). Monoterpenes are present in the grape either in the free state or bound to sugars in the form of glycosides. Free monoterpenes include the volatile aroma compounds, known as terpenols, and the odorless terpenoid hydroxylated linalool compounds (polyols). The monoterpene precursors of terpenols are the bound terpenes and the polyols. The bound terpenes are a mixture of disaccharide glycosides of various monoterpene alcohols. It has been shown (Dubourdieu and others 1988; Hock and others 1984) that only trace amounts of terpenes are produced (<15 µg/L) as yeast by-products during fermentation, and hence almost no contribution of monoterpenes is attributed to yeast during fermentation (Seung 1996). Most of the monoterpenes exist in bound forms in juice; these include geraniol, linalool and nerol. In this form they are odorless. Their sensory thresholds can increase significantly if they are released from their bound saccharides. Enzyme treatment of the wine in this case causes a tremendous increase in the terpenes, resulting in a characteristic change of the original wine aroma.

2.3.4.4 Aldehydes

Several authors have reported the variation of total aldehyde with the type of yeast used (Then and Radler 1971; Fleet and Heard 1993). Romano and others 1994, divided 86 *S. cerevisiae* wine strains into groups producing low, medium and high amounts of acetaldehyde. The low and high phenotypes also differed considerably in the production of acetic acid (<500 mg/L), and a higher total content of higher alcohols (>300 mg/L). Wines resulting from fermenting with high producers showed a different pattern, containing detectable amounts of acetoin, elevated amounts of acetic acid (522 to 1185

mg/L) and a lower content of higher alcohols (256 to 270 mg/L). Aldehydes, chiefly acetaldehyde, are present in small amounts in brandy. Other compounds such as butyraldehyde benzaldehyde have also been reported. During distillation and wood maturation some acetaldehyde is produced by the oxidation of ethanol, a reaction which is facilitated by charcoal or aeration, hence the slow increase of aldehyde content noted in cognacs during aging. Ethanol and acetaldehyde react slowly to form acetal, a compound of pronounced odor. From the sensory point of view acetals are important because of their screening effect on the undesirable sensory effects of the parent carbonyls.

2.4 ENZYMES IN FRUIT WINEMAKING

Wine quality is measured by finesse and intensity, and by microbial and physicochemical stability and originates in taste and smell. Enzymes play a definitive role in the process of winemaking; wine can be seen as the product of enzymatic transformation of the grape juice. During winemaking, enzymes are desired as early as the pre-fermentation stage. Their activities originate not only from the grape itself but also from yeasts and other microorganisms (fungi, bacteria). The activity of endogenous enzymes alone is insufficient; to overcome this problem exogenous enzyme preparations are used (Canal-Llauberes 1992). Several factors must be considered when choosing a commercial enzyme preparation: wine type, processing conditions, desired effect and the cost/benefit relationship. Enzyme activity is a function of pH, temperature and contact time. Knowledge of the pH and temperature profile of the particular enzyme preparation determines the enzyme dosage and the cost to the winery. Decreases in pH, temperature and/or contact time, along with increases in other chemical characteristics of the must/wine (e.g. SO₂), increases the need for enzyme usage (and conversely) (Haight 1995).

Treatment of fruit pulp with pectic enzymes has made it possible to press soft fruits such as strawberries, ripe pears and soft cold-storage apples. Combinations of pectinases, pectic lyases and cellulases are being used for liquefying fruit, with the resulting advantages of increased yields and increased Brix. Enzyme effectiveness varies with the differences in cell wall composition of different fruits, hence the need to understand the cell wall structure in order to effectively solubilize and hydrolyze the cell-wall polysaccharides of different fruits (Wrolstad and others 1994). **Fig. 2.7** shows a general flow diagram for the processing of fruit and vegetable juices. The arrows indicate the points in the process at which enzymes are applied usefully.

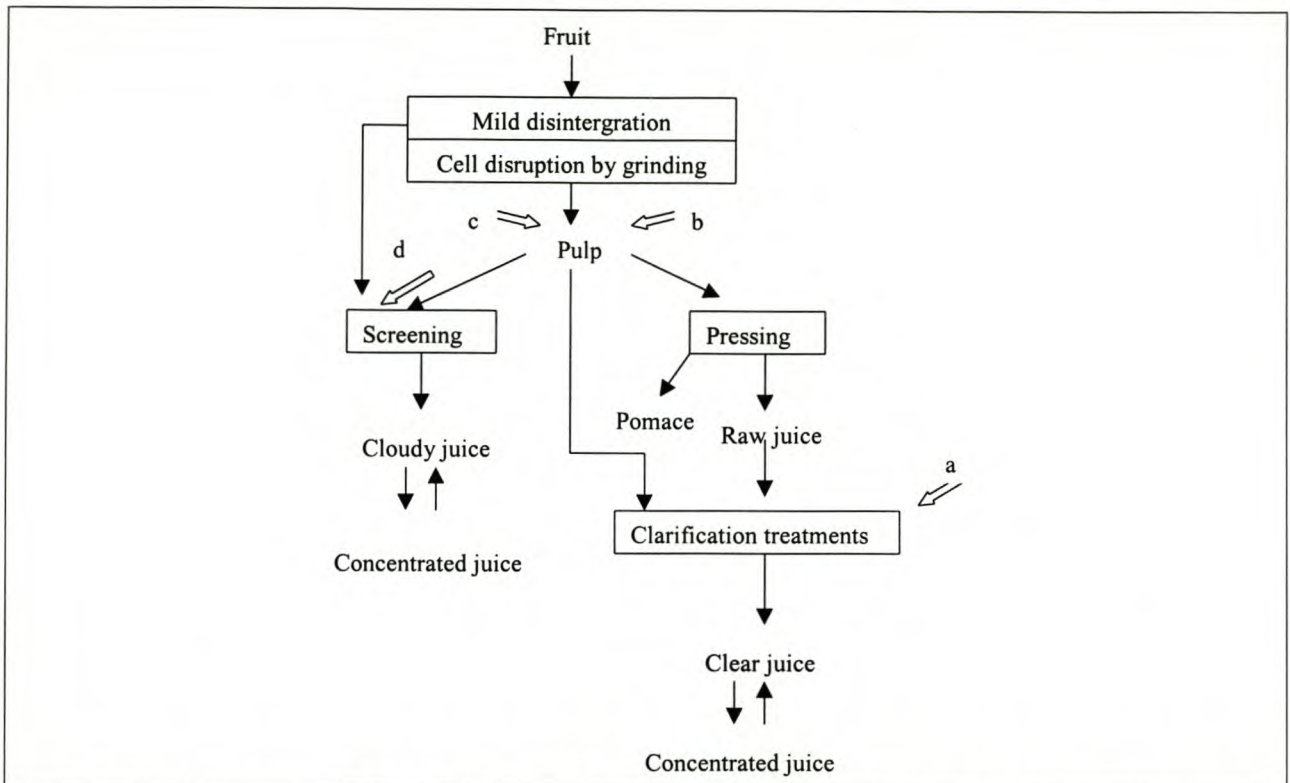


Figure 2.7 Flow diagram of fruit juice manufacture. Arrows indicate eventual enzyme treatments by (a) pectinases for clarification; (b) pectinases for pulp enzyming; (c) pectinases and C,-cellulases for liquefaction; and (d) polygalacturonase, pectin lyase, or pectate lyase for maceration (Pilnik and Voragen 1989).

Enzymatic treatments have a multiple purpose in that the changes made to the must and the wine have an influence on the processes of clarification and filtration, the process of extraction of color and the aroma and stability of the wine (Canal-Llauberes 1989).

2.4.1 Fruit juice clarification

Fruit juice clarification (**Fig. 2.7** arrow a) has been reported to be the oldest and still the largest use of pectinases, applied mainly to deciduous juices and grape juice. The traditional way of preparing such juices is by crushing and pressing the pulp. This gives a highly viscous juice with a persistent cloud of cell wall fragments and complexes of such fragments with cytoplasmic protein (Pilnik and Voragen 1989). Pectins are complexes of acids (homogalacturonan and rhamnogalacturonan) and neutral polysaccharides (arabinan, galactan, and arabinogalactan) that are found in the colloid form. They act as protective colloids, causing problems in the clarification and stabilization process (Feuillat 1987).

The addition of pectinases has been shown to lower viscosity by de-esterifying (pectin esterases) or depolymerising (polygalacturonases, pectin and pectate lyases) specific substrates. Pectinases facilitate aggregation of cloud particles to larger units

("break") which subsequently sediment and are easily removed by centrifugation or ultra filtration (Pilnik and Voragen 1989).

Volatile C₆ alcohol compounds such as hexanol are responsible for the grassy taste of juices and wines. Crushing of the grapes leads to a greater formation of C₆ compounds than the pressing of uncrushed grapes. Juice clarification reduces the amount of C₆ compounds in the juice (Dubourdieu and others 1986).

Also available for clarification of fruits affected by *Botrytis cinerea* are glucanases. The origin of serious problems in clarification and filtration is the presence of the beta-glucan, a polymer of glucose with a high molecular weight (Mr 10⁵-10⁶) synthesized by *Botrytis cinerea* in botrytised grapes. This compound prevents particles (including yeast and bacteria) from settling, resulting in very turbid wines (Haight 1995). Fleet (1991) reported the production of β -(1,3)-D-glucanases in many yeast species including *S. cerevisiae*. These enzymes show endo or exo activities. They are constitutive glycoproteins and their activities vary independently during the cell cycle (growth, mating, and sporulation).

Lubberes and others (1987) have shown that the effect of the enzyme endo- β -(1,3)-glucanase on the cell walls explains the release of mannoproteins during wine aging on lees. When using botrytised grapes, beta-glucanases can be added at either juice clarification or post fermentation. The dosage rate must be increased when the enzymes are added to chilled juice or wine, due to the unfavorable conditions of temperature and shorter contact times. Optimal results have been recorded when the enzyme preparation is added post-primary fermentation after the racking stage. Tannins as well as bentonite can irreversibly inactivate enzymes. This explains why red wines need higher dosages than white wines (Haight 1995).

2.4.2 Enzyme treatment of pulp for juice extraction

In must rich in pectic substances (e.g. from Muscat grapes), the addition of pectic enzymes to crushed grapes can improve juice extraction. Commercial preparations contain diverse enzymatic activities which are active at low pH: pectin methyl esterases (PME), polygalacturonases (PG), pectin lyases (PL) and hemicellulases. The mode of action of these enzymes is shown in **Fig. 2.8**. These pectolytic preparations can also contain diverse glycosidases and proteases responsible for secondary transformations. It is important that their degree of purity be assured (Ribereau-Gayon and others 2000).

Pectin methyl esterases (PME) de-esterify pectin, producing methanol and pectin with a lower degree of esterification. These are specific for galacturonic acid and are not able to de-esterify the pectin completely. They are characterized by an exo-mode of action. Polygalacturonases (PG) hydrolyze the glycosidic linkages between non-esterified galacturonic units. Endo-polygalacturonases hydrolyze low esterified pectins in a more or less random fashion, allowing a swift reduction in viscosity (endo-mode of action). Pectin lyases (PL) and pectic acid lyase catalyze beta-elimination reactions

between esterified and non-esterified galacturonic acid units following an endo-mode of action (Silva and others 1989).

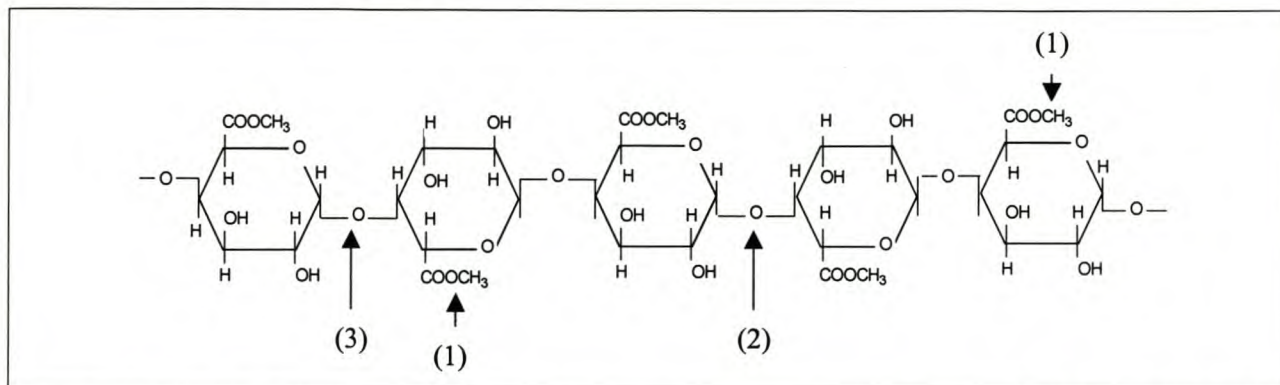


Figure 2.8 Portion of a pectin polymer showing sites of pectinase activity: (1) PME activity, (2) PG activity, and (3) PL activity (Haight 1995).

Over the years, work has been done to show that pulping of soft fruits like the marula with much soluble pectin results in a semi-gelled mass that is very difficult to press (Pilnik and Voragen 1993). Thin free-run juice and a pulp with good pressing characteristics is yielded as a result of enzymatic pectin degradation. Several authors have attributed increases in grape juice yields to the use of pectinases; Ough and Berg (1974) reported a 15% increase. These enzymes also participate in enhancing the filterability of wines. The treatment of crushed grapes with pectolytic enzymes in the production of red wine leads to a more rapid and complete breakdown of the cell wall of the skin and hence the extraction of anthocyanins that in turn enhance the color (Pilnik and Voragen 1993).

Enzymes can be inactivated by polyphenols; hence the need to aerate the pulp to allow oxidation of the polyphenols by the endogenous polyphenol oxidases. This results in polyphenol polymerization into high molecular weight compounds that are unable to inhibit the added enzymes. This also reduces the danger of a polyphenol haze in the finished product (Pilnik and Voragen 1993). It has been observed that enzymes that perform well in juice clarification are also suitable for the enzyme treatment of pulp for juice extraction.

2.4.3 Liquefaction

Enzymes can also be used for liquefaction; in this case pressing of the pulp as shown in **Fig. 2.7** is not necessary. The enzymes specific for this process are pectinases and exo-glucanases (C_1 -cellulase, cellohydrolase). Exo- β -glucanases work in conjunction with endo- β -glucanases (C_x -cellulase) that in most enzyme preparations are *Aspergillus*-derived. Complete liquefaction of a juice is reached when cell walls disappear. Examples of fruits and vegetables that can be liquefied are papaya, cucumber (which become almost clear on enzyme treatment), apples and peaches, which give a cloudy juice and

carrots, which give a pulpy juice depending on the accessibility of the cell wall compounds to the enzymes (lignin). The breakdown products thus increase the soluble solids content of the juices resulting in high juice yields. Liquefaction is an interesting process for vegetables and fruits that do not yield juice on pressing or for which no presses have yet been developed. Liquefaction is also advantageous in that it eliminates the costly waste disposal process (Pilnik and Voragen 1993).

2.4.4 Maceration

This is the transformation of organized tissue into a suspension of intact cells, resulting in pulpy products used as base material for pulpy juices and nectars, as baby foods and as ingredients for dairy products such as puddings and yogurts. Enzymatic maceration aims at transforming tissue into a suspension of intact cells and to achieve this, the pectin degradation should affect the middle lamella pectin only (Pilnik and Voragen 1993). The enzymes that carry out maceration (macerases) are preparations with only polygalacturonase and pectin lyase activity. Macerases are being used on various other substrates like vegetables, e.g. carrots. In this case the restricted pectin degradation solubilizes rather than degrades the middle lamella pectin, which may improve the creamy mouth feel.

Macerated products with only limited pectin degradation may gain consumer appeal due to the fact that most uses of exogenous pectinases for fruit juice extraction and clarification are based on the destruction of pectin and other desirable dietary fiber components. Macerated products thus promote the production of purified polygalacturonases and lyases (Pilnik and Voragen 1993).

2.4.5 Enzyme treatment for aroma extraction

These treatments help release aroma precursors and terpenyl glycosides responsible for the varietal character through hydrolysis (Rapp and Mandery 1986). Aroma of grape juice includes volatile free odorous substances, especially terpenes (linalool, geraniol, nerol, citronellel, α -terpineol, and linalool oxide) which play a fundamental role in giving character to certain cultivars, for example muscat. Recent studies have shown that enzymic hydrolysis occurs in 2 stages, following a sequential mechanism illustrated in **Fig. 2.9**.

In the first stage, the inter sugar linkage is cleaved by arabinofuranosidase, rhamnopyranosidase, or apiofuranosidase, (depending on the structure of the aglycon moiety), and the corresponding monoterpenyl β -D-glucosides are released. In the second stage, the liberation of mono-terpenols takes place after the action of a β -glucosidase on the previous monoterpenyl β -D-glucosidases (Gunata and others 1988; 1990a).

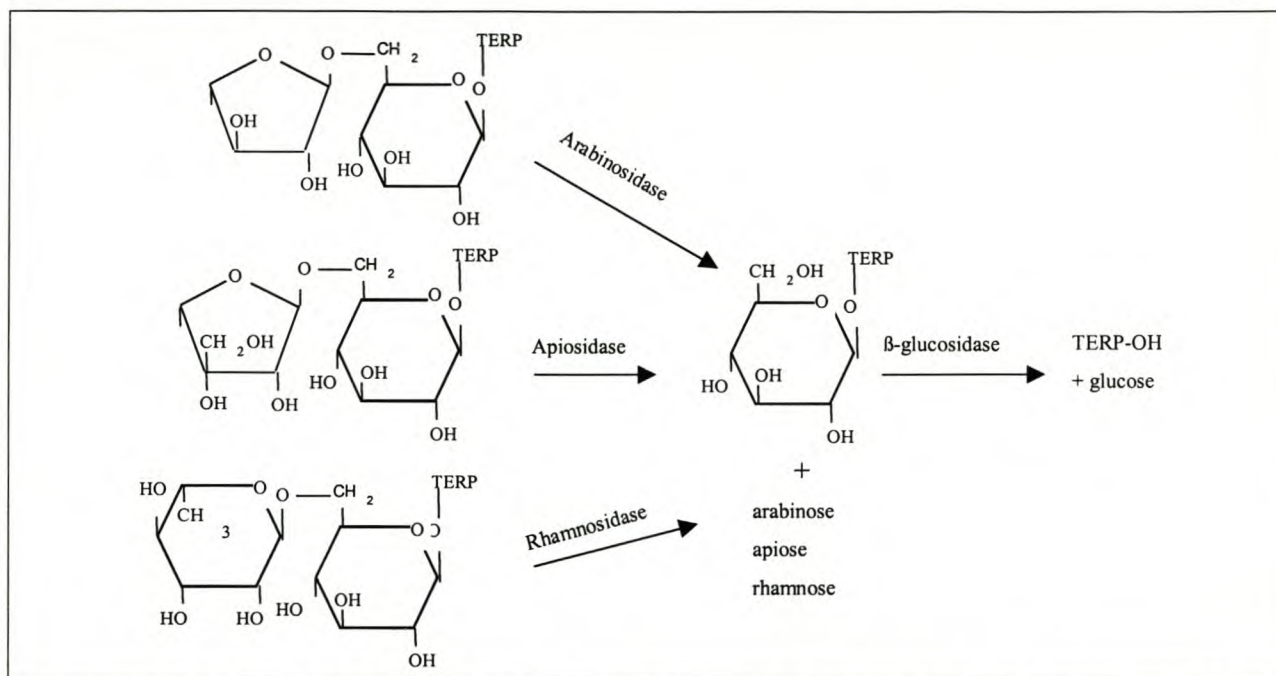


Figure 2.9 Sequential mechanism of terpene glycoside enzymic hydrolysis (Bayonove and others 1992). TERP: geranyl, neryl, linalyl, α -terpinyl, citronellyl.

Most studies have focused on β -glucosidases because of their wide occurrence in plants, fungi and yeasts. This enzyme, despite its capacity to liberate aroma substances in wine, is less effective in must. According to Dubourdieu and others (1988) the liberation of terpenols during fermentation can be explained by yeast β -glucosidase activity. They showed that the *S. cerevisiae* β -glucosidase was not inhibited by glucose, in contrast to what has been found for the grape glucosidase.

Proteases can have an intra or extracellular origin. Exocellular proteases have been shown to play an important role during aging. However, because of the particular conditions found in wine (pH in the range 3-4), only a few proteases are active (Lurton 1987). Yeast proteolytic activity has been shown to have a potential in protein haze reduction. These enzymes cause hydrolysis of the proteins into amino acids or short-chain peptides that are completely soluble in wine. However, intervention with exogenous enzymes is not free of problems (Cologrande and others 1994).

2.4.6 Effects of enzyme activity

The flavor intensity of many fruit juices is frequently enhanced during clarification. The glycoside flavor precursors identified include glycosides of aliphatic and aromatic alcohols, terpenes, lactones, carbohydrates and isoprenoids.

Enzymes are also used for production of low alcohol wine. Through enzyme treatment of the juice, glucose is converted into gluconic acid, which cannot be metabolized by the yeast. This results in a wine with reduced alcohol and higher acidity.

An additional beneficial effect of enzymes is the removal of phenolic compounds to prevent oxidation and hence stabilization of color. Cantarelli and others (1989) reported that the effect of conventional fining agents can be enhanced by laccase treatment (Canal-Llauberes 1992).

The use of enzyme preparations can also have adverse effects. Use of enzymes can result in the release of objectionable odors that are detrimental to the flavor quality (Chatonnet and others 1993). For example in citrus juices, it has been established that bound and free ferulic acid serves as a precursor to p-vinylguaiacol (PVG), which gives a rotten flavor. β -glucosidase liberates ferulic acid, hence its presence in orange juice should be avoided, whether its source is from commercial enzymes or from microbial contamination (Wrolstad and others 1994).

2.5 DISTILLATION

The sensory character of alcoholic beverages is changed as the absolute and relative concentrations of volatiles are altered by distillation. The partition coefficients of volatiles vary considerably with ethanol concentration, further altering the headspace volatile composition from the starting wine or wort. Non-volatiles, including polyphenols or organic acids, are virtually eliminated by distillation. The composition of distillate is determined by many factors, including still type, the degree of rectification, and the selection of fractions taken for inclusion in the distilled beverage. The concentration of individual compounds also varies within a particular spirit type and this can be ascribed to differences in the genetic composition of the raw material used, climatic factors and differences in technological production factors (Postel and Adam 1990). Distillation also notably affects the quantities of aroma compounds. The proportions of the compounds in the fermentation medium have been seen to be different to those in distillates. Consequently the small differences caused by different yeasts or fermentation conditions are not as significant in distilled beverages as in undistilled beverages.

Different methods of distillation are used all over the world depending on the type of beverage to be produced. Broadly, they fall into one of two groups: continuous stills, e.g. the "Armagnacais", and pot stills (Charante distillation).

2.5.1 Continuous stills

The boilers, distillation columns, wine-heater and cooler constitute the still's main parts as shown in **Fig. 2.10**. The column of the boiler ranges from 5 hL to a maximum of 35 hL; it is divided into two or three sections by separation plates. An open fire, propane gas or wood using indirect steam are different energy sources used to heat the wine. The column has 5 to 15 plates. These plates are fitted with differently shaped bubble-through devices, bubble caps, bell-shaped tunnels or oil grooves. The plates above the

wine arrival pipe are called 'dry plates' and help reduce tailing while increasing the alcohol concentration.

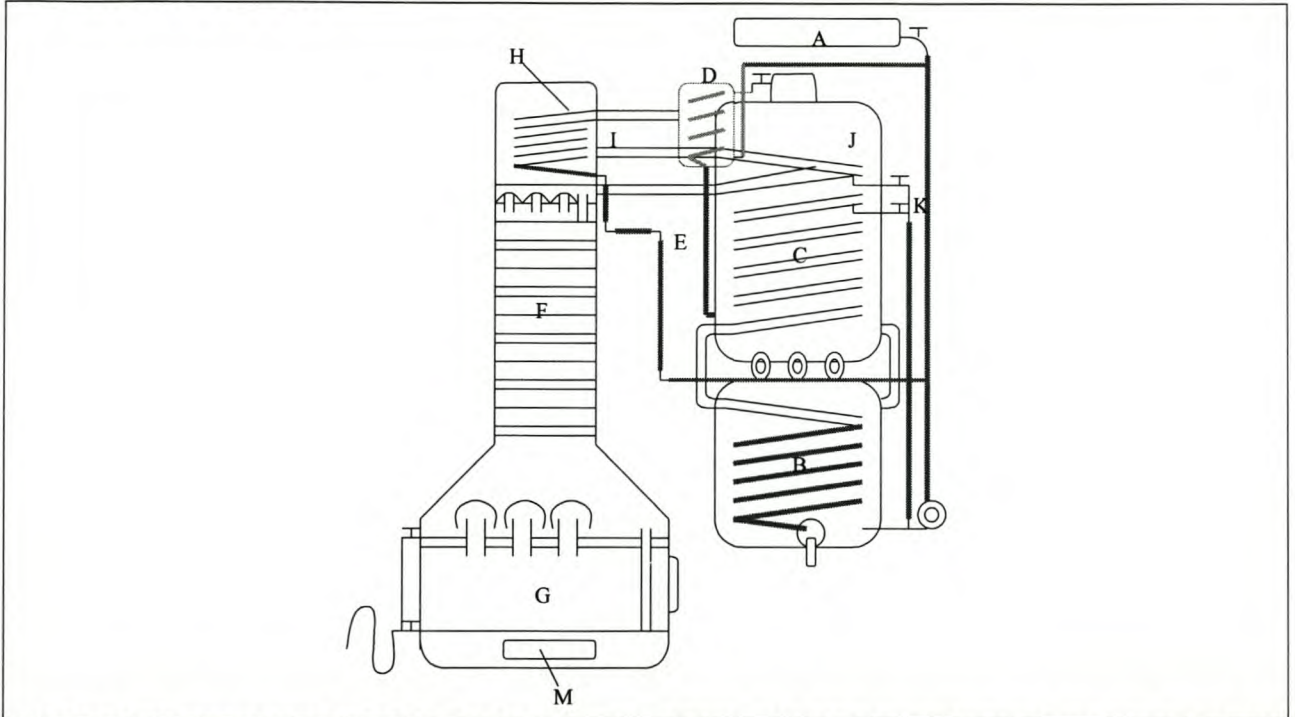


Figure 2.10 A, head of wine; B, cooler; C, wine heater; D, head condenser; E, wine arrival; F, column; G, boilers; H, head column coil; I, swan neck; J, coil; K, drawing and recycling of tailings; L, alcoholmeter holder; M, furnace (Bertrand 1989).

The wine is heated in the preheater using counter current heat exchange from a coil (which runs through the preheater) which is used to condense the alcoholic vapors arising from the top of the column.

Sometimes a head-foreshots condenser system can be fitted to the still above the wine-heater; more frequently though, a tail-products condenser is placed level with the alcoholic vapor pipe, between the column and the wine-heater. Tailings can also be collected in the first turns of the coil. The condensed fractions can be returned to the wine and recycled.

Continuous distillation is more economical than two-stage distillation and is also three times faster. With this method, the volatile substances are either entirely distilled (higher alcohols), or are more or less rectified (2-phenyl ethanol, ethyl lactate, 2,3-butanediol), according to their polarity (Wildbolz 1986). The fatty acid ethyl esters and fatty acids with a high molecular weight are released by heating the yeasts, which means that the quantity of these acids depends on the wine's yeast content.

To modify the composition of the spirit, the distiller can control two main parameters: wine flow and heating. Still adjustments play an essential role in the composition of the spirits: lowering heat or increasing wine-flow brings down the temperature at the base head column coil and results in a higher alcohol concentration at the base of the column. The amount of 'tailings' decreases exponentially when the percentage of alcohol

increases (Bertrand 1989). For prolonged aging, a large quantity of tailings is an advantage because of the 'winey' character of the tails fraction; but if the beverage (e.g. Armagnac) is to be marketed rapidly, it is preferable to make a clean high-proof distillate to limit the amount of such substances (Bertrand 1992).

2.5.2 The Charante distillation

This method is used for the production of Cognac spirits. The total capacity of the still is limited to 30 hL, corresponding to 25 hL of useful load. The heating must be by open fire: gas being the energy commonly used. Two successive distillates are necessary to produce a spirit with alcohol strength of no more than 72% vol. The first distillation of the wine produces the 'brouillis' of general alcohol strength of between 27% and 30% vol., depending on the initial strength of the wine. The brouillis in turn is submitted to distillation: this is the second distillation or "*bonne chauffe*" which generates three fractions: the heads, the heart and the tails. The rate of volatility of each constituent of the wine or of the brouillis depends on its physiochemical characteristics (Cantagrel and others 1990).

- The heads contain the most volatile elements (SO₂, aldehydes and other sulfur compounds) which are often detrimental to the quality of the spirit. Their volume represents 1-2% of the volume of the batch.
- The heart contains the most noble constituents of the aroma in their ideal proportions. It constitutes the authentic cognac/brandy that will be submitted to maturation.
- The tails are still rich in longer chain acids and alcohols, but also contain less-volatile components that must be removed, to avoid spoiling the quality of the spirit.

The use of pot stills promotes production of higher congener concentrations and heavier flavors. Fusel oil concentrations have been shown to be high in pot-distilled malt whisky and brandy, whereas column-distilled gin, vodka and grain-whisky have lower levels. Column still distillation strips flavor whilst only concentrating alcohol.

2.5.3 Technological factors influencing the quality of the product

2.5.3.1 Distillation

(i) *Pot still distillation without dephlegmation*

Acetaldehyde and the very volatile esters, for example ethyl acetate, to a lesser extent ethyl butyrate and some other esters are heads fraction compounds. Therefore, with a large heads fraction cutoff, not only will the unwanted acetaldehyde be removed, but many of the sensorically favorable esters will be removed as well. Typical tails fraction compounds include furfural, 2-phenethyl ethanol, benzyl alcohol, ethyl lactate and diethyl succinate. Thus separation of the tails fraction largely diminishes the concentration of these compounds (Postel and Adam 1990).

(ii) *Discontinuous distillation without a dephlegmator*

A dephlegmator or fractionating column is a device used in fractional distillation to cool the vapor mixture, thus condensing less volatile boiling fractions. The aroma compounds behave in much the same way as in a potstill without a dephlegmator. The stronger the dephlegmation, however, the more heart fraction compounds (such as higher alcohols and longer chain esters and volatile acids) are lost along with the tails fraction compounds (Postel and Adam 1990).

(iii) *Continuous distillation-Influence of the alcoholic cutoff point*

Postel and Adam (1990) have shown that the typical heads compounds acetaldehyde, ethyl acetate and methanol will remain in the spirit up to the cutoff value of 95% vol., the middle (heart) fraction compounds remain in the spirit up to a cutoff value of 87% vol. At cutoff values higher than 87%, a strong decrease in the concentration of these compounds becomes apparent. At a cutoff value of 77-79% the higher esters (beginning at ethyl myristate) as well as the typical tails fraction components already start to decrease in concentration. The cutoff value of 86% is the optimum cutoff point in a continuous distillation, practically no losses in heads and heart fraction components are incurred. However some of the longer chain esters will already be lost and most of the tails fraction will be largely eliminated in the resultant distillate.

2.5.3.2 Influence of yeast lees fraction

Increasing the fraction of yeast lees distilled along with the wine lead to increased concentration of ethyl caprate, -caprylate and -laurate. To a lesser extent, the concentrations of ethyl myristate, caproate and palmitate as well as acetoin, may also increase. Otherwise the yeast lees fraction has no influence on the concentration of remaining carbonyl compounds or terpenes. Storing the wine on its lees for 5 to 10 months has no significant effect on the concentration of esters during later distillation. Thus an increase in ester concentration is only achieved when the yeast lees is distilled along with the wine. However, excessively large fractions of yeast lees added to the distillation would not significantly (more than 0.1 mg/100 mL AA) affect the concentration of compounds like ethyl heptanoate and nonanoate (both possessing a distinctly "wine-like" aroma) in the distillate (Postel and Adam 1990).

2.5.3.3 Influence of mash pasteurization

Methanol concentration is greatly influenced by mash pasteurization. By rendering all the enzymes inactive at the heat of pasteurization, the methanol content is greatly reduced, for example in cherry mash. Postel (1984) showed that the content of methanol (a result of cleavage of pectin by pectinases) was reduced from 630-200 mg/100 mL of pure alcohol. Pasteurization has been shown to lead to a reduction in carbonyl

compounds and an increase in esters. Heat treatment does however lead to problems with mash fluidity.

2.5.3.4 Influence of juice fermentation

Experiments with cherries have shown that fermentation of free run juice reduces the methanol content by 80% when compared to fermentation with mash pulp. The concentration of fusel alcohols, carbonyl compounds, methyl and ethyl acetate and ethyl benzoate were also reduced, while the concentrations of esters such as isoamyl-, hexyl, benzyl and 2-phenethylacetate as well as ethyl caproate, caprylate, laurate and phenethyl acetate all increased. However, the distillates obtained from free run juice proved to be very neutral and of lesser sensory quality than those distilled from mash.

2.5.3.5 Influence of storage time of fermented mashes

Based on work done by Postel and Adam on cherry mash, the longer the fermented mash is stored the higher the concentration of propanol in the distillate. This is due to bacterial action that takes place during storage.

2.6 STRATEGIES FOR OPTIMISATION OF FERMENTATION FOR PRODUCTION OF MARULA WINE

The structure of the marula fruit pulp makes it a strong candidate for the use of enzymes: for juice extraction, improvement of pressing, improvement of clarification and filterability of both juice and marula wine. If the fruit is to be used for making a wine as the end product, it is more important that pectolytic enzymes be a part of the process. Furthermore, Wörner and others (1998) have reported that enzyme-treated pulp is easily fermentable, easier to pump and distill, and the equipment is easier to clean. Highly purified enzymes are advisable for use on the marula pulp in order to avoid side activities that may result in production of off-flavors.

The low sugar content in the fruit makes it suitable for the production of a low alcohol beverage; however if wine is the targeted end-product, it is definitely advisable to add sugar to attain the required alcohol concentration. Due to the high viscosity of the pulp, fermentation of the pulp without water dilution causes a high risk of stuck-fermentations. It is advisable to dilute the pulp at a ratio of at least 1:2 for a successful fermentation.

The marula fruit has a very high vitamin C content, hence a good balance between SO₂ and ascorbic acid must be found in order to limit the oxidation of the product. In this study the average vitamin C content was 496 mg/L; this makes the marula wine highly liable to oxidation. If applied in excess, SO₂ has negative side effects both to the wine and the consumer. Thus other sanitary methods and anti-oxidants should be considered. Fermentation at relatively lower temperatures is advisable for the marula fruit in order to get a fruity wine.

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CHAPTER 3

RESEARCH RESULTS I

Comparison of commercial enzymes on the processing of marula pulp, wine and spirits

RESEARCH RESULTS I

COMPARISON OF COMMERCIAL ENZYMES FOR THE PROCESSING OF MARULA PULP, WINE AND SPIRITS

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Commercial enzymes were used in this study to improve the yield and clarification of marula fruit (*Sclerocarya berria* sub. *caffra*) juice. An increase of up to 12% in juice treated with the enzyme Rapidase Filtration was recorded. A 15-fold improvement in juice clarity was observed after treatment with pre-fermentation processing enzymes. Marula wine fermented with *Saccharomyces cerevisiae* strain N96 was treated with enzymes after alcoholic fermentation to hydrolyze bound monoterpenes. An increase in the free monoterpenes of at least 92% was observed in enzyme treated juice. The distilled wines were analyzed for free monoterpenes. A panel of 10 judges was used to rank the juice, wine and distillate according to the 'nose' and the ability to portray a typical marula flavor of the end product. Enzymes had both positive and negative effects on the flavor of the juice, wine and distillate.

3.1 INTRODUCTION

Enzymes play a pivotal role in the winemaking process. In addition to enzymes which occur in pre- and post-fermentation practices, there are at least ten different enzymes driving the fermentation kinetics that convert grape juice to wine. It is therefore of key importance to understand the nature and behavior of these enzymes and to create the optimal conditions to exploit those enzymes that are beneficial, while inhibiting those that may be detrimental to wine quality. Many of these enzymes originate from the grape itself, the indigenous microflora on the grape and the microorganisms present during winemaking. Commercial enzyme preparations are widely used as supplements since the endogenous enzymes of grapes, yeasts and other microorganisms present in must and wine are often neither efficient nor sufficient under winemaking conditions to

effectively catalyze the various biotransformation reactions (for a detailed review on enzymes in wine making see Van Rensburg and Pretorius 2000).

Pectolytic enzyme preparations are used in many fields of food technology with great success and for many years (Ough and Berg 1974). In wine and fruit juices these enzyme preparations are mainly used to yield more juice and increase the press capacity (Wörner and others 1998). In addition, research has also shown the important influence of these enzymes on the flavor and quality of wines. The use of pectolytic enzyme preparations has been reported to affect the components related to the sensory quality because these enzyme preparations often also contain other enzyme activities (e.g. cinnamylesterase, glucosidase, oxidase) that can have a negative effect on wine (Lao and others 1997).

The available aromas in a fruit impart and define the characteristics and to a larger extent, the final quality of the end product. The maceration process promotes the extraction of aroma compounds since a considerable amount of aroma substances are located in the skin of the berries. Cordonnier and Bayonove (1974) reported that some of the aroma compounds found in grapes are in a volatile and odorous form, while others are glycosidically bound aroma precursors. Terpenic compounds constitute the greater part of these aroma compounds and most are the odorless terpenic precursors (Günata and others 1985; Gueguen and others 1996; Park 1996). These odorless monoterpenes are transformed completely into odorous compounds by enzymes either during juice extraction, alcoholic fermentation or post fermentation. Although there are numerous reports on the presence of bound monoterpenes and use of commercial enzymes for their hydrolysis in grapes and wines very little research has been reported on the marula fruit juice, wine and spirits (Pretorius and others 1985). The marula is one of the most commonly utilized wild fruit of Southern Africa. Drought resistance, exceptional yield per tree and the possibility to utilize both the fruit and nut contained within the seed. Also the ease of harvesting, the exotic flavor and nutritional value of the fruit are some of the characteristics that have led recently to its commercial utilization (Weinert and others 1990).

Dubourdieu and others (1988), studied the β -glucosidase activity in their investigation of the effect of the contribution of monoterpenes by yeasts and Hock and others (1984) investigated the formation of terpenes by yeasts during alcoholic fermentation. In both cases only trace elements of terpenes were produced ($<15 \mu\text{g/L}$) showing the small contribution the yeast strains make towards the production of terpenes during fermentation. On the other hand King and Dickinson (2000) showed that yeasts (on grapes and hops), are able to transform a range of monoterpene alcohols using a variety of reactions. These reactions are therefore likely to influence the organoleptic properties of wine and beer.

In this study we report the effect of commercial enzyme preparations on juice yield, clarification and flavor, specifically the enhancement of the typical marula flavor in marula juice, wine and spirits by hydrolysis of bound monoterpenes (non-volatile and water insoluble) using different commercial enzymes.

3.2 MATERIALS AND METHODS

3.2.1 Fruit juice

Chilled marula pulp was collected from South Africa's Northern Province in the 1999 and 2000 seasons. The pulp had on average 5° Brix sugar concentration and pH 3.7.

3.2.2 Enzymes

Enzymes were used in two parts, pre-fermentation, for juice extraction and clarification and post fermentation for the enhancement of wine aroma. The enzymes are shown in **Table 3.1**. All experiments were performed in duplicate and also repeated in the 1999 and 2000 seasons.

Table 3.1-Enzymes used for juice extraction, clarification and aroma enhancement

Pre-fermentation processing enzymes			
Enzyme	Description	Dose	Producer
Rapidase filtration	Pectinases & β -glucosidase activities	0.03 g/L	Gist-brocades
Rapidase X-press	Pectinases,	0.03 g/L	Gist-brocades
Rapidase TF	Pectinase, hemicellulase, cellulose activities	30 μ /L	Gist-brocades
Rapidase Adex-P	Pectinases, rhamnogalacturonases hemicellulases	30 μ /L	Gist-brocades
Rohapect DA6L	Pectinase, arabinase activities	30 μ /L	Röhm
Post fermentation enzyme preparations for the liberation of varietal aromas			
Enzyme	Description	Dose	Producer
AR2000	Pectolytic enzymes with glucosidases	0.03 g/L	Gist-brocades
Novoferm	Pectolytic enzyme	0.2 g/L	Novo Novodisk Ferment Ltd.
Trenolin Bukett	Pectolytic enzymes with β -glucosidase activity	0.1 g/L	Erbloslöh-Geinsenheim.

3.2.3 Juice extraction

Commercial enzymes were added to 1 L of marula pulp and 15 mg/L of velcorin was added to inhibit most bacterial and all yeast activity. Non-enzyme treated pulp was used as the control. After the addition of enzymes the pulp was left to stand for 72 h. After 72 h the pulp was centrifuged at 2 500 rpm for 10 min in order to remove the solid particles of the pulp. The juice collected was measured and weighed.

3.2.4 Enzyme addition to hydrolyze bound monoterpenes

The aroma liberating enzyme preparations were added directly to wines that had been fermented to dryness with untreated marula juice using the yeast strain N96. The wines were left at 20°C for 4 weeks to allow the enzymes to hydrolyze the bound monoterpenes. After 4 weeks, the wines were analyzed for terpene composition using gas chromatography (GC). The wines were stored at 4°C until the panel of judges did sensorial analysis.

3.2.5 Winemaking

The thickness of the marula pulp, resulted in problematic fermentations and hence the marula pulp was diluted with water in the ratio 1:1 to reduce the viscosity of the juice. Sugar was added to the juice at a concentration of 35% per liter of water added. Diammonium phosphate (DAP) was added to the juice to adjust the nitrogen concentration of the juice depending on the free available nitrogen (FAN) concentration of the pulp. The amount of DAP added was calculated using the formula

$$\frac{43.9 - \text{FAN}/^{\circ}\text{B} \times 0.5}{0.108} = \text{g/hL DAP}$$

The juice was inoculated with the commercial wine yeast, *S. cerevisiae* strain N96, at a 0.2 g/L concentration and fermented at 20°C. Fermentation was performed in 4.5 L bottles. The fermentation process was followed by measuring the decrease in the weight of the bottles and alcoholic fermentation was considered complete when the weight of the bottles stabilized. Upon completion of alcoholic fermentation, the wines were racked and centrifuged at 2500 rpm for 10 min and the clear wine was stored at 4°C until distillation, chemical and sensory analysis.

3.2.6 Analysis

On completion of alcoholic fermentation, the wine was analyzed for residual sugars (RS), alcohol (% v/v), volatile acid (VA), total acids (TA) and pH. The average results are shown in **Table 3.2**.

Table 3.2 Average routine results from analysis of marula wine after fermentation

Component	Concentration
Residual sugars (g/L)	4.1
Alcohol % (v/v)	13.03
Volatile acidity (mg/L)	0.24
Total acidity (mg/L)	8.69
pH	3.72
Ascorbic acid (mg/L)	495.9

3.2.7 Terpene extraction and analysis of monoterpenes

50 mL of wine sample was used in a liquid-liquid extraction procedure using 30 mL n-pentane and 2 mL ethyl pelargonate as the internal standard. Continuous liquid-liquid extraction was carried out at 60 rpm in a rotary evaporator (without vacuum) for 30 min after which the n-pentane layer was collected and concentrated. The concentration of the n-pentane phase was performed in a water bath at 55°C until 2 mL was left. One mL of the concentrate, was collected and analyzed for terpenes. The analysis of terpenes was performed on a Hewlett Packard HP5890 series II gas chromatograph coupled to an HP7673 auto sampler and injector and an HP3396A integrator. The Lab Alliance capillary column was used, with dimensions 60 m x 0.321D x 0.5 µm. Hydrogen was used as the carrier gas with the FID detector at 250°C; injector temperature: 200°C; split ratio: 20 mL/min; oven temperature program: 35°C for 15 min, thereafter increasing at 6°C/min to 230°C; run time: 75 min.

3.2.8 Distillations

The wines were double distilled in electrically heated round bottomed flasks capable of holding 4.5 L of liquid. In order to simulate the conditions of a copper pot still, 0.67 g/L copper sulfate and two strips of copper metal were added. Boiling stones were added to ensure homogenous heat distribution during the distillation process. In the first distillation the wines were brought slowly to evaporation after which the flow rate was maintained at 5 mL/min throughout the distillation process which lasted for 10 h. Distillation was stopped at 30% (v/v) alcohol. The second distillation was divided into two phases. The first phase was the collection of the first 1% of the distillate at a flow rate of 1 mL/min. This fraction was discarded. The second phase proceeded with an increased flow rate of 5 mL/min. Distillation proceeded until the distillate (or the heart fraction) reached an alcohol concentration of 70% (v/v) alcohol. This fraction was collected and kept at 4°C for further analysis.

3.2.9 Sensory Evaluation

Sensory evaluation of the resultant wines and distillates was based on a line scale and a 4 point ranking scale. The panel of 10 judges comprised of brandy and marula liqueur producers. All judges possessed extensive commercial brandy and marula base wine and wine distillate tasting experience. Samples of 50 mL were presented in random order at 15°C in randomly numbered, clear, 125 mL tulip shaped glasses. Samples were evaluated at a room temperature of 22°C ± 1°C under white light. Evaluations took place in the mornings between 09.00H and 10.00H. The wines were not diluted or pre-treated. The 70% distillates were diluted using water to an alcohol strength of 23% v/v alcohol.

3.2.10 Statistical Analysis

Analysis of variance (ANOVA) was performed for all the juice yields and the turbidity of the juices. The average linkage cluster analysis was performed on the enzyme treated juice and on the enzyme treated wines and the resulting spirits using their terpene compositions. This was done using the STATISTICA processing package.

3.3 RESULTS AND DISCUSSION

3.3.1 The influence of pre-fermentation processing commercial enzymes on juice

3.3.1.2 Juice yield

Application of enzyme to the pulp for enzyme extraction resulted in an increase in juice yield as shown in **Table 3.3**.

Table 3.3 Influence of pre-fermenting processing enzymes on juice

Enzyme	Juice yield (mL)	Turbidity (NTU)	Total terpenols (µg/L)
Rapidase Filtration	597.1 ^a	19 ^a	53.12 ^{ns}
Rapidase Xpress	593.7 ^b	7 ^a	379.50 ^a
Rapidase TF	590.5 ^b	9 ^a	60.54 ^{ns}
Rapidase Adex P	591.4 ^b	10 ^a	75.43 ^{ns}
Rohapect DA6L	569.5 ^b	45 ^a	519.86 ^a
Control	533.4	742	27.71

^a denotes a 99.99% significance level, ^{ns} denotes not significant

^b denotes a 99% significance level.

An increase of at least 6.7% yield was observed for the enzyme Rohapect DA6L. Rapidase Xpress gave an increase in yield of 10.66%. The highest yield obtained was 12% by treating the pulp with the enzyme Rapidase Filtration.

3.3.1.3 Clarification

Commercial enzymes have been marketed due to their improvement of clarification or the extraction of color and aroma. The majority of these commercial preparations are pectolytic enzymes that act on the skin by degrading it hence facilitating sedimentation of colloidal particles resulting from the degradation of pectins, arabanes, galactanes and arabino-galactanes (Chatonnet and others 1992; Haight and Gump 1994). The control gave the most turbid juice of 742 nephelometric turbidity units (NTU) compared to the juice treated with enzymes. Among the enzyme treated juices, Rohapect DA6L gave the most turbid juice of 45 NTU. This can possibly be ascribed to the fact that the enzyme preparation has pectinase and arabanase activity compared to the others that have hemicellulase activities (**Table 3.1**). The enzymes, Rapidase Adex-P, Rapidase TF and Rapidase Xpress gave the lowest turbidity and hence the most clear juice. These enzymes are pectinase plus hemicellulase preparations and this can explain why they both performed better in the marula pulp which is very thick and hence has a high degree of hemicellulose and cellulose linkages. The clarity of the juice has a direct impact on the filterability of the juice as has been reported by Brown and Ough (1981) and Berg (1959).

3.3.1.4 Aroma enhancement

The five pre-fermentation processing commercial enzymes were evaluated on their action on monoterpene hydrolysis (**Table 3.4**). The olfactory perception thresholds of monoterpenes are rather low with the most odoriferous being citronellol (18 µg/L) and linalool (50 µg/L). Terpeneol and nerol have the lowest threshold of 400 µg/L and that of geraniol (rose flavor) is 130 µg/L. It is important to note that the olfactory impact of terpene compounds is synergistic and hence it is difficult to say exactly which compound contributed to the perceived flavor.

Table 3.4 Liberation of volatile terpenols from marula fruit juice by enzymic treatment

Terpenols (µg/l)	Control	Rapidase filtration	Rapidase tf	Rapidase adex p	Rapidase xpress	Rohapect DA6L
Linalool	14.36	20.65	32.24	29.31	179.31	200.94
Terpeneol	10.48	27.88	17.57	36.21	147.70	284.87
Citronellol	28.38	-	-	-	-	-
Nerol	-	2.27	5.42	5.42	28.15	-
Geraniol	2.88	3.45	8.02	4.43	24.35	34.05
Total	27.71	53.12	60.54	75.43	379.50	519.86

Increases in total terpenes of 1269.5%, 1776%, 172%, 118% and 92% by treating the pulp with enzymes Rohapect DA6L, Rapidase Xpress, Rapidase Adex-P, Rapidase TF and Rapidase Filtration, respectively, were observed. These results are in agreement

with those reported by Gueguen and others (1996) on treatment of different fruits (mango, strawberry, apple juice and Sauvignon blanc wine) with β -glucosidases. Their results indicated that free volatile compound (terpenes, cyclic alcohols) concentrations increased significantly in enzyme treated fruit juices and wines. It is interesting to see that these processing enzymes that are normally used to improve filtration and juice extraction do contain side activities that can improve the monoterpene content of the juice. Normally, the β -glucosidase activity would not work before fermentation, because it is repressed by glucose but in the marula juice, the natural glucose concentration seems to be low enough not to repress the enzymes. The results of the average linkage cluster performed on the volatile terpenes of the juice are shown in **Fig. 3.1**.

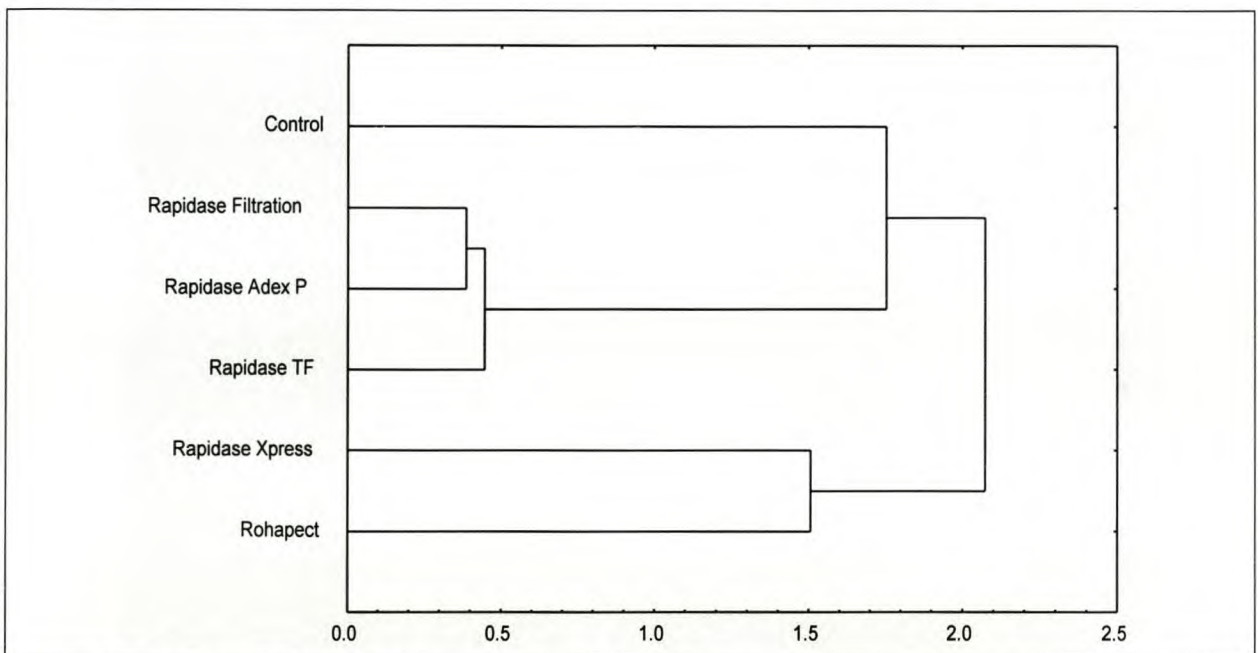


Figure 3.1 Average Linkage Cluster analysis for terpenes of enzyme treated juice.

It is clear that Rohapect DA6L and Rapidase Xpress had a different flavor profile compared to the Rapidase Filtration-, Rapidase Adex-P- and Rapidase TF-enzymes and that of the control. When sensory evaluation was done on the treated juice, the panel of judges agreed that Rohapect DA6L and Rapidase-Xpress gave a slightly more intense typical marula flavor profile compared to the control. On the other hand, juice treated with the enzyme Rapidase Filtration was found to have a more intense and better profile compared to the control. Rapidase Adex-P and Rapidase TF gave an intense flavor profile but generally this was perceived as bad and not typical of the marula flavor. Several authors have reported that apart from producing desirable reactions such as increased yield and improved stability, enzymes can also catalyze undesirable reactions resulting in pigment degradation or off-flavor formation (Wrolstad and others 1994; Miklósy and Pölös 1995). It is thus important to avoid having reactions that result in adverse quality changes.

3.3.2 Influence of post-fermentation aroma releasing commercial enzymes on marula wine

Fruit aroma includes volatile free odorous substances, especially terpenes (linalool, terpineol, citronellol, nerol and geraniol) and bound glycosides (non-volatile precursors as terpenglycosides) that are the precursors of the flavoring aglycons when hydrolytic reactions take place. The β -glycosidases responsible for the release of bound monoterpenes have been reported to be strongly inhibited by glucose (Park 1996) hence the need to add them towards the end of fermentation or after fermentation. Miklósy and Pölös (1995) reported the increase in activity of β -glucosidases by 5-15% ethanol. The enzymes, AR2000, Novoferm and Trenolin Bukett were added to the wines after fermentation for maximum activity. The results of gas chromatography analysis of the terpene volatiles are shown in **Table 3.5**.

TABLE 3.5 Liberation of volatile terpenols from marula wine by post-fermentation aroma releasing enzymes

Terpenols ($\mu\text{g/L}$)	Control	Trenolin bukett	Novoferm	AR2000
Linalool	116.20	128.46	119.38	220.45
Terpineol	65.55	69.75	68.96	122.76
Citronellol	4.79	4.52	4.66	6.61
Nerol	8.64	12.89	9.39	16.20
Geraniol	21.39	23.58	25.45	44.55
Total	213.47	239.19	227.83	410.57

The major contributor of the floral characteristic of the control wine and the enzyme treated wines is linalool. The presence of terpene levels in the control can be attributed to the fact that the fermented marula pulp contains some skins from the fruit and hence a higher level of naturally occurring enzymes. Yeasts also contain enzymes that liberate terpenes to a certain extent. The addition of pectolytic enzymes resulted in striking increases in the monoterpene profile of at least 7% for the wine treated with Novoferm and 92% for the wine treated with AR2000 being observed. There are no significant changes in minor free monoterpenes, citronellol and nerol, for the enzyme treated wine except for those treated by the enzyme AR2000. In grapes, the minor monoterpenes have been reported to be present and their contribution to the grape or wine is highly unlikely without synergistic or additive effects (Park 1996). Free linalool increased from 14 $\mu\text{g/L}$ to a maximum of 116 $\mu\text{g/L}$ in the wine without enzyme treatment. This increase might have been due to yeast glucosidases present during fermentation and it is also possible that chemical hydrolysis might have taken place. Treating the wine with the enzyme AR2000 resulted in a 90% increase in terpenes. There was no significant difference in aroma and taste between the control and the wine treated with Novoferm.

There was, however, a significant difference between the control and the wines treated with AR2000 and Trenolin Bukett. This is clearly shown in **Fig. 3.2** by the average linkage cluster.

The panel ranked the wines in terms of aroma intensity and typicality of the marula flavor. Wines treated with enzymes were ranked as having a higher overall flavor intensity. Enzymic hydrolysis helped release aroma precursors and terpenyl glycosides responsible for the character of the fruit.

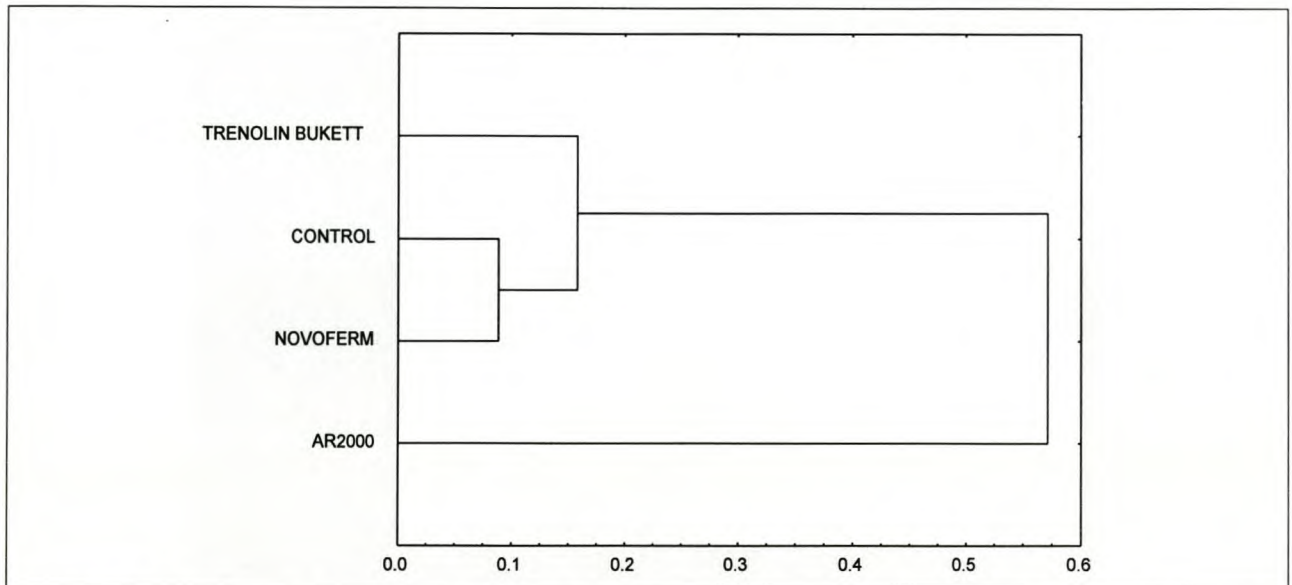


Figure 3.2 Average linkage cluster analysis for terpene volatiles of enzyme treated wines

3.3.3 Influence of post-fermentation aroma releasing enzymes on spirits

In this study, an overwhelming increase in terpenes was observed after distillation as shown in **Table 3.6**. This can be attributed to the concentration of the volatile compounds on distilling. However, the percent increase proportions in the distillate are different from those in the wines. This suggests that heat treatment during distillation might have had a great influence on the hydrolysis of the terpenes. On distillation, the average linkage cluster in **Fig. 3.3**, shows a different grouping of spirits.

TABLE 3.6 Volatile terpenes in spirits of enzyme treated wines

Terpenols $\mu\text{g/L}$	Control	Trenolin bukett	Novoferm	AR2000
Linalool	291.25	319.74	441.34	575.15
Terpeneol	168.05	201.25	279.15	310.67
Citronellol	20.23	20.28	48.20	48.10
Nerol	19.46	21.67	24.10	30.88
Geraniol	756.90	683.83	984.59	810.76
Total	1255.9	1246.8	1777.4	1775.6

High temperature acid treatment may lead to an extensive rearrangement of the terpenols. Di Stefano (1989), studied the transformations of the terpenic compounds and showed that as the pH of the aqueous medium diminishes, the hydration and cyclisation of linalool increases.

The difference in the linkages is not that different for all the wines. Even though the distillation process affected the terpene levels, the highest levels were found in spirits from enzyme treated wines (Novoferm and AR2000). It is tempting to speculate that this increase can be explained by the increase in activity of the enzymes during heating, before denaturing, since enzyme activity is highest at temperatures 40°C to 60°C. The enzymes still present in the wines are thus activated by the increase in temperature and hydrolyze the bound terpeneglycosides thereby increasing the concentration of the corresponding free terpenes in the spirits or the heat itself can denature these bound terpeneglycosides.

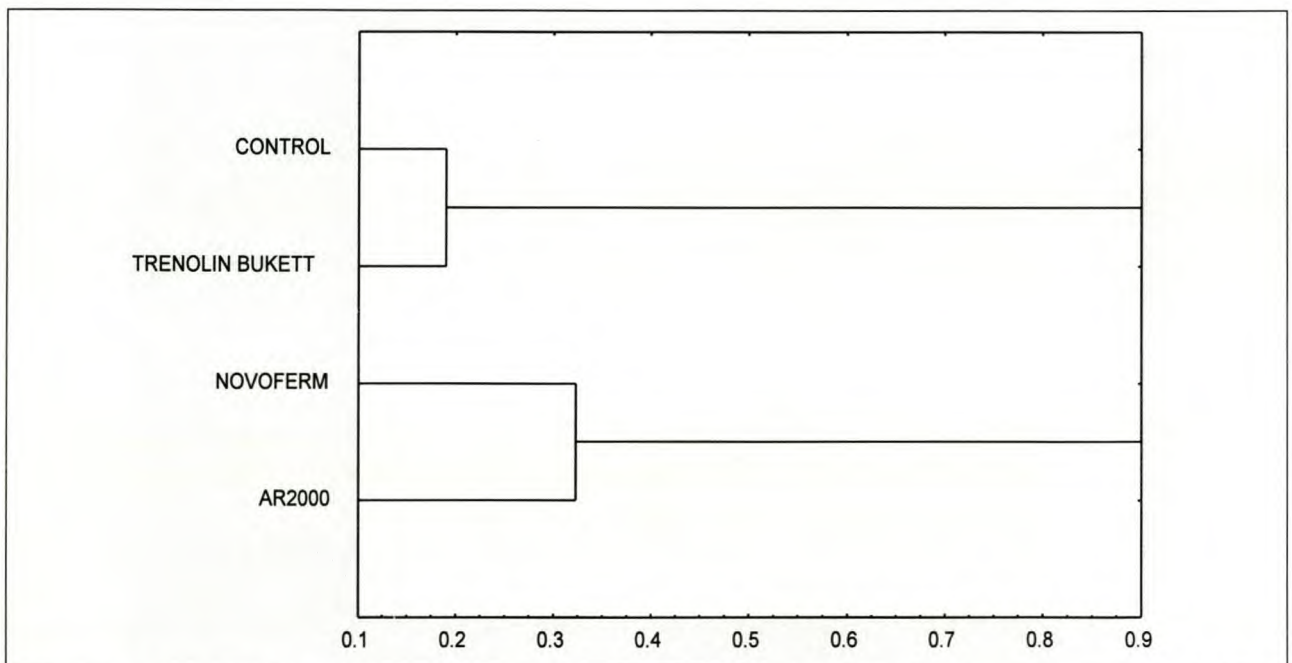


Figure 3.3 Average linkage cluster analysis for terpene volatiles of spirits from enzyme treated wines.

Sensory evaluation of the spirits made from enzyme treated wines showed an appreciation of the spirits treated by Trenolin Bukett and the control. Spirits from wines treated with the enzymes, Novoferm and AR2000 were ranked as possessing very little typical marula characters.

3.4 CONCLUSIONS

Enzyme treatment of marula juice had a significant influence on the yield, clarification and the aroma profile of the juice and the wine. The commercially available enzyme preparations contain different side activities and hence the differences in their

performance. The enzyme Rohapect DA6L, although not resulting in enough yield, does have some side activities that result in the enhancement of the aroma through release of terpenes and also gives a significantly clear juice compared to the control without changing the typical marula flavor profile. The enzyme Rapidase Xpress is excellent in increasing the juice yield (12%), results in an extremely clear juice (7 NTU) and a phenomenal increase in total terpenes that enhance the typical marula flavor profile. The enzymes Rapidase Adex-P, Rapidase Filtration and Rapidase TF gave juice that had a flavor profile significantly different from the control with Rapidase Adex-P and Rapidase TF perceived as having negative flavor notes in them.

Post-fermentation treatment of wine with enzymes enhances the aroma profile of wines and spirits. The process of distillation per se has an influence on the aroma profile of the spirits. These results show that it is advisable to use the enzyme Trenolin Bukett for post-fermentation treatment because, although as it increases the aroma profile of the wine, it still is closely related to the control which in this case should portray the unaltered marula profile. AR2000 has an overwhelming effect on the flavor profile but the risk of deviating from the typical marula flavor on using this enzyme is high.

This work has shown that the commercial pectolytic enzyme preparations influence differently the flavor profile when used on the same fruit and hence it is important to first do trials on a small scale with different preparations and then choosing and optimizing the conditions for the best performing enzyme preparation.

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CHAPTER 4

RESEARCH RESULTS II

Optimization of fermentation processes for the production of indigenous fruit wines and distillates

RESEARCH RESULTS II

EFFECT OF DIFFERENT YEAST STRAINS ON THE FLAVOR PROFILE OF THE MARULA FRUIT WINES AND DISTILLATES

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Keywords: marula juice, commercial yeast strains, volatile compounds, wine, distillate

Juice of the *Sclerocarya birrea* sub. *caffra* (marula) fruit was fermented by indigenous microflora and different commercial *Saccharomyces cerevisiae* yeast strains at different temperatures, namely 15°C and 30°C. Volatile acids, esters and higher alcohols were quantified for the wine and distillates and the results were interpreted using a multivariate analysis of variance (MANOVA) and an average linkage cluster analysis. Significant differences, between 15°C and 30°C, and also between yeasts with respect to volatile compounds, were observed. The yeast strains VIN7 and FC consistently produced wines and final distillates significantly different from the other strains. The panel of tasters comprising of marula and brandy producers was asked to select wines and distillates that had an acceptable and typical marula 'nose' and also to detect the differences between wines and distillates fermented with the same yeast strain at different temperatures.

4.1 INTRODUCTION

The use of selected yeast cultures as starters for wine fermentation has led to the production of more consistent wines. Yeast and fermentation conditions are claimed to be the most important factors that influence the flavors in wine. Aroma compounds play a significant role in the quality of our food and luxury products due to their pronounced effect on our sensory organs. Several authors have reported on the influence of yeast added to an alcoholic fermentation on wine quality (Heard and Fleet 1986; Kunkee and Vilas 1994; Giudici and others 1993; Bertolini and others 1996; Atienza and others 1998; Antonelli and others 1999; Steger and Lambrechts 2000), because higher alcohol and ester contents in the wine depend on yeast and fermentation temperature (Killian and Ough 1979; Aragon and others 1998). Dubourdieu and Chatonnet (1993), reported that different yeast strains have different enzyme activities that can vary on their action on the precursors. Yeast form and modify the important components of fermented

beverages namely volatile organic acids, aldehydes, alcohols and esters (Soles and others 1982). The production levels of these by-products are variable and yeast strain specific. Thus the yeast strain used during fermentation can have a great influence on the ultimate quality of the end-product. This makes the choice of yeast strain crucial if good quality fruit wines and distillates are to be assured.

Literature on the influence of yeast on volatile composition of wines, shows that different yeast strains have great differences in volatile compound production (Di Stefano and others 1981; Soles and others 1982; Steger and Lambrechts 2000). The concentration of wine aroma compounds can be influenced by various factors, amongst others the environment (climate, soil), grape variety, degree of ripeness, fermentation conditions (pH, temperature, yeast flora), wine production (oenological methods, treatment substances) and aging (bottle maturation) of wine (Rapp 1998). Pretorius and others 1985, studied the volatile flavor components of marula juice and showed that sesquiterpene hydrocarbons and benzyl alcohol are the major aroma components. The marula aroma extracts could be separated into 153 compounds. An odor assessment after GC separation of the aroma compounds showed the absence of a character impact compound. They concluded that the constituents of the aroma extracts contribute to the over-all flavor according to their aroma value.

The aim of this work was to study the effect of different commercial yeast starter cultures on the flavor of marula wines and distillates. This study focused on significant differences between alcoholic fermentation secondary products in particular volatile composition. The aromas of the wines obtained by fermentation of marula fruit pulp were evaluated by a panel of experienced judges. This study contributes towards a collaborative programme aimed at the enhancement of the quality of products derived from one of Africa's most popular wild fruits, the marula.

4.2 MATERIALS AND METHODS

Fruit juice: Chilled marula pulp was collected from the Northern Province of South Africa in the 1999 and 2000 seasons. The pulp had on average 5° Balling and pH 3.7. Due to the thickness of the pulp, fermentations of the undiluted pulp resulted in stuck fermentations hence the marula pulp was diluted with water in a 1:1 ratio to reduce the turbidity of the juice.

Experimental design: The investigations were carried out in parts; the effect of fermentation temperature (15°C, 30°C) and effect of the yeast strain on the resultant wine and distillate. Ten randomly selected commercial yeast strains and spontaneous fermentations were studied. In order to compare the effects of yeast, fermentation temperature and distillation process the fermentations were done in triplicate twice for each analysis during the seasons of 1999 and 2000. The yeast strains used are shown in **Table 1**.

Table 4.1 Commercial yeast strains used in this study

<i>Saccharomyces cerevisiae</i> strains	Source
DY10	Anchor Yeast, South Africa
DY502	Anchor Yeast, South Africa
Fermol Clariferm (FC)	AEB Africa
VIN7	Anchor Yeast, South Africa
VIN13	Anchor Yeast, South Africa
N96	Anchor Yeast, South Africa
WE14	Anchor Yeast, South Africa
228	Anchor Yeast, South Africa
WE372	Anchor Yeast, South Africa
NT116	Anchor Yeast, South Africa

Analysis: Conventional parameters such as balling, reducing sugars (RS), alcohol, volatile acidity (VA), total acids (TA) and pH were measured, these are shown in **Table 4.2**.

Table 4.2 Average routine analysis of resultant marula base wines fermented at 15°C

Yeast strain	Residual sugar (g/L)	Ethanol %	Volatile acidity (mg/L)	Total acidity (mg/L)	pH	Ascorbic acid
DY502	2.70	11.00	0.17	6.60	3.92	412.50
WE372	3.50	11.40	0.19	6.40	3.99	434.50
WE228	2.80	11.40	0.17	6.50	3.95	440.00
DY10	2.70	12.30	0.19	6.60	4.04	440.00
WE14	1.90	11.40	0.19	7.50	3.77	440.00
VIN13	2.90	11.90	0.20	6.60	4.00	440.00
NT116	2.10	12.30	0.19	6.90	4.03	448.30
N96	2.70	11.60	0.15	6.60	3.99	453.80
SPONT	3.20	11.00	0.17	6.40	4.01	448.00
VIN7	2.40	13.80	0.26	6.40	3.97	423.50
FC	6.80	13.80	0.20	6.90	3.63	192.50

Winemaking: Sugar was added to the diluted juice at a concentration of 35% per liter of water added. Di-ammonium phosphate (DAP) was added to the juice to adjust the nitrogen concentration of the juice depending on the FAN concentration of the pulp. The amount of DAP added was calculated using the formula

$$\frac{43.9\text{-FAN}^{\circ}\text{B} \times 0.5}{0.108} = \text{g/hL DAP.}$$

The juice was inoculated with a *Saccharomyces cerevisiae* yeast strain at 0.2 g/L concentration and fermented at the desired temperature. Fermentation was done in 4.5 liter bottles. The fermentation process was followed by measuring the decrease in weight of the bottles and alcoholic fermentation was considered complete when the weight of the bottles stabilized. Upon completion of alcoholic fermentation, the wines were racked and centrifuged at 2500 rpm for 10 minutes and the clear wine was stored at 4°C until distillation, chemical and sensory analysis.

Distillations: The wines were double distilled in electrically heated round bottomed 4.5 liter flasks. In order to simulate the conditions of a copper pot still, 0.67 g/L copper sulfate and two strips of copper metal were added. To ensure a homogenous heat distribution during the distillation process boiling stones were added. In the first distillation, the wines were brought slowly to evaporation after which the flow rate was maintained at 5 ml/min throughout the distillation process which lasted for 10 h. Distillation was stopped at 30% (v/v) alcohol. The second distillation was divided into two phases. The first phase was the collection of 1% of the first distillate at a flow rate of 1 mL/min, this fraction was discarded. The second phase proceeded with an increased flow rate of 5 mL/min. Distillation proceeded until the distillate (or the heart fraction) reached an alcohol concentration of 70% (v/v). This fraction was collected and kept at 4°C until analysis.

Extraction of volatile compounds and their analysis: 50 mL of wine sample was used in a liquid-liquid extraction procedure using 30 mL diethylether and 4 mL of a 2.2 mg/L solution of 4-methyl-2-pentanol serving as the internal standard. Continuous liquid-liquid extraction was carried out at 60 rpm in a rotary evaporator (without vacuum) for 30 min after which 1 mL of the diethylether layer was collected and analysed for volatile compounds. For 70% (v/v) alcohol distillates, volatile extraction was done by taking a 5 mL sample to which 0.25 mL internal standard was added and mixed before analysis. The analysis for volatile compounds was run on a Hewlett Packard HP5890 series II gas chromatograph coupled to an HP7673 auto sampler and injector and an HP3396A integrator. The column used was a Lab Alliance capillary with dimensions, 60 m x 0.321D x 0.5 µm; hydrogen was used as the carrier gas and an FID detector at 250°C; injector temperature: 200°C; split ratio: 20 mL/min; oven temperature programme: 35°C for 15 min, thereafter increasing at 6°C/min to 230°C; run time: 75 min.

Sensory Evaluation: Sensory evaluation of the wines and distillates was based on flavor quality and intensity on a line scale. The judges were asked to determine the acceptability of the samples considering the 'nose' and mark on the line scale the intensity of the flavor profile. The panel of judges comprised of brandy and marula

liqueur producers from a major brandy producing company in South Africa. All the judges possessed extensive commercial brandy and marula base wine and distillate tasting expertise. Samples of 50 mL were presented in random order at 15°C in randomly numbered, clear, 125 mL tulip-shaped glasses. Samples were evaluated at room temperature of 22°C ± 1°C under white light. Evaluations took place in the mornings between 09.00 and 10.00. The wines were not diluted or pre-treated. The 70% distillates were diluted using distilled water to an alcohol strength of 23% v/v alcohol.

Statistical analysis: Analysis of Variance (ANOVA), Principal Component Analysis and the UPGMA cluster analysis were calculated for all the base wines and distillates using the gas chromatographic data pertaining to their respective volatile compound compositions. Cluster analysis and PCA are useful for finding natural groups among the samples. These were calculated using the STATISTICA program. Results of the sensory evaluations were processed by the SYSTAT program using the Kolmogorov-Smirnov test. For none of the judges rankings was the distribution significantly different from the normal. A triangular test was done on the wines and distillates to determine the effect of fermentation temperature. Results of the triangular test were analyzed using a Triangular testing program (Randall 1998).

4.3 RESULTS AND DISCUSSION

Effect of fermentation temperature on volatile concentrations: Several authors have reported on the influence of fermentation temperature on the volatile concentration and hence quality of wines (Killian and Ough 1979; Amerine and Ough 1980; Aragon and others 1998). In this study, the concentrations of carbonyl compounds like acetaldehyde and higher alcohols tended to increase with increasing temperature (**Table 4.3**). Total esters and total volatile acids increased at lower fermentation temperature. The production of acetaldehyde increased at a fermentation temperature of 30°C for all the yeast strains used in this experiment except for the VIN7 yeast strain where 13.62 mg/L acetaldehyde was produced at 15°C and 8.27 mg/L was produced at 30°C. In wine samples fermented with Fermol Clarifiant (FC) no acetaldehyde was detected at either 15°C or 30°C. For the rest of the yeast strains acetaldehyde was not detected at 15°C. At 30°C the concentrations varied considerably, from 2.75 mg/L in wines fermented with strain WE372 to 21.72 mg/L in wines fermented with strain DY502. These results disagree with Amerine and Ough (1980), who reported that temperature does not have an effect on acetaldehyde formation.

There was a significant reduction of total esters – ethyl acetate at a higher fermentation temperature. An increase of at least 2-fold in the esters was observed at 15°C with the exception of VIN13 that produced more esters at 30°C than at 15°C. The production of ethyl acetate did not show much variation between the different temperatures.

Table 4.3 Comparison of volatile flavor compound production (mg/l) between fermentations of 15°C and 30°C

Yeast	Acetaldehyde		Total esters		Total esters-ethyl acetate		Propanol		Total HAs		Total Volatile acids	
	15°C	30°C	15°C	30°C	15°C	30°C	15°C	30°C	15°C	30°C	15°C	30°C
DY502	-	21.72	52.08	26.84	20.25	5.17	101.17	189.48	896.8	571.63	1508.44	241.23
WE372	-	2.75	43.9	26.59	13.41	5.22	81.8	169.93	542.16	531.14	342.4	177.42
WE228	-	7.51	42.53	29.6	13.05	5.96	100.4	193.08	725.39	646.47	545.62	206.95
DY10	-	6.49	30.48	33.86	10.27	6.88	80.84	180.39	614.23	753.92	490.48	253.83
WE14	-	7.88	37.01	37.07	11.49	8.83	88.74	216.11	655.06	941.58	488.66	299.5
VIN13	-	9.38	30.7	49.54	11.14	10.67	72.89	341.79	581.4	1201.37	458.95	402.79
NT116	-	12.03	48.56	34.79	15.82	6.91	110.19	232.01	886.18	972.29	676.52	300.04
N96	-	10.4	28.82	31.68	11.35	6.93	73.55	264.25	560.34	1110.16	455.11	229.38
SPONT	-	6.16	39.2	28.05	11.08	5.87	88.82	166.27	635.73	654.47	654.4	201.48
VIN7	13.26	8.27	28.92	39.62	8.71	7.11	69.27	215.88	499.77	632.25	357.06	343.42
FC	-	-	63.22	33.73	14.93	6.39	325.25	191.07	674.95	643.36	386.96	272.45

Wine fermented with yeast strains apart from DY502, WE372 and FC exhibited increased levels of higher alcohols (HA) at 30°C. Propanol concentrations showed distinct increases with increase in temperature. Low concentrations were recorded at 15°C with the exception of FC that had a lower concentration at 30°C.

Total volatile acid concentrations were higher at 15°C than at 30°C for all the strains. At 15°C the highest recorded concentration of volatile acids of 1 508.44 mg/L was exhibited in the wine fermented with the strain DY502 and the lowest concentration was recorded in the wine fermented with WE372, that had 342 mg/L. At 30°C the total volatile acids ranged from 177.42 mg/L exhibited in wine fermented with WE372 to 402.79 mg/L in wine fermented with VIN13. This may be due to the interaction between temperature, yeast strain and other factors. From these results the choice of a suitable fermentation temperature would be in favor of 15°C as this gave more esters and less higher alcohols which can result in undesirable odors if in excess.

A triangular test was done on the wines fermented at 15°C and at 30°C and their resultant distillates. There was a significant difference between the wines and the distillates fermented at different temperatures as shown in **Table 4.4**. For the yeast strains VIN7, VIN13, DY10, DY502, N96, WE228, and WE14 the preferred temperature was 15°C. Strain WE372 was preferred at 30°C fermentation temperature. For strain FC the differentiation between the two fermenting temperatures was not significant, however, the overall preferred temperature was 15°C. On distillation of the wine fermented with the strain FC, there was a distinct difference at 99.99% confidence level of preference at 30°C. Samples with native microflora had an irregular behaviour with respect to temperature. This is in agreement with work done by Aragon and others (1998), on grape wine. Due to the preference of the tasters for wines and distillates fermented at 15°C, we decided to make a comprehensive analysis of the volatile compounds in wines on the 15°C fermentations. Therefore the results presented from now onwards were based on the 15°C fermentations.

Table 4.4 Effect of fermentation temperature on the performance of yeast strains in the marula fruit

STRAIN	WINE		DISTILLATE	
	SL-Value	Preferred treatment	SL-Value	Preferred treatment
VIN7	96.14	15°C	99.12	15°C
DY10	96.14	15°C	99.99	15°C
N96	99.12	15°C	99.84	15°C
VIN13	96.14	15°C	87.79	15°C
DY502	99.12	15°C	99.84	15°C
FC	87.79	15°C	99.99	30°C
NT116	99.99	30°C	89.65	15°C
WE228	99.99	15°C	99.98	15°C
WE14	99.99	15°C	99.84	15°C
WE372	99.12	30°C	99.99	30°C
SPONT	96.14	15/30°C	96.53	15/30°C

Influence of yeast type on higher alcohols: For juice with high turbidity like the marula pulp, the production of higher alcohols during fermentation is of great significance on the sensory evaluation of the wine and distillate produced. Higher alcohols themselves have little impact on sensory properties of wine, however concentration of the HA fraction during distillation can be high enough to render the flavor of the product unpleasant due to their strong, pungent smell and taste. This is particularly true for *iso*-amyl-alcohol which is the component usually produced in largest amounts (Nykänen and Suomalainen 1983; Boulton and others 1995). The average HAs of wines fermented at 15°C and their distillates are shown in **Tables 4.5** and **4.6**. It was clear from these tables that different yeast strains produced different concentrations and ratios of HAs. Strains VIN7, WE372 and N96 exhibited the lowest relative total HA concentrations, DY502 and NT116 produced the highest total HAs in the base wines and in turn had high levels of total HA in their distillates. The spontaneous fermentation exhibited intermediate concentrations of total HA in the base wine which consequently produced a relatively low concentration of total HA in the final distillate.

VIN7 and N96 had a 6-fold increase respectively in total HA concentration from the wine to the distillate concentration. These two strains in turn produced wines whose propanol levels increased significantly with a 19.3-fold and 28-fold increases respectively. The spontaneous fermentation and WE228 had a 4.8-fold and 4.7-fold increase in their total HA concentrations respectively. DY10, WE372 and VIN13 had a 5.2, 5.5 and 5.6-fold increase in their HA concentrations respectively. Strains NT116 and FC exhibited a 3.4-fold increase in their total HAs. DY502 produced the lowest increase, 3.6-fold in the total HA on distillation. The wines fermented using the yeast strain WE14 exhibited a 5-fold increase of total HAs on distillation.

Methanol concentrations ranged from 116.53 mg/L for wines fermented with N96 to 206.99 mg/L for wines fermented with strain FC. The boiling point for methanol is 65°C and hence on distillation it was collected in the heads fraction which explains its absence in the hearts fraction.

The increase in propanol from the base wine to the concentration in the second distillate varied from a 5.8-fold increase in strain FC to a 28-fold increase in distillate resulting from wine fermented with N96. Such a difference in the increase in propanol can be attributed to the individual strain characteristics, different yeast lees exhibit different effects on higher alcohols (Giudici and others 1990). This can be attributed to the cell wall polysaccharides that have the ability to bind to particular compounds (Steger and Lambrechts 2000). The yeast cell wall is made up of 50% glucans and 50% mannoproteins and these are able to bind with compounds like β -ionone, ethyl hexanoate and octanal whose hydrophobicity plays an important role. N96 produced wine with 73.55 mg/L propanol whereas strain FC produced wine with 325.20 mg/L. However, after distillation N96 had a higher propanol concentration of 2 060.60 mg/L than that of strain FC which was 1879.70 mg/L. This clearly showed how misleading it could be to choose a yeast strain for the production of a distillate based on the

Table 4.5 Volatile compound profile of marula base wines made from the 10 commercial strains and a spontaneous fermentation (mg/L)

COMPONENT	VIN7	N96	SPONT	WE14	WE228	DY10	VIN13	WE372	DY502	NT116	FC
Acetaldehyde	13.63	-	-	-	-	-	-	-	-	-	-
Ethyl acetate	20.22	17.47	28.12	25.52	29.48	20.21	19.56	30.48	31.83	32.74	48.30
Ethyl butyrate	0.57	0.96	0.96	0.83	0.90	-	0.78	1.06	1.11	1.13	1.33
<i>i</i> -amyl acetate	0.22	0.46	0.46	0.34	0.39	-	0.21	0.36	0.38	0.37	1.79
Ethyl caproate	0.36	0.37	0.42	0.47	0.51	0.35	0.35	0.51	0.58	0.52	1.01
Hexyl acetate	0.13	0.41	-	-	-	-	-	0.50	-	-	-
Ethyl lactate	3.26	3.84	3.74	3.79	4.56	4.06	3.86	5.72	9.49	5.56	5.90
Ethyl caprylate	0.32	0.33	0.39	0.44	0.47	0.33	0.33	0.49	0.54	0.49	0.99
Ethyl caprate	0.48	1.02	0.99	1.19	1.32	1.00	0.97	1.54	1.80	1.66	2.70
Di-ethyl succinate	1.96	3.84	3.84	4.20	4.65	4.30	4.43	2.97	6.10	5.82	1.21
2-Phenethyl acetate	1.41	0.13	0.26	0.25	0.25	0.23	0.20	0.27	0.25	0.27	0.00
Total esters	28.92	28.82	39.20	37.01	42.53	30.48	30.70	43.90	52.08	48.56	63.22
Total esters - ethyl acetate	8.71	11.35	11.07	11.49	13.05	10.27	11.14	13.41	20.25	15.82	14.93
Methanol	151.81	116.53	181.79	165.52	182.17	131.51	124.28	123.04	243.94	221.70	206.99
Propanol	69.27	73.55	88.82	88.74	100.39	80.84	72.89	81.80	101.17	110.20	325.25
<i>i</i> -butanol	35.28	40.63	36.43	38.04	43.00	44.53	42.18	81.04	44.36	56.12	23.73
<i>n</i> -butanol	1.15	1.20	1.58	1.33	1.47	1.26	2.01	1.12	1.84	1.91	1.47
<i>i</i> -amyl alcohol	242.26	251.82	250.39	278.54	309.57	269.91	266.77	191.61	387.32	382.81	104.81
Hexanol	-	-	-	-	-	-	-	5.72	-	-	-
2-Phenethyl ETOH	84.41	76.60	76.72	82.89	88.79	86.19	81.26	57.83	118.17	113.45	12.70
Total HAs	499.77	560.34	635.73	655.06	725.38	614.23	589.40	542.16	896.80	886.18	674.95
Acetoin	1.55	1.43	1.36	9.17	24.28	1.74	1.16	21.93	145.18	3.82	0.97
Acetic acid	348.05	442.97	646.80	479.83	535.86	482.61	450.69	331.44	1495.81	665.97	369.81
Propionic acid	0.73	1.48	1.22	1.32	1.36	1.42	1.43	1.86	2.07	1.92	1.53
<i>i</i> -butyric acid	1.27	1.29	1.18	1.43	1.57	1.39	1.30	1.77	1.57	1.68	0.71
<i>n</i> -butyric acid	0.22	2.04	0.12	0.13	0.12	-	0.10	0.75	0.17	0.17	0.28
<i>i</i> -valeric acid	0.92	0.14	-	0.09	0.12	0.11	0.12	0.13	0.11	0.12	0.12
<i>n</i> -valeric acid	0.10	0.23	0.24	0.22	0.20	0.17	0.19	0.19	0.24	0.28	0.34
Hexanoic acid	1.41	1.79	1.43	1.91	2.14	1.41	1.95	1.19	2.99	2.06	4.13
Octanoic acid	2.02	2.67	1.90	2.54	2.90	2.07	1.97	2.90	3.89	2.80	5.22
Decanoic acid	2.34	2.49	1.51	1.19	1.35	1.31	1.20	2.17	1.59	1.52	4.82
Total volatile acids	357.06	455.11	654.40	488.66	545.62	490.48	458.94	342.40	1508.44	676.52	386.96

Table 4.6 Volatile data on 70% v/v spirits obtained from distillation of marula base wines made from the different yeast strains (mg/L)

COMPONENT	WE228	VIN13	FC	VIN7	SPONT	N96	DY10	NT116	DY502	WE372	WE14
Acetaldehyde	90.08	100.48	115.10	69.38	96.40	205.97	102.63	91.08	89.58	93.76	101.97
Ethyl Acetate	41.55	32.38	64.93	26.86	42.26	38.64	33.96	41.06	35.48	40.75	38.04
<i>i</i> -amyl Acetate	1.62	1.60	4.19	1.37	1.70	0.99	1.39	0.90	1.13	1.78	1.92
Ethyl Caproate	1.56	1.93	3.04	1.44	2.31	1.89	2.01	2.28	2.08	2.11	1.71
Hexyl Acetate								0.11			
Ethyl lactate	17.48	18.34	24.20	17.38	19.99	19.92	18.63	16.53	19.03	18.00	19.39
Ethyl caprilate	2.09	1.89	2.55	1.53	2.20	1.77	1.92	2.16	2.00	1.92	2.23
Ethyl caprate	1.50	1.49	1.56	1.23	1.65	1.34	1.40	1.42	1.48	1.34	1.11
Di-ethyl succinate	1.37	1.32	0.82	1.49	1.43	1.40	1.46	1.44	1.35	1.32	1.32
2-phenethyl acetate	1.48	0.93	0.63	0.76	1.02	0.79	0.89	0.85	0.90	0.87	0.87
Total esters	68.65	59.88	101.91	52.06	72.58	66.74	61.65	66.76	63.45	68.09	66.60
Total esters-ethyl acetate	27.10	27.50	36.98	25.20	30.31	28.10	27.69	25.69	27.97	27.34	28.56
Propanol	1496.38	1557.45	1879.74	1339.87	1410.64	2060.62	1448.74	1898.76	1501.49	1367.07	1468.84
<i>i</i> -butanol	175.76	145.27	140.88	135.01	148.29	140.56	156.42	136.54	157.13	136.92	165.85
<i>n</i> -butanol	7.91	8.98	9.39	8.80	8.75	7.46	9.06	8.91	8.69	9.78	7.57
<i>i</i> -amyl alcohol	1648.64	1517.75	659.89	1440.25	1417.25	1399.40	1549.07	1449.45	1520.00	1425.35	1606.81
Hexanol	0.68	0.49	0.27	0.34	0.65	0.41	0.43	0.51	0.62	0.50	0.56
2-phenethyl ETOH	54.34	59.46	16.29	54.25	58.59	63.81	55.43	49.37	62.71	57.11	54.89
Total HAs	3383.71	3289.40	2706.47	2978.52	3044.16	3672.26	3219.14	3543.54	3250.64	2996.73	3304.52
Acetic acid	11.98	10.54	11.53	9.74	6.12	7.12	13.97	14.48	7.16	10.81	13.93
<i>i</i> -butyric acid	3.90	2.43	0.96	0.82	5.06	1.66	1.99	2.64	3.13	2.77	3.94
Hexanoic acid	8.39	7.57	7.16	7.01	8.51	7.55	6.68	5.88	7.86	6.30	6.09
Octanoic acid	0.67	0.93	0.58	0.62	0.77	0.71	1.05	0.76	0.74	0.84	0.92
Decanoic acid	5.08	7.09	5.39	4.02	5.84	4.91	4.11	4.57	5.40	5.35	5.99
Total volatile acids	30.01	28.56	25.62	22.22	26.30	21.95	27.81	28.34	24.29	26.06	30.87

performance of the yeast in the wine. The process of distillation and yeast lees present in the wine at the time of distillation contribute greatly to the resultant product. The HAs that were quantified in this study (*n*-propanol, isoamyl alcohol, *n*-butanol and *iso*-butanol) all have boiling points lower than 200°C and are soluble in alcohol. They are also completely or partially soluble in water. They therefore distill mainly into the heart fraction of the distillate with only a small fraction, mainly methanol distilling first into the heads fraction (Steger and Lambrechts 2000).

iso-butanol production also varied with the yeast strain and an increase in its concentration was observed after the second distillation of the base wines. Strain WE372 gave the lowest increase of 1.7-fold on distillation whereas strain FC had a 5.9-fold increase. The concentrations for *n*-butanol observed were very low compared to the other HAs. The lowest concentration in *n*-butanol of 1.12 mg/L was observed in the wine fermented with the strain WE372 and the highest concentration of 2.01 mg/L was observed for the wines fermented with strain VIN13. Trace concentrations of hexanol were observed in the wines fermented from pulp collected in the 2000 season with only wines made with strain WE372 exhibiting some hexanol. Hexanol concentrations were not detectable in the wines and on distillation these concentrations increased to amounts detectable. The highest concentration of hexanol, 0.7 mg/L was detected in the wine fermented with strain WE228. Concentrations of 2-phenethyl alcohol ranging from 14 mg/L in the distillate resulting from wine fermented with strain NT116 to 19.2 mg/L in the distillate resulting from a spontaneous fermentation were observed. The 2-phenethyl alcohol concentrations decreased after distillations which is in agreement with the fact that its boiling point is 219°C (Ribereau-Gayon and others 2000) and hence it is more likely to go into the tails fraction.

The results showed how fruit fermented under the same conditions gave a completely different volatile profile of a product, due to the use of different yeast strains that produced different proportions of the various volatiles. More or less the same trend in the proportions of volatile increases were observed during the two harvest seasons 1999 and 2000. However the 1999 season had highly odorous fruit compared to the 2000 fruit which was exposed to floods at the end of maturation. This resulted in the dilution of the fruit giving it a low aromatic intensity and the heavy character of the HAs was thus more pronounced.

Ester production by the different strains: Esters impart a pleasant smell. Young wines derive their fresh, fruity aroma characteristics largely from the presence of a mixture of esters produced during fermentation. The most significant esters are those of higher alcohols which are ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl hexanoate and 2-phenethyl acetate and esters of straight chain saturated fatty acids.

Ethyl acetate has been reported by various authors to be the main ester in wine (Ribereau-Gayon and others 2000; Steger and Lambrechts 2000) in agreement to these reports, the same trend was observed in the marula wines fermented at 15°C and 30°C. The concentrations, however, varied significantly from season to season;

1999 had concentrations ranging from 100 to 193 mg/L whereas in the year 2000 ethyl acetate concentrations varied from 17.50 to 48.30 mg/L.

Low concentrations of ethyl butyrate ranging from undetectable in DY10 to 1.30 mg/l in FC were observed in the wines and the distillates for all the yeast strains had undetectable concentrations. Ethyl butyrate might have been included in the heads fraction and hence could not be detected in the heart fraction which was analyzed for volatiles. Ribereau-Gayon and others (2000) have reported the olfactory perception of ethyl acetate as being approximately 160 mg/L, at high levels, it can spoil the wine bouquet with an unusual, unpleasant, pungent tang, whilst at very low doses, (50-80 mg/L) ethyl acetate contributes to a wine's olfactory complexity and thus has a positive impact on quality. Isoamyl acetate levels in DY10 were not detectable, FC had the highest concentration of 1.80 mg/L. The second distillation of FC exhibited the highest concentration of iso amyl acetate of 4.20 mg/L. Ethyl caproate concentrations did not vary that much between the different strains in the wine except for FC that had 1.00 mg/L which was at least double the concentrations contributed by the other strains. The distillate also gave a high concentration of 3.00 mg/L compared to the lowest which was exhibited in wine fermented with strain VIN7 with a concentration of 1.40 mg/L.

Hexyl acetate was recorded at very low levels in the wines and was not detectable in the second distillates except for strain NT116 that had a concentration of 0.10 mg/L. Di-ethyl succinate concentrations also varied considerably from 1.20 mg/L for the base wine fermented with strain FC, to 6.10 mg/L in wine fermented with strain DY502. The resultant second distillates portrayed a similar trend with di-ethyl succinate levels from wine fermented with strain FC having a low concentration of 0.80 mg/L and that of strain DY502 having a relatively high concentration of 1.40 mg/L. Other esters (ethyl caprylate, ethyl caprate and 2-phenethyl acetate) did not vary considerably in their concentration among the different yeast strains. The presence of ethyl lactate in the wines and distillates may be linked to malolactic fermentation, and in this case we cannot rule out the involvement of an esterase of bacterial origin. Total esters varied considerably from N96-derived wines with 28.80 mg/L to 63.20 mg/L obtained from wines fermented with strain FC.

Selection of high performance yeast strains:

UPGMA Cluster Analysis: UPGMA cluster analysis of the volatiles was done on both base wines and final distillates resulting from fermentation with the different strains. **Figures 4.1 and 4.2** show the average clusters for volatile compounds of wines fermented with the different strains and their final distillates, respectively. From the clusters it was clear that some of the base wines and distillates fermented with the different strains were significantly different from each other. It is interesting to note that strains FC and VIN7 consistently produced wines and final distillates significantly different from the other strains. The rest of the strains were clustered differently for the wines and the final distillates. This reflects how distillation and yeast lees present in the wine during the distillation process affect the volatile profiles of the distillates.

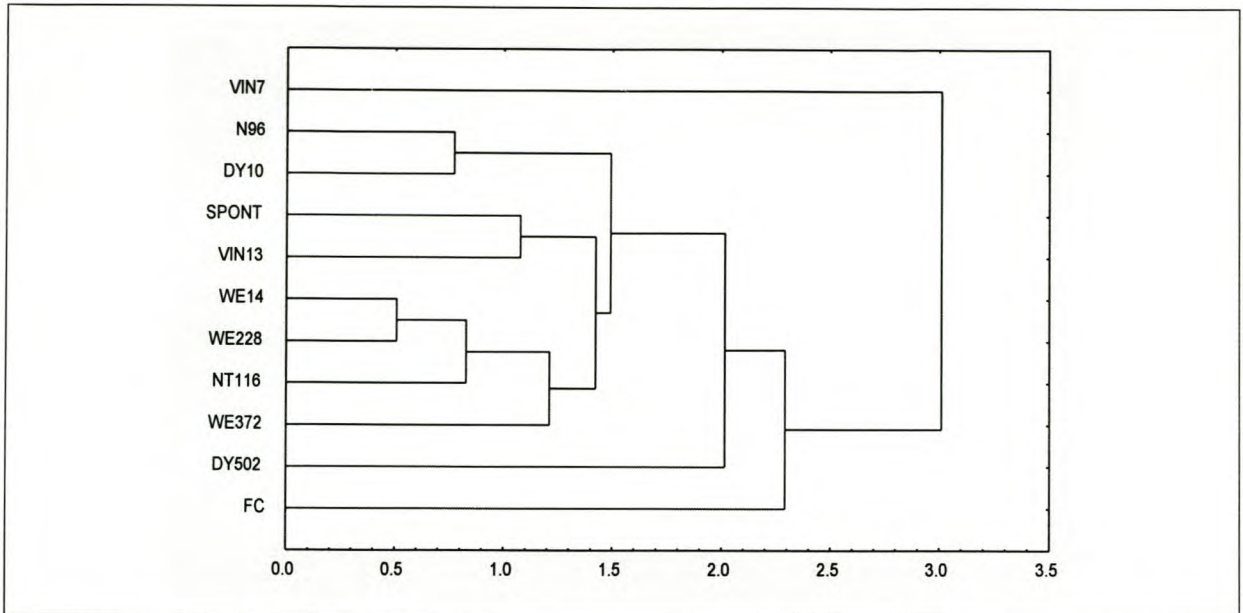


Figure 4.1 Average linkage cluster analysis of marula base wines resulting from fermentation with 11 strains.

It is interesting to also note that strains WE14 and WE228 consistently produced wines and distillates that had a similar profile and hence they were clustered together in both situations (**Figures 4.1 and 4.2**). The volatile profiles of the wines changed on distillation, hence the differences in the clustering, with the exception of WE14, WE228 and FC. Thus, when one is selecting for a yeast strain for the production of a distilled beverage, it is recommended to judge the performance of the strains after distillation. This is in agreement with the report of Steger and Lambrechts (2000), who selected yeast strains for the production of premium quality South African brandy base products. In their study, they concluded that it was important to use the quality of the distillate as a basis for evaluation of the yeast.

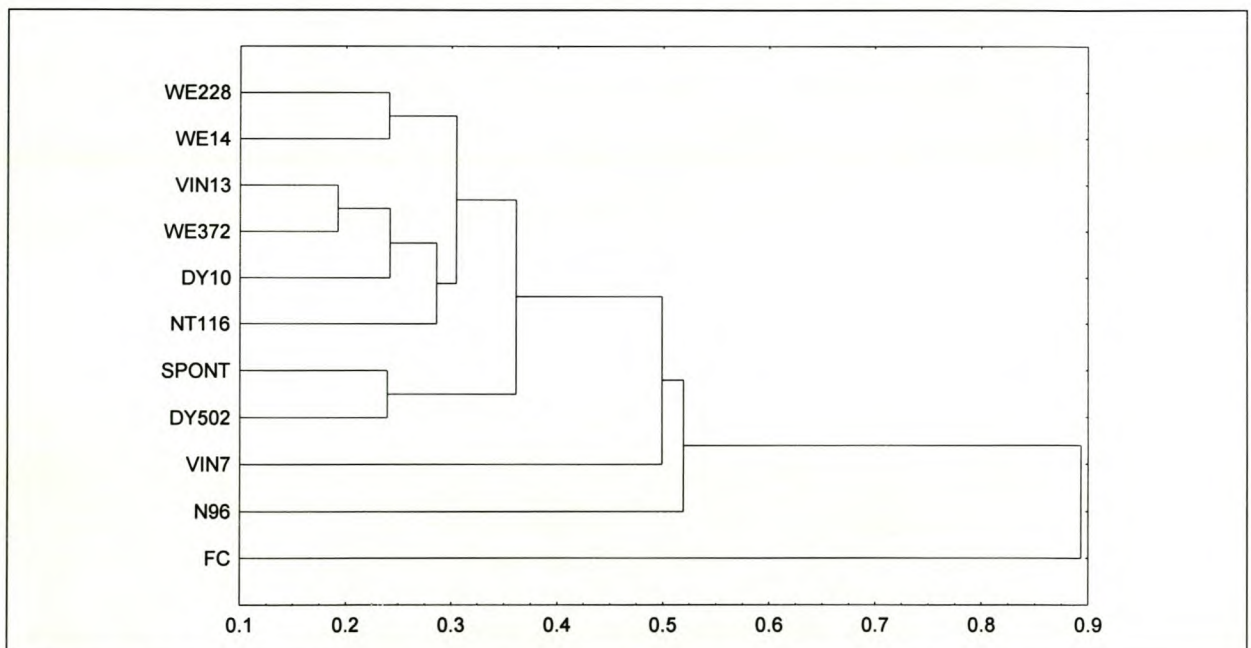


Figure 4.2 Average linkage cluster analysis of the 11 final distillates.

Principal Component Analysis (PCA): PCA was also performed on the volatile compounds, esters, higher alcohols and volatile acids of all the wines fermented with different strains to find yeast grouping at each temperature. PCA at 15°C showed that volatile compounds, higher alcohols, esters and volatile acids yielded a grouping of yeast strains similar in all cases for the wines as shown in **Fig. 4.3**. For wine, the first two principal components (PC) accounted for 42.7% and 26.6% of the variance, respectively. The first PC (PC1) separated WE372 from the rest of the strains and the second PC separated VIN7 and FC. Ethyl caprate explained most of the variability between the yeast strains. For the distillates at 15°C, the esters yielded a grouping with FC being separated from the other strains. No apparent grouping was observed when considering the volatile acids.

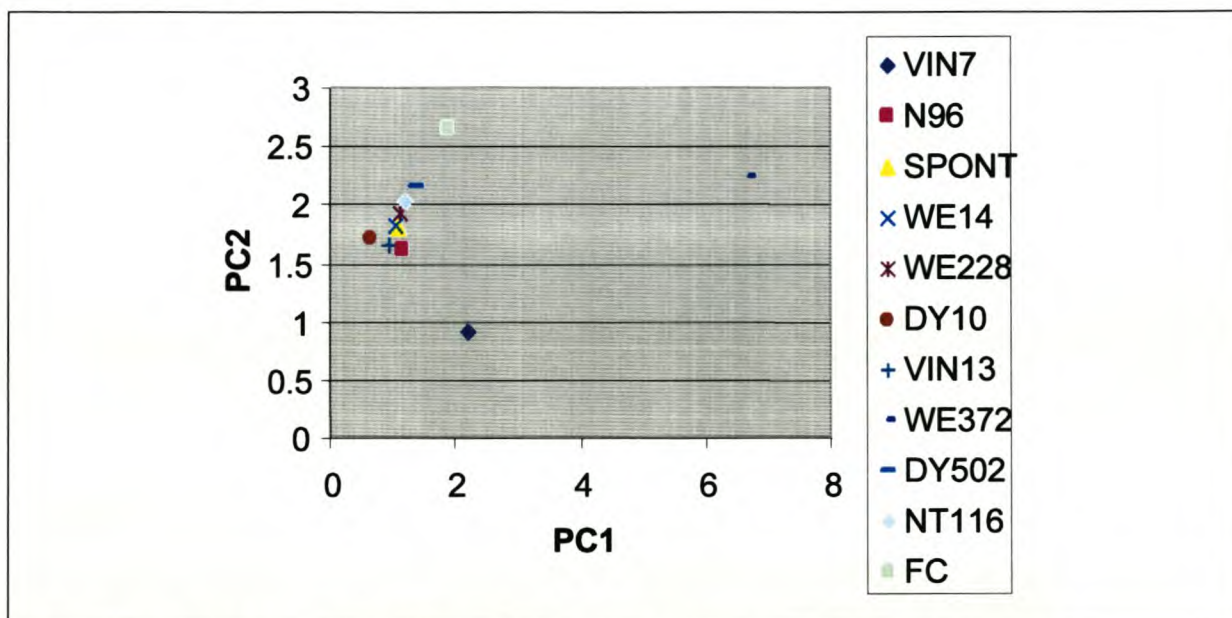


Figure 4.3 Principal component analysis for esters produced in the wines.

At 30°C no apparent grouping was observed when considering any of the volatile components in the wines. In the distillates no apparent grouping was observed for higher alcohols and volatile acids. When PCA was applied to esters, FC, NT116 and DY502 were clearly separated from the other strains. When considering all the volatile compounds only FC was separated from the other strains (**Fig. 4.4**). Considering the two seasons, 1999 and 2000, no apparent trend of yeast strains was observed. More work needs to be done over a longer period of time to establish any correlation if possible between climatic conditions and yeast performance. It is therefore very important, at the beginning of each season to analyze the fruit extensively and choose the yeast strain accordingly.

Sensory Analysis: The perceived quality of the individual marula base wines and distillates as assessed by the tasters is depicted by Duncan's rankings. A sensory

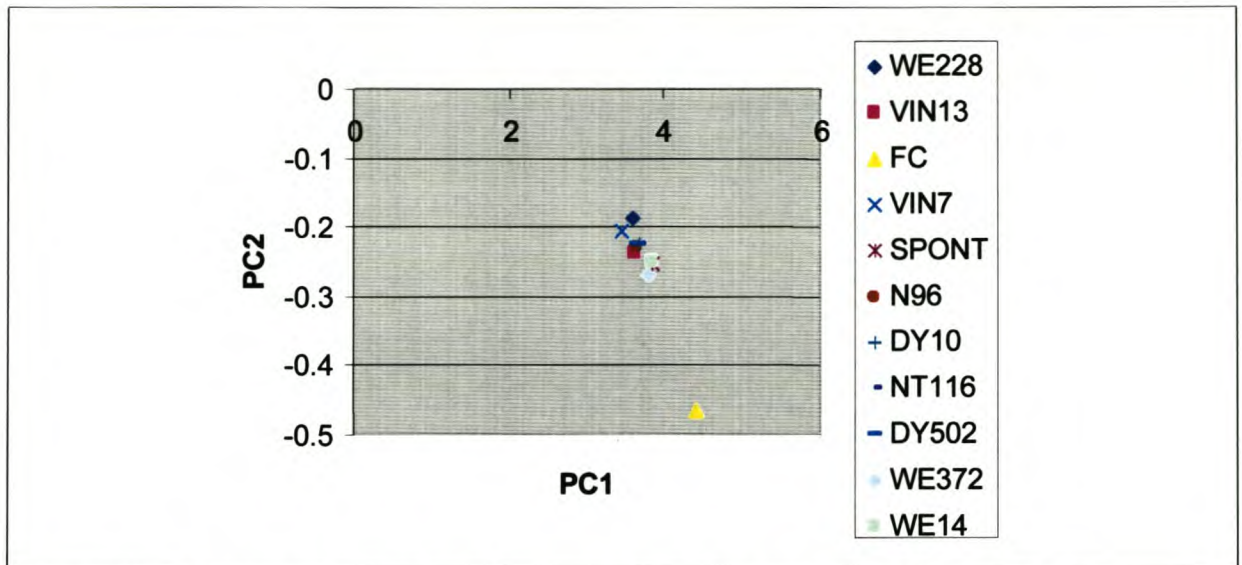


Figure 4.4 Principal component analysis for esters produced in the final distillates.

analysis was done on all the wines and distillates using a panel of 10 judges. For differences in flavor intensity and acceptability, differences were obtained according to ANOVA for the yeast strains. For none of the tasters was the Kolmogorov-Smirnov test for distribution significantly different from normal. Hence, no data transformation was necessary. As shown in **Table 4.7**, the tasters differed in their criteria in judging the wines but there was no significant difference among the wines produced by the different strains. However, after distillation, there was a significant difference among wines ($p < 0.00003$). The mean values of the sensory analysis were grouped according to Duncan's grouping (**Table 4.8** and **4.9**) for wines and distillates respectively. The wines were grouped into three groups with wine fermented by the strain DY520 performing the worst. The wines fermented with strains N96 and WE14 were ranked as the wines with typical good marula flavor complex. The sensory evaluation performed on the resultant distillates also gave three main groups with strains WE372 and FC performing the worst. It is interesting to note that strains DY10, NT116 and VIN13 were ranked highly. These strains have similar volatile profiles, as shown in **Fig. 4.3**. The clustering of WE372 with strains VIN13, DY10 and NT116 could not be explained.

Table 4.7 Two-way ANOVA for the marula base wines and their distillate assessed by a panel.

Effect	Marula wine			Final distillates		
	df	F-value	p-value	df	F-value	p-value
Taster	9	2.02	0.046	9	2.8687	0.0051
Strain	19	1.49	0.1546	10	4.5464	0.00003

Table 4.8 Sensory rating for wines

Duncan Grouping		Mean	N	Strains
	A	56.40	10	DY10
	A			
	A	55.60	10	NT116
	A			
	A	55.10	10	VIN13
	A			
	A	54.70	10	WE14
	A			
	A	54.40	10	VIN7
	A			
	A	53.20	10	SPONT
	A			
	A	45.20	10	WE22
	A			
	A	44.70	10	N96
	A			
B	A	27.90	10	DY502
B				
B	C	12.67	9	WE372
	C			
	C	-1.80	10	FC

Table 4.9 Sensory ratings for distillates

Duncan Grouping		Mean	N	Strains
	A	50.60	10	N96
	A			
	A	45.30	10	WE14
	A			
B	A	43.70	10	DY10
B	A			
B	A	40.80	10	VIN13
B	A			
B	A	40.00	10	WE372
B	A			
B	A	39.20	10	NT116
B	A			
B	A	34.20	10	WE228
B	A			
B	A	32.30	10	VIN7
B	A			
B	A	18.30	10	SPONT
B	A			
B	A	10.10	10	FC
B	A			
B	A	2.30	10	DY502

Correlation between sensory evaluation results and volatile compound composition

The marula base wine fermented with the yeast strain N96 and DY10 were ranked as the best. The wine fermented with strains FC and DY502 were rated as having a low

quality. Comparing the volatile compounds of these strains it was shown that, despite the very high ester levels in DY502 which are almost double those of N96, DY502 had a very high HA concentration. In this case the VA levels that were 3-fold those in N96 might have clearly contributed to the overall performance of the strain. Strain FC performed the worst after DY502 as shown in **Table 4.8**. In this case the wine had low VA with very high levels of esters and relatively high total HAs. It is important to note that FC and DY10 had the highest concentrations of methanol compared to the rest of the strains. Methanol gives a cooked cabbage odor and its threshold is 1.20 g/L (Ribereau-Gayon and others 2000). This could have contributed to the disagreeable olfactory flaws in the wines. On the other hand, the distillate of strain DY10 was judged to be the best despite having intermediate total ester concentrations of 27.69 mg/L. This distillate had low propanol levels whilst the isoamyl alcohol levels were very high in the wines. In contrast the distillation of FC and WE372 were rated as least performers. They both had relatively high ester levels with FC having the highest concentration of 101.90 mg/L. It is interesting to note that these two had low total HA concentrations and the total VA was also low. In the distillates, too high propanol levels seemed to mask all the other positive notes in the distillates, and thus gave it a solvent note as described by the panel. Given the fact that strains with high concentrations of esters did not necessarily perform best both in the wines and the distillates, one is tempted to speculate on the effect of too much esters in the marula fruit. It has been reported that marula fruit that is too ripe exhibits a repulsive odor (Schäefer and McGill 1986).

4.4 CONCLUSIONS

From the study, it is apparent that fermentation temperature had a significant influence on the production of a typical marula aroma complex in both the wines and the distillates. It can thus be recommended to carry out fermentations for the marula fruit at low fermenting temperatures. However, more work still needs to be done in order to determine the effect of temperatures between 15°C and 30°C. The different yeast strains produced different volatile compound concentrations. It is thus clear that depending on the type of end-product, the choice of yeast strain should be based on the quality of either the wine or distillate. Seasonal variations of the fruit proved to have a significant effect on the performance of the different yeast strains. More work needs to be done with respect to seasonal changes.

The volatile profile ratios of wines change on distillation, therefore when selecting for a yeast strain for the production of a distilled beverage, it is recommended to judge the performance of the strain after distillation.

The performance of the spontaneous fermentations in this study highlights the importance of untapping the hidden wealth of indigenous yeast species present on the marula and the selection and genetic development of yeast starter cultures with improved flavor profiles.

At this point we can recommend the strains that performed well in this study, however, these experiments should in future be taken a step further by scaling up the fermentations and distillations in order to get a representative picture of their performance on a large scale.

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CHAPTER 5

**GENERAL DISCUSSION
AND CONCLUSIONS**

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Over the years, it has been acknowledged that indigenous food resources such as the marula have been adapted to the food habits, tastes and needs of traditional societies and to the agro-ecological situation where they occur. It is therefore imperative that efforts be made to investigate and document the lesser known of plant and animal origin and to conserve those with promising and proven food potential; and to improve the yield and quality of these foods through research. The work presented herein is a result of dedicated research from the industry and the university in order to understand indigenous products.

To date, no work has been done towards the optimization of the fermentation processes of the marula fruit. This study was divided into two parts; firstly, the pre-fermentation phase, which consisted of selecting different commercial enzymes for juice extraction and aroma improvement; secondly, was the fermentation phase and post fermentation phase, whereby different commercial yeast strains were used to ferment the fruit with the aim to get the best typical marula aroma complex.

In order to optimize the fermentation processes of any fruit it is important to understand the properties of the fruit in question, hence a review of the marula fruit was given in chapter 2. Having understood these properties it was then imperative to apply the different technologies accordingly to suit the processing of the fruit. In this chapter we discussed the alcoholic fermentation and how it links with the quality of the fermented beverage. The chapter emphasized aroma as a measure of quality, as this formed the basis of this study.

The study on the pre-fermentation phase showed the great potential that can be offered by the use of commercial enzyme preparations. There was a marked increase in the juice yield as shown in chapter 3. Addition of enzyme preparations also resulted in an increase in the terpene composition of the juice. From this work we recommend the use of the enzyme Rapidase X-press which increased yield by 12%, clarity was 7 NTU and a 13.7-fold increase in terpenes was observed. Above all, the taste panel confirmed that the juice treated with this enzyme had a typical marula flavor. The positive results observed in this study merit further investigations pertaining to the influence on these enzymes on resultant wines and distillates. Secondly, we recommend Rohapect, which has a shortcoming of having a low impact on the juice yield but a large impact on the terpene levels.

The use of commercial enzymes after fermentation resulted in the enhancement of the aroma profile of the wines which was also detectable in the corresponding distillates. This shows the possibility to manipulate the fruit, in order to obtain a certain character of the end product. From this study, the enzyme Trenolin bukett is recommended for post-fermentation aroma enhancement. It is important to note however, that some enzyme preparations led to the release of excessive terpenes

that proved detrimental to the end-product. Therefore, extensive research needs to be done before choosing the enzyme, as not all enzymes give the desired results.

Fermentation of the fruit at different temperatures resulted in wines and spirits that had different volatile profiles and differences in aroma. There was a significant difference in the aroma of the wines and the spirits for the different yeast strains used in this study. The work showed at very high significance levels that the preferred fermentation temperature was 15°C compared to 30°C. The yeast strains whose wines were preferred at 15°C are VIN7, DY10, N96, VIN13, WE228 and WE14. The strains NT116 and WE372 were preferred at 30°C. The significance level for wine fermented with the yeast strain FC was lower than 95% and hence we cannot conclude with confidence the preferred temperature in this case. More work should be carried out in order to establish the best fermenting temperature for this strain. The change in preference of the fermentation temperature after distillation portrayed by some strains, like NT116, that was preferred at 30°C for the wine and 15°C for the distillate may be attributed to factors other than temperature. There was no specific temperature preference for wines and spirits that underwent spontaneous fermentations. This shows how inconsistent spontaneous fermentations can be and the reason why winemakers have moved away from the spontaneous fermentations for consistent quality. It is also important to note at this point that the yeast strains used in this work were selected from grapes and hence the selection of indigenous marula strains could go a long way in giving an even better product.

The results of the volatile composition of the different wines and distillates clearly shows that different yeast strains are responsible for the production of different levels of volatile compounds. For example, FC produced high amounts of esters compared to the other strains. This can be attributed to the fact that FC contains a pool of pectolytic enzymes (polygalacturonases, pectinlyases and pectin methylesterases), which could have possibly contributed highly to the release of aromatic compounds. The variation in volatile compounds from strain to strain are given in chapter 4. The differences have a direct impact on the overall volatile profile of the wine or distillate and thus leading to differences in their quality.

On distilling the wines a change in the volatile profile ratios was observed with the different yeast strains. This explains the differences in the rankings by the panel of tasters of the various strains. For example N96, although it was grouped among the best performing yeasts in wines, it was not at the top of the list, after distillation this strain was ranked as producing the best distillate. In this case it is highly likely that the yeast cells of N96 played a great role in influencing the final flavor of the distillate. From these results we recommend judging the performance of the yeast strain for purposes of producing a distilled product from marula fruit after the distillation process. The process of distillation and the yeast cells available have a great influence on the quality of the final distillate. The top four yeast strains preferred for wine production by the panel were DY10, NT116, VIN13 and WE14 and for distillates they were N96, WE14, DY10 and VIN13. As much as the process of

distillation and the cells available have an effect on the profile of the distillates, the study also showed that a good distillate comes from a good base wine. The strains DY10, VIN13 and WE14 that performed well for the wines were in turn ranked among the best distillates.

This work serves as a basis for more work that still remains to be done towards the understanding of the fermentation processes of the marula fruit and the production of the typical flavor from the wines and distillates and we recommend that it should be repeated at a larger scale. The nutritional factor of the marula fruit is of great importance and should not be overlooked. From the routine analysis that we carried out on the wines soon after fermentation, the levels of ascorbic acid still present were overwhelmingly high, on average, 400 mg/L. More work should be done to see the evolution of this vital vitamin over time and also after distillation.