ANTIOXIDANT EFFECTIVENESS OF SOME SOUTH AFRICAN WINES

CATHARINA ELIZABETH BRAND

Thesis submitted in fulfilment of the requirement for the Degree

MAGISTER TECHNOLOGIAE

BIOMEDICAL TECHNOLOGY

in the

Faculty of Applied Sciences

Department Paramedical Sciences

at the

Technikon Free State

Supervisor: Mr F Cloete [M.Sc. (Biochemistry) RAU]

Co-supervisor: Prof JG Barnard [D.Phil. (Exercise Physiology) UOFS]

Bloemfontein

December 1998

TABLE OF CONTENT

CHAPTER 3

MATERIALS AND METHODS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ADP	Adenosine diphosphate
BHT	Butylated hydroxytoluene
BMI	Body mass index
C_{12}	Fatty acid with carbon chain of 12
C_{16}	Fatty acid with carbon chain of 16
CAD	Coronary artery disease
cHCl	Concentrated hydrochloric acid
CHD	Coronory heart disease
$CuCl_2$	Copper chloride
$CuSO_4$	Copper sulphate
CVD	Coronary vascular disease
DHA	Docosahexanoic acid
DNA	Deoxyribonucleic acid
EPA	Eicosapentanoic acid
FH	Familial hypercholesterolemia
FOX2	Ferrous xylenol orange, Version II method / metode
g	Gravitation (x g = times gravitation)
GAE	Gallic acid equivalent
HDL	High-density lipoprotein
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
H_2SO_4	Sulphuric acid
LDL	Low-density lipoprotein / Lae-digtheid-lipoproteïene
mg	Milligram
ml	Milliliter

mmol	Millimol
mmol/l	Millimol per liter
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NaOH	Sodium hydroxide
n-3	Omega 3 family of fatty acids
$OH^{\cdot}$	Hydroxyl radical
$O_2^{\cdot -}$	Superoxide radical
PBS	Phosphate buffered saline
PMS	5-methylphenazinium methosulphate
PVPP	Polyvinylpolypyrrolidone
Plt	Platelet
PUFA	Polyunsaturated fatty acid
$PUFA^{\cdot}$	Polyunsaturated fatty acid radical
rpm	Revolutions per minute
TC	Total cholesterol
µg	Microgram
µl	Microliter
UOFS	University of the Orange Free State
UV	Ultra violet
V. vinifera	*Vitis vinifera*
Vis	Visible
VLDL	Very-low-density lipoprotein

vol %	Volume percentage
vol / vol	Volume per volume
WCC	White cell count
$X^{\cdot}$	Oxidising species
$X - H^{\cdot}$	Reduced species

VERKLARING TEN OPSIGTE VAN SELFSTANDIGE WERK

Ek, CATHARINA, ELIZABETH BRAND, verklaar hiermee dat die navorsingsprojek wat vir die verwerwing van die MAGISTER TECHNOLOGIAE: TOEGEPASTE WETENSKAPPE: BIOMEDIESE TEGNOLOGIE, aan die Technikon Vrystaat deur my voorgelê word, my selfstandige werk is en nie voorheen deur my of enige ander persoon, by enige ander instelling, ter verwerwing van enige kwalifikasie, voorgelê is nie.

..... 28/1/99

HANDTEKENING VAN STUDENT DATUM

DECLARATION OF INDEPENDENT WORK

I, CATHARINA ELIZABETH BRAND, do hereby declare that this research project submitted for the degree MAGISTER TECHNOLOGIAE: APPLIED SCIENCES: BIOMEDICAL TECHNOLOGY, is my own independent work that has not been submitted before, to any institution by me or anyone else as part of any qualification.

..... 28/1/99

SIGNATURE OF STUDENT DATE

SUMMARY

The most important health problems of the Western society are diseases such as coronary heart diseases (CHD), cancer and stroke, of which CHD is the major cause of death. Dietary factors may contribute to the development and progress of these conditions. A lower incidence of CDH is found in people following a Mediterranean diet. The French, who follow a diet rich in fats, are not keen on exercise and are heavy smokers, have a much lower incidence of CHD in comparison to the rest of the world. This phenomenon is called the French Paradox and is attributed to the French population consuming large amounts of red wine.

Phenolic compounds in wine may protect the low-density lipoprotein (LDL) against oxidative modification, thereby reducing the risk of cardiovascular morbidity. *In vitro* studies were performed measuring the antioxidant effect of South African wines and juices in prolonging the inhibition period of LDL oxidation.

Twelve wines, six red and six white and two grape juices, one red and one white were analysed for antioxidant activity. Wines and juices were fractionated into neutral and acid fractions though solid phase column chromatography. Total phenolic levels were determined using the Folin-Ciocalteu method. The phenolic concentrations in red wines ranged between 2550 - 2750 mg/l in Cabernet Sauvignon, 2050 - 2325 mg/l in Merlot and 1645 - 1650 mg/l in the blended red wines. The white wines gave total phenolic concentrations of 290 - 315 mg/l in Chardonnay, 240 - 250 mg/l in Sauvignon Blanc, 230 mg/l in a blended white wine and 225 mg/l in the Riesling. The Red Grapetiser gave a phenolic concentration of 410 mg/l and the White Grapetiser a phenolic concentration of 180 mg/l.

The lag periods preceding LDL oxidation was determined using the conjugated diene method and confirmed by the Ferrous Xylenol Orange, Version II method (FOX2). The percentage biological activity was calculated from the lag periods.

Biological activity due to the presence of ascorbic acid in the beverages ranged from 10% in Red Grapetiser, 2% in White Grapetiser and between 1-5% in five of the six white wines. In the one white wine and the red wines there was no ascorbic acid present and could thus not have influenced the biological activities of the wines. Alcohol had no influence on the determination of total phenolic level and on the percentage biological activity of either the wines or the juices.

The red wines gave the highest biological activity, with the Merlot wines at 1620% and 1190%, Cabernet Sauvignon at 970% and 880% and the blended red wines at 820% and 660% respectively. In the white wines the percentage biological activity was much lower ranging from 306% and 162% in the Sauvignon Blanc, 226% and 169% in the Chardonnay, 171% in the Riesling and 155% in the blended white wine. The Red Grapetiser gave a biological activity of 170% and the White Grapetiser 74%. The biological activity of red and white wine, and different phenolic fractions were compared. When comparing white wine with red wine in the same 5 mg/l phenolic concentration, white wine gave a much higher percentage biological activity. From these results it is evident that phenolic levels are important in the determination of the antioxidant activity of a wine, but the phenolic concentration is not directly proportional to the biological activity of the wine.

OPSOMMING

Die belangrikste gesondheidsprobleme in die Westerse samelewing is koronêre hartvatsiektes, kanker en beroertes, waarvan hartvatsiektes die algemeenste voorkom. Eetgewoontes kan bydra tot die ontstaan en verloop van die siektetoestande. 'n Laer insidensie van hartvatsiektes kom voor by mense wat 'n Meditereense dieet volg. Die Franse daarinteen, eet kossoorte ryk aan vette, is nie besonder lief vir oefeninge nie, en daarby is hulle strawwe rokers, nogtans is hul insidensie van hartvatsiektes van die laagste in die wêreld. Hierdie verskynsel staan bekend as die Franse Paradoks en word toegeskryf aan hul voorliefde vir rooiwyn.

Fenoliese komponente in wyn kan moontlik voorkomend optree in die beskerming van lae-digtheid-lipoproteïene (LDL) teen oksidasie en daardeur die gevare van kardiovaskulêre siektes verlaag. Die inhiberende uitwerking wat Suid-Afrikaanse wyne en sappe op LDL oksidasie het, is *in vitro* bepaal.

Twaalf wyne, ses rooi en ses wit en twee sappe, een rooi en een wit is vir antioksidant aktiwiteit geanaliseer. Wyne en sappe is met behulp van soliede fase kolomchromatografie in neutrale en suur fenole gefraksioneer. Totale fenoliese bepalings is volgens die Folin Ciocalteu-metode uitgevoer. Fenoliese konsentrasies in rooiwyne het gewissel tussen 2550 - 2750 mg/l in Cabernet Sauvignon, 2050 - 2325 mg/l in Merlot en 1645 - 1650 mg/l in die versnitte rooiwyn. Die witwyne se fenoliese konsentrasies het gewissel van 290 - 315 mg/l in Chardonnay, 240 - 250 mg/l in Sauvignon Blanc, 230 mg/l in die versnitte witwyn en 225 mg/l in die Riesling. Rooi Grapetiser het 'n fenoliese konsentrasie van 410 mg/l en Wit Grapetiser 'n fenoliese konsentrasie van 180 mg/l gelewer.

Die vertragingsperiode voordat LDL-oksidasie in aanvang neem, is volgens die gekonjugeerde diene-metode bepaal en volgens die "Ferrous Xylenol Orange", weergawe II-metode (FOX2) bevestig. Die persentasie biologiese aktiwiteit is vanaf die vertragingsperiode bereken.

Biologiese aktiwiteit as gevolg van die teenwoordigheid van askorbiensuur in die wyne en sappe het gevarieer van 10% in Rooi Grapetiser, 2% in Wit Grapetiser en vanaf 1–5% in vyf van die ses witwyne. Die een witwyn en die rooiwyne het geen askorbiensuur bevat nie en kon dit dus nie die biologiese aktiwiteite van die wyne beïnvloed nie. Alkohol het geen invloed gehad op die bepaling van fenoliese vlakke en ook nie op die persentasie biologiese aktiwiteit van die wyne en sappe nie.

Rooiwyn het die hoogste biologiese aktiwiteit getoon. Die heel beste was die Merlot wyne met 1620% en 1190%, Cabernet Sauvignon met 970% en 880% en daarna die versnitte wyne met 820% en 660%. In die witwyne was die persentasie biologiese aktiwiteit baie laer. Biologiese aktiwiteite het gewissel vanaf 306% en 162% in die Sauvignon Blanc, 226% en 169% in the Chardonnay, 171% in die Riesling en 155% in die versnitte witwyn. Die Rooi en Wit Grapetiser het onderskeidelik biologiese aktiwiteite van 170% en 74% getoon. Die persentasie biologiese aktiwiteit tussen rooiwyn en witwyn en die verskillende fenoliese fraksies is onderling met mekaar vergelyk. Deur die biologiese aktiwiteit van witwyn en rooiwyn, by 5 mg/l fenoliese konsentrasies, met mekaar te vergelyk, is gevind dat witwyn 'n baie hoër biologiese aktiwiteit toon. Uit die resultate kan afgelei word, dat fenoliese vlakke belangrik is vir die bepaling van antioksidant aktiwiteit, maar dat die fenoliese konsentrasie van 'n wyn nie direk in verhouding tot die biologiese aktiwiteit van die wyn is nie.

CHAPTER 1

INTRODUCTION

"An apple a day keeps the doctor away", and the motherly instruction to "eat your greens" are well-worn phrases that were originally based upon intuition rather than scientific facts (Mera, 1994). As is often the case with medical folklore, scientific investigation has revealed that there is at least a germ of truth underlying these assertions. Nutritional status affects our predisposition to a wide variety of diseases, both acute and chronic. The principal causes of death in Western countries are coronary heart disease (CHD), cancers and stroke. These disorders are largely influenced by diet.

The French population who follow a diet rich in fats, are not to keen on exercise and are heavy smokers should have a high incidence of coronary heart disease, but this is not the case. This phenomenon is known as the French Paradox (Renaud & de Lorgeril, 1992). The French are consumers of large amounts of red wine. In addition to this, the French also eat large amounts of fresh fruit and vegetables (Demrow, Slane & Folts, 1995). Moderate consumption of alcoholic beverages acts in a protective manner against CHD (Siemann & Creasy, 1992). Alcohol is believed to protect against CHD by preventing arteriosclerosis through the action of high-density lipoprotein (HDL) cholesterol.

It is known that other factors, especially in red wines, might contribute to this preventive action of alcoholic beverages against CHD. It has been described that certain antioxidants may contribute to this preventative action. The phenolic levels in some wines and also in fresh fruit are fairly high. Some red wines contain ten times more

phenolics in comparison to white wines (Frankel, Waterhouse & Teissedre (1995). These phenolics present in wines can act as antioxidants against free radicals. Aerobic metabolism entails the production of reactive oxygen species, even under basal conditions, hence there is a continuous requirement for inactivation of these reactive oxygen species. Free radicals can be implicated in the origin and the development of cancers and cardiovascular disease. Antioxidants, such as vitamin C, vitamin E, beta-carotene and phenolics are responsible for the destruction of these reactive oxygen intermediates (Sies, 1993). Polyunsaturated fatty acids present in membrane phospholipids are the main target for free radical damage and results in lipid peroxidation. Maxwell, Cruickshank & Thorpe (1994) demonstrated that in subjects who ingest red wine, their serum showed an increase in antioxidant activity responsible for the inhibition of low-density lipoprotein (LDL) oxidation *in vitro*. A study done by Whitehead, Robinson, Allaway, Syms & Hale (1995) compared the antioxidant effect of serum after subjects consumed red wine, white wine and high doses of vitamin C. The authors concluded that red wine had a high antioxidant capacity *in vitro* in addition to its ability to increase antioxidant capacity of serum *in vivo*. In a study done on dogs by Demrow *et al.* (1994) it was demonstrated that the platelet inhibitory properties of red wine and grape juice, without high levels of ethanol, can be contributed to the biological activity of the phenolic compounds present in the beverages. According to Frankel, Kanner, German, Parks & Kinsella (1993) the phenolic substances in red wine inhibits the copper-catalysed oxidation of LDL.

Renaud, Beswick, Fehily, Sharp & Elwood (1992) demonstrated that the phenolics in red wine inhibit platelet aggregation. Seigneur, Bonnet, Dorian, Benchimol, Drouillet, Gouverneur, Larrue, Crockett, Boisseau, Ribereau-Gayon & Bricaud (1990) compared a group of subjects consuming red wine, white wine and synthetic wine, alternatively. The authors concluded that while drinking red wine, platelet aggregation studies showed a reduced activity against adenosine diphosphate (ADP) induced aggregation

and an increase in HDL cholesterol levels. Resveratrol is a specific phenolic compound present in the skin of grapes (Siemann & Greasy, 1992) and acts as a general defence mechanism to infection and stress of the grape vine (Jeandett, Bessis, Maume & Sbaghi, 1993). Factors influencing the amount of resveratrol in wines could be the geographical origin of the grape, the weather conditions during the growing season, the grape cultivar and the period the wine spent on the skins. This could explain why red wine shows a higher level of resveratrol in comparison to white wine. The polyphenolics isolated from *Lonicera japonica* have a similar inhibitory effect on platelets as compared to aspirin (Chang & Hsu, 1992). Pattichis, Louca, Jarman, Sandler & Glover (1993) reported that phenolics cause the release of 5-hydroxytryptamine from platelets *in vitro*. This release could be the cause of clinical migraine attacks.

Goldberg (1995a) raised the question whether wine consumption should be advocated as a health promoting indulgence? He stated that if adults in North America drink two glasses of wine each day, CVD that accounts for almost 50% of deaths in their population could be reduced by 40% and $ 40 billion could be saved annually.

1.1 AIM OF THE STUDY

The aim of this study was to determine the antioxidative properties of certain South African red and white wines as well as fruit juices.

1.2 HYPOTHESIS

South African red and white wines contain phenolic compounds with antioxidative properties.

CHAPTER 2

LITERATURE REVIEW

2.1 FRENCH PARADOX

Paul, the apostle, warned Timothy: "Drink no longer water, but use a little wine for thy stomach's sake and thine often infirmities" (1 Timothy 5:23).

2.1.1 HEALTH STATUS

In the Western countries CHD, cancer and stroke are the main causes of death, with CHD being the principle cause (Mera, 1994). Coronary vascular disease (CVD) is the major cause of death among South African white, coloured and Indian populations and about the third most common cause among black South Africans. As the lifestyle of black South Africans changes, the incidence of CHD increases. The Westernised South African population fall into a category with high total cholesterol (TC) values (Rossouw, Steyn, Berger, Vermaak, Seftel & Gevers 1988). Between 1:50 to 1:100 Afrikaans speaking South Africans are heterozygous for familial hypercholesterolemia (FH). CVD in the age group 15-64 causes the death of 34% of men in England (Mera 1994). Women have hormonal protection against CHD, but after menopause they stand an equal chance to men in developing CHD (Klatsky & Armstrong 1992; Goldberg, 1995a). Eskimos in Greenland, the French and inhibitants of the Mediterranean area have a low rate of CHD (Sanders, 1991; Buist, 1996).

2.1.2 COST IMPLICATIONS

CVD has a severe impact of the economy in South Africa and is the reason for a great loss in productivity. Pestana, Steyn, Leiman & Hartzenberg (1996) estimated that the total cost of CVD in South Africa was between R4.135 and R5.035 billion during 1991.

The direct health costs were estimated to be approximately 42% of the total cost. The rest reflects the indirect cost of earnings foregone as a result of premature morbidity and mortality.

2.1.3 EATING AND DRINKING HABITS

Mera (1994) postulated that: "We are what we eat". High intake of fat leads to a high concentration of plasma LDL. The type of fat intake is more important than the overall fat intake (Sanders, 1991; Kinsella, Frankel, German & Kanner, 1993). Experimental studies have shown that saturated fatty acids C12 - C16 increase plasma cholesterol levels (Sanders, 1991). The relationship between the intake of saturated fats and plasma cholesterol levels is probably related to genetic differences rather than environmental influences (Sanders, 1991).

In the United Kingdom (UK) 40% of the energy intake consists of fat of which 16% are saturated fatty acids. The Health of the Nation target in the UK is to reduce these figures to around 35% and 11% respectively (Mera, 1994). French subjects have a intake of saturated fatty acids of approximately 15% (Kinsella *et al.*, 1993). The Mediterranean diet consists of a high intake of total fats, in the order of 35% of the energy intake (Sanders, 1991). The European Atherosclerosis Society, 1987, and the National Research Council, 1989, suggested that the intake of saturated fatty acids should be decreased to below 10% of the energy intake (Sanders, 1991).

The main component of polyunsaturated vegetable oil, linoleic acid, may be the major risk factor for coronary artery disease (CAD). Polyunsaturated fatty acids (PUFA) are unstable, and oxidise easily to form lipid peroxides, that damage arteries.

Eskimos have a relatively high consumption of n-3 fatty acids with an increased bleeding time and reduced platelet function. An example of an n-3 fatty acid is α-

linolenic acid, found in chloroplasts or green vegetables. This can be metabolised to eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). EPA and DHA can be obtained directly from consumption of fatty fish such as sardines, mackerel, herring, trout and salmon (Sanders, 1991; Mera, 1994; Buist, 1996).

The Mediterranean diet consists of large amounts of fat in the form of olive oil (rich in oleic acid), sardines, anchovies, bread, vegetables, fruit and cheese together with wine (Sanders, 1991; Buist, 1996). Sanders (1991) recommend that a Mediterranean diet is beneficial in preventing CHD. It is suggested people should eat five or more servings of fresh fruits and vegetables daily and consume large amounts of onions, garlic and green tea and drink red wine (Mera, 1994; Buist, 1996).

Goldberg, Hahn & Parkes (1995d) described the epidemiological links between alcohol and CHD. The incidence of myocardial infarction (MI) is higher in total abstainers than in moderate to heavy drinkers. Klatsky & Armstrong (1992) collected data from health examination questionnaires from 128,934 patients. They confirmed that people who consume wine, especially red wine were at lower risk of cardiovascular or CAD death in comparison to beer or other liquor drinking subjects and also in comparison to nondrinkers. The beverage types differ in alcohol concentration. Wine in particular is often ingested with food, slowly, and with regularity (Klatsky & Armstrong, 1992).

2.1.4 PEOPLE OF FRANCE

The French follow a lifestyle of high fat consumption, high frequency of smoking and they do have a lower exercise consciousness in comparison to the clean-living North Americans (Goldberg, 1995a). In contradiction the French people have a much lower incidence of CHD and this anomaly is known as the French Paradox (Renaud & de Lorgeril, 1992; Frankel *et al.,* 1993). They attribute this anomaly to the regular consumption of red wine by the French.

2.2 UNDERLYING MECHANISM OF THE DEVELOPMENT AND PROGRESS OF CARDIOVASCULAR DISEASE

2.2.1 FREE RADICALS

Free radicals are chemical entities that have single, unpaired electrons (Zoecklein, Fugelsang, Gump & Nury, 1995: 15-17). They are unstable, short lived and highly reactive by virtue of the fact that, in order to gain chemical stability, they seek to loose or gain an electron. Free radicals vary widely in their thermodynamic properties ranging from very oxidizing to very reducing (Buettner, 1992). The formation of free radicals is the result of chemical, physical and enzymatic processes. Peroxidative reactions are also increased by infection, tissue damage and inflammation (Kinsella *et al.*, 1993).

Free radicals are able to interact with molecules such as membrane lipids and deoxyribonucleic acid (DNA) causing damage to these molecules (Buettner, 1993). Each oxidized species is capable of stealing an electron (or hydrogen atom) from a reduced species. Oxygen binds onto the radicals to form peroxy radicals and hydroperoxides. This causes a chain reaction that results in damage to membranes and perturbation of many metabolic pathways. It causes damage to blood vessels, leading to atherogenesis and increases thrombotic events. Each cell in the body is exposed to numerous free radical attacks, capable of forming damaging peroxides and hydroxyl radicals.

In principle a singlet oxygen molecule could react with PUFA to form $O_2^{\cdot -}$ and PUFA$^{\cdot}$. However, the kinetically preferred reaction is not electron transfer, but rather an addition reaction of a single oxygen to the double bonds of PUFA, to form lipid peroxides (Buettner, 1993). The most oxidizing radical which is likely to arise in a biological

system is the hydroxyl radical $HO^{\cdot}$. Any oxidizing species with a higher oxidation potential than PUFA can, in principle, bring about lipid peroxidation;

$PUFA\text{-}H + X^{\cdot} \rightarrow PUFA^{\cdot} + X\text{-}H^{\cdot}$.

Buist (1996) stated that although pure cholesterol and PUFA in vegetable oil do not damage arteries their oxidised metabolites could do damage. Fortunately the body has evolved an array of protective mechanisms to control peroxidation and rapidly quench, eliminate and/or inactivate free radical generators.

2.2.1.1 Pro-oxidants

A pro-oxidant is a compound that can cause the oxidation of a target molecule. Whether a compound acts as an antioxidant or as a pro-oxidant depends on its oxidation state and that of other species with which it can interact (Zoecklein *et al.*, 1995: 17-18). It is therefore important that a spent antioxidant must be deactivated immediately after performing its function, otherwise it might conceivably do harm as an oxidised species.

Oxidant functions are carried out by different types of radicals, with X-irradiation generating the hydroxyl radical, and irradiation with ultraviolet light generating electronically excited states with subsequent radical formation (Sies, 1993). Ultrasound and microwave radiation can also generate reactive oxygen species. Even shear stress e.g. in homogenisation, is known to generate radicals. Smoking and a smoking environment act as a pro-oxidant which also results in an increase in the fibrinogen level (Mera, 1994).

2.2.2 ANTIOXIDANTS

Scavenging enzymes and antioxidants are involved in the protection of tissues against radical induced damage. Chemically antioxidants work in a number of ways (Zoecklein *et al.*, 1995:18-19). One of the simplest ways is by being able to effectively scavenge reactive species. The other mechanisms are by providing a hydrogen atom or a hydride onto a target substrate. An antioxidant will sacrifice its own structure to prevent damage to other sensitive biomolecules.

The defense strategies of an antioxidant include three levels of protection namely: prevention, interception and repair (Sies, 1993). The function of an antioxidant can be under the influence of the biological system and the biochemical environment. The antioxidant must reach the target cell or tissue in sufficient concentrations to have a positive insult (Zoecklein *et al.*, 1995: 17). After the antioxidant has fulfilled its task, it must be removed from the scene of interaction. Organisms have developed many ways to protect themselves from free radical processes (Buettner, 1993). Antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase are preventive antioxidants, as they eliminate species involved in the initiation of the free radical chain reactions. In contrast small molecule antioxidants, such as ascorbate, tocopherol, reduced co-enzyme Q_{10}, urate and glutathione are able to "repair" oxidising radicals directly and therefore are chain-breaking antioxidants. In addition, it is now apparent that ascorbate and tocopherol function together to protect membrane lipids from damage. It is a well known that grapes are a poor source of vitamins.

Endogenous antioxidants, consumed in the form of food compounds containing a benzene ring, or analogs, are among the most suited antioxidants. Phenolics in a simple or complex form are ubiquitous in plants, especially in grapes. Flavonoids in wine react with pro-oxidant superoxide anions, hydroxyl radicals ($OH^{\cdot}$) and lipid peroxy radicals. The large variety of compounds with varying redox potentials present in

wines, phenolics, flavonols, flavonoids and phytoalexine may interact in a synenergistic manner and provide effective protection against lipid oxidation.

2.2.2.1 Synergism of alcohol and wine antioxidants

Wine is unique among beverages in that it contains both alcohol and antioxidants (Zoecklein *et al.,* 1995:19). Antioxidants are protected once they enter the body by the very same process that our body uses to detoxify ingested ethanol. In the liver and to a lesser extent in the rest of the organs, two nicotinamide adenine dinucleotide (NAD^+) dependent oxidizing enzymes, are active alcohol dehydrogenase which converts ethanol to acetaldehyde and aldehyde dehydrogenase, which converts acetaldehyde to acetate produce nicotinamide adenine dinucleotide (NADH) in each step. This NADH is then capable of recycling spent antioxidants by reducing them and in the process, regenerate NAD^+ which in turn is required to detoxify more ethanol. Most table wines contain about 12% ethanol (vol/vol), a plethora of reducing equivalents is produced during the ethanol detoxification process. Excess reducing equivalents might be deleterious in cases of abuse by keeping moieties in a reduced instead of an oxidized state required to maintain tissue homeostasis.

The health implications of synergism between antioxidants and ethanol are:

a.) antioxidants are recycled, minimizing formation of pro-oxidants thus preventing further oxidative damage to tissues

b.) antioxidants are available in their reduced state

c.) excessive levels of NADH, generated by detoxification of ethanol, are lowered, thus minimizing reductive damage to their systems.

Other alcoholic beverages do not share these unique properties of wine. These facts might help explain (at least in part) why wine drinkers do not become as easily inebriated as those who consume equivalent amounts of ethanol from other sources.

Dealcoholised wine, while still plethoric with antioxidants, might not offer these combined benefits, because no (or very little, e.g. 0,05%) alcohol is present, antioxidant absorbtion from the gut might not be so facile.

2.2.3 CHOLESTEROL

Cholesterol, the principal body sterol is a complex alcohol formed of four fused rings and a side chain. It is solid at body temperature, present in all tissues and can be converted by the adrenals and gonads into steroid hormones. Lipoproteins are micellar structures consisting of an outer membrane of apolipoproteins interspersed with phospholipid and cholesterol, and an inner core packed with triglycerides and cholesterol esters. There are four major classes of lipoproteins: chylomicrons, very-low-density lipoprotein (VLDL), LDL and HDL. Approximately 60% of the total cholesterol in male plasma is carried by LDL, 20% by HDL, 15% by VLDL and 5% by chylomicrons (Kaplan, Szabo & Opheim, 1988: 302-308).

The total serum cholesterol ratio is based on epidemiological rather than on a statistical approach. The adult populations at low risk of CHD usually have a total serum cholesterol value of 4 - 5 mmol/l. South African TC action limits are age-specific. Screening test for TC is relatively inexpensive (Rossouw *et al.*, 1988). It is, however acknowledged, that the determination of elevated levels of LDL could improve CHD prediction. Blood fibrinogen level is also a good predictive indicator of CHD. The lower concentrations of HDL are associated with more cardiovascular events (Levy, 1981).

In theory each 1% reduction in total serum cholesterol should lead to a 2% reduction in the risk of CHD. In practice this is not always the case (Mera, 1994). Death due to CHD is not necessarily due to elevated cholesterol levels. Cholesterol levels are divided into five ranks. Reducing the cholesterol level from rank 1 to rank 2 would only prolong the life span of a human being with one year (Buist, 1996). Buist described that

in 170 patients with severe or moderate arteriosclerosis, 72% had serum cholesterol levels of less than the recommended 5,2 mmol/l concentration.

2.2.4 ARTERIOSCLEROSIS

Arteriosclerosis is the thickening and toughening of arterial walls. It is associated with damage to the endothelial lining and the formation of lipid deposits in the tunica media. This may be the result of the peroxidation of lipids (Kinsella *et al.*, 1993) which is the most common form of arteriosclerosis.

High levels of LDL are associated with a high incidence of arteriosclerosis (Mera, 1994). LDL becomes modified through oxidation. Oxidised LDL is taken up by macrophages to give foam cell characteristics. Accumulation of macrophage foam cells within the aorta intima is characteristic of early arteriosclerosis, as demonstrated *in vitro* by Bolton, Jessup, Stanley & Dean (1993). Through the years these lipid-laden cells progressively accumulate and the adjacent smooth muscle cells proliferate causing a raised lesion forming a fibrous plaque impeding blood flow (Kinsella *et al.*, 1993) in the vessels.

2.2.4.1 Platelets and thrombosis

Renaud *et al.* (1992) performed platelet aggregation studies on 1600 subjects and concluded that alcohol decreases secondary aggregation to ADP. Seigneur *et al.* (1990) concluded that pure alcohol led to enhance ADP or adrenalin induced platelet aggregation. The authors also noted a reduction in LDL cholesterol and an increase in apoprotein A-1 levels in serum. In contrast, ingestion of red wine for 15 days reduced ADP induced platelet aggregation and increased HDL cholesterol levels.

Demrow *et al.* (1995) concluded that red wine was effective as an anti-platelet and an anti-thrombotic compound. Antioxidants in the red wine, acting as inhibitors, are very effective in reducing thrombosis and blocking of arteries by plague formation, which is

fatal in more than 90% of deaths from CHD (Kinsella *et al.*, 1993). Thrombi formed mostly as a result of aggregated platelets.

Pace-Asciak, Hahn, Diamandis, Soleas & Goldberg (1995) demonstrated that *trans*-resveratrol and quercetin showed a dose dependent inhibition of both thrombin induced and ADP induced platelet aggregation, whereas ethanol inhibited only thrombin induced aggregation. Of the phenolics tested, catechin and epicatechin had little effect but quercetin inhibited both thrombin induced and ADP induced aggregation.

2.3 WINE AND HEALTH

Alcohol has a cardioprotective effect, confirming a relationship between alcohol consumption and death from CHD. This is independent of age or sex (Goldberg *et al.*, 1995d). The array of compounds in red wine, grapes and fruit may be partly responsible for the lower incidence of inflammatory reactions and CHD. It reduces peroxidative reactions such as oxidation of lipids, particularly LDL, modulates plasma and tissue peroxide tone and down regulates the activity of peroxidative enzymes involved in arteriosclerosis and thrombosis (Kinsella *et al.*, 1993).

Phenolics in red wine provide additional protection by altering eicosanoid metabolism in favour of increased prostacyclin and decreased thromboxane synthesis and have antioxidative functions which prevent the peroxidation of LDL (Gryglewski, Korbut, Robak & Swies, 1986). Epidemiological evidence shows that the plasma levels of the natural antioxidants are higher in European populations where there is a low incidence of CHD (De Whalley, Rankin, Hoult, Jessup & Leake, 1990).

2.3.1 Grapes and grape products

Vitis vinifera, or the common English name grapevine, belongs to the Vitaceae family. More than 8,000 types of grapes have been described of which 20% are cultivated in

vineyards, gardens and a variety collection centres (Bombardelli & Morazzoni, 1995). Culturing grapes requires long, dry, warm to hot summers and mild winters. The cultivation of *V.vinifera* requires sandy or gravelly clay loam that is most suitable for good drainage. Correct pruning is important for the crop. Grape is the world's largest fruit crop. Italy, France and Spain account for approximately 40% of the world's acreage of grapevines. Products produced from grapes are wines, juices, jams, jellies, raisons and canned grapes (Larrauri, Puperez & Calixto, 1996). It is also popular as a fresh fruit.

A by-product of wine production is wine pomace. It represents up to 20% of the weight of grapes processed. Pomace consists of pressed skins, seeds and stems. Pomace is used for the feeding of livestock and for the production of ethanol, tartrates, citric acid, grape seed oil, hydrocolloids, anthocyanins and food fibre (Larrauri *et al.*, 1996).

Grapes are considered a poor source of vitamins. Ascorbic acid is present in concentrations of 100 mg/kg in fresh grapes (Boulton, Singleton, Bisson & Kunkee, 1996:52). The most important compounds found in wines are alcohol, acid polyphenols like anthocynins (red wine), catechin, procyanidines and tannins. Grape juice, obtained from coloured or white grapes, presents a chemical composition similar to that of grapes, except for the content of crude fibre and fat that are primary present in skin and seeds and therefore absent in the juice.

2.3.1.1 Use of wine

Wines have played an important role in civilization and religious ceremonies since Biblical times. As Noah landed on the slopes of Mount Ararat, he planted a vineyard. The Psalms of King David of Israel speak often of wine. Christian missionaries carried the vines into Europe. They needed sacramental wines and wherever they built a church they planted a vineyard.

An alcohol intake of 2 units per day would provide approximately 6-8% of the total daily energy intake (Sanders, 1991). Wine has been an integral part of the human diet. Goldberg (1995a) suggested that consuming two glasses of wine each day should decrease the incidence of CVD by 40%. Not only does it form a main part of a formal meal, but is just as acceptable with an informal braai or a picnic. Individuals who are more serious about their wines form wine clubs, where the members obey to very strict rules.

2.3.1.2 Alcohol addiction

Treat alcohol with caution and moderation. The ugly face of alcohol is displayed among those to whom it has become a form of addiction. The harmful effect of alcohol abuse should not be overlooked. Six or more drinks per day could result in liver cirrhosis. It could also result in death due to unnatural causes or tobacco-related cancer (heavy drinkers are usually smokers as well) (Goldberg *et al.*, 1995d).

2.3.2 Phenolics in wine

Phenols are responsible for the colour, taste and body of the wines (Bombardelli & Morazzoni, 1995). They contribute to the olfactory profile, serve as important oxygen reservoirs and as substrates for browning reactions. Phenolic compounds are derivatives of phenol (hydroxybenzene). Two distinct groups occur in grapes and wines: the nonflavonoid and flavonoid phenols (*Zoecklein et al.*, 1995: 115-122). Sixty percent of the phenolic compounds present in the grapes are extracted into wines. This concentration may be increased through microbial activity, fermentation and storage in oak.

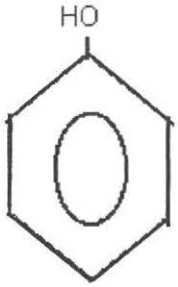
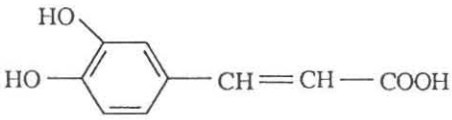
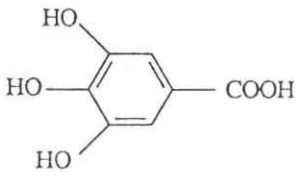


FIGURE 2.1 **Basic structure of a phenol (hydroxybenzene).**

Nonflavonoid phenols are derivatives of hydroxycinnamic and hydroxybenzoic acids. These are esterified to sugars, organic acids or alcohols. The phenolic content of juice is largely nonflavonoids. The nonflavonoid levels are relatively constant in red and white wines because of the extraction of grape pulp. During alcohol fermentation, slow hydrolysis of nonflavonoid ester occurs, resulting in free acid and ester forms.



(a) Caffeic acid

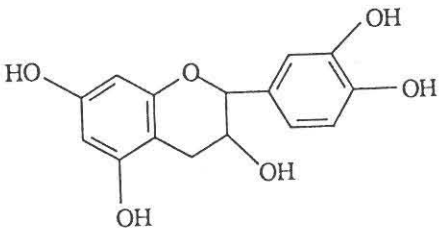


(b) Gallic acid

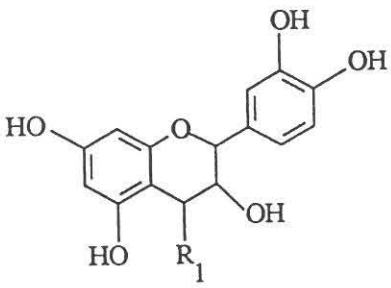
FIGURE 2.2 **Representative examples of nonflavonoid (a) caffeic acid and (b) gallic acid.**

Much of the structure and colour of wine are due to flavonoids that are found in skins, seeds and pulp of the grape. The base structure, aglycone, of flavonoids may exist

either in the free form or in combination (polymerized) with other flavonoids or a combination of both. Flavonoid phenols usually account for 80-90% of phenolic content from conventionally produced red wines and 25% in white wines. Catechins contribute to the browning of white wines and also to the browning and bitterness of red wines. In white wines catechins contribute significantly to the flavour profile.



(a) Catechin



(b) Procyanidins

FIGURE 2.3 **Representative examples of flavonoids (a) catechin and (b) procyanidins.**

2.3.2.1 Specific phenolics

Resveratrol, a nonflavonoid (phytoalexin) present in the skin of *Vitis viniferas* grapes and in wines (Siemann & Creasy, 1992), induced platelet hypoaggregation and increased high-density lipoprotein (HDL) cholesterol (Seigneur *et al.*, 1990). *Trans*-resveratrol in the skin of specific grapes may play a crucial role in modulating lipoprotein metabolism, eicosanoid synthesis, oxidation and coagulation (Goldberg, 1995a). Resveratrol has also been identified in groundnuts and is an active ingredient of Japanese and Chinese folk medicine.

Jeandet, Bessis, & Gautheron (1991) measured the levels of resveratrol in grape berries during fruit ripening. They concluded that the level of resveratrol was relatively

high in irradiated immature clusters, but reached a low level in the ripe fruit. They confirmed that resveratrol was synthesized mainly in the skin cells and was absent from or low in the fruit flesh. There was a clear negative correlation between the resveratrol content of grape skin and the developmental stage of the berries.

The level of resveratrol in wines ranged between 0,003 – 2,861 µM in red wines and was much lower, between 0,001 - 0,438 µM, in white wines (Siemann & Creasy, 1992). Goldberg, Yan, Ng, Diamandis, Karumanchiri, Soleas & Waterhouse (1995c) did a global survey of *trans*-resveratrol concentrations in commercial wines, and found that Pinot noir consistently gave the highest concentrations. The levels of resveratrol in white wines were increased *ca.* 10-fold when maceration occurred (Jeandet, Bessis, Maume, Meunier, Peyron & Trollat, 1995). Different resveratrol levels were present in different cultivars (Jeandet *et al.*, 1995). Both the *trans* and *cis* forms were present in all the wines analysed (Jeandet *et al.*, 1993), with higher levels in wines produced from red grapes than from white grapes.

The analyses for *cis*- and *trans*-resveratrol levels in wines, was determined by high performance liquid chromatography (HPLC) and spectrophotometry (Siemann & Creasy, 1992; Lamuela-Raventós, Romera-Pérez, Waterhouse & de la Torre-Bononat, 1995). Some authors used gas chromatography and gas chromatography-mass spectrometry (Jeandet *et al.,* 1993; Goldberg, Karumanchiri, Ng, Yan, Diamandis & Soleas, 1995b).

Wines were protected from ultra violet (UV) light to avoid photochemical isomerisation of *trans*-resveratrol to the *cis* form (Jeandet *et al.*, 1993). Trela & Waterhouse (1996) found *trans*-resveratrol to be stable for months, except in a high pH-buffer. The *cis*-resveratrol was stable at a neutral pH, but both the *cis* and *trans* form had to be protected from light.

Resveratrol has antifungal activities. It is produced by the grapevines in response to a pathogen infection, *B. cinerea* (Jeandet *et al.*, 1995). It was expected that the more the grapes were infected with *Botrytis*, the higher the resveratrol levels should be. Contrary to what was expected, wines obtained from grapes infected 40-80% by *Botrytis*, showed low resveratrol levels.

Geographical origin appears to be a factor in determining the level of resveratrol in wine (Sieman & Creasy, 1992). The influence of different vinification techniques affected the concentrations of resveratrol in wines (Vrhosek, Wendelin & Eder, 1997).

White grapes are straw coloured due to the flavone compound, quercetin (Bombardelli & Morazzoni, 1995). Quercetin, catechin and epicatechin were analysed in wines by the Council for Scientific and Industrial Research (CSIR). Quercetin was absent in the South African white wines analysed (Wynboer, 1997).

Teissedre, Waterhouse & Frankel (1995) extracted, isolated and purified catechin oligomers as well as the procyanidin dimers and trimers from grape seeds. These compounds were tested for their inhibition of LDL oxidation, along with other monomeric wine phenolics. The procyanidin dimers B_2 and B_8 and trimer C_1, as well as the monomers catechin, epicatechin and myricetin had the highest antioxidant activity. Gallic acid, quercetin, caffeic acid and rutin had a lower antioxidant activity. According to Larrauri *et al.* (1996) some constituents other than phenolic substances, which are unique in red wine are responsible for antioxidant activity. In the same study it was confirmed that resveratrol and quercetin were present in red wine in higher concentrations than in white wine.

2.3.3. BIOLOGICAL ACTIVITY OF PHENOLICS *IN VITRO*

An *in vitro* comparison of red wine, white wine and various fruit juices showed a high antioxidant capacity of red wine, in addition to its ability to increase the antioxidant capacity of serum *in vivo* (Whitehead *et al.*, 1995). Serum antioxidant capacity was determined by means of a chemiluminescent reaction.

Abu-Amsha, Croft, Puddey, Proudfoot & Beilin (1996) investigated the possible antioxidant effects of various beverages, including red wines, white wines, beers and red grape juices on metal ion-dependent (copper) and -independent oxidation of isolated human LDL. The higher the polyphenolic content of the beverage the greater was its antioxidant effects. When stripping the polyphenolics from beverages the lag time returned to control levels. The authors concluded that phenolics in both alcoholic and non-alcoholic beverages can give dose dependent protection against oxidation of LDL.

Vinson & Hontz (1995) concluded that red wine had a much higher phenolic content than white wine, but the phenolics in white wine presented with more potent antioxidants. In contrast to this Frankel *et al.* (1995) determined the activities of 20 selected California wines in inhibiting the copper-catalysed oxidation of LDL. They concluded that the relative inhibition of LDL oxidation varied from 46-100% with red wines and 3-6% with white wines. When compared at the same total phenolic concentration, the oxidation inhibition varied from 37-65% in the red wines and from 27-46% in the white wines.

Teissedre *et al.* (1995) compared the antioxidant activities of different red wines and concluded that Merlot gave the highest percentage, followed by Cabernet Sauvignon. Goldberg, Tsang, Karumanchiri, Diamandis, Soleas & Ng (1996) isolated *cis*- and *trans*-isomers of resveratrol, and their glucosides, catechin, epicatechin, quercetin and rutin.

All of the phenolics measured were reasonably stable in opened wines, which were protected against ultra violet light (UV) for up to 1 week at room temperature or 4°C, but most showed losses of 10-14% when stored for 6 weeks at either temperature.

2.3.4 BIOLOGICAL ACTIVITY OF PHENOLICS *IN VIVO*

De Rijke, Demacker, Assen, Sloots, Katan & Stalenhoef (1996) did *in vivo* studies on 13 healthy volunteers to disprove antioxidant activity of wines. The volunteers were divided into two groups. On a daily basis, one group received 550 ml of red wine and the other group received 550 ml of white wine. The alcohol levels were reduced to 3,5%. The subjects were on a strict diet throughout the experimental period. Data showed that neither red nor white wine influenced the concentration of either lipophilic or hydrophilic antioxidants. LDL oxidisability was not influenced. According to this research group, the French paradox does not exists at all, or could be due to other factors, other than the effects of wine on LDL oxidation.

In another study ten subjects consumed a standard meal with and without red wine intake (Maxwell *et al.,* 1994). Antioxidant activity was measured on blood samples taken during the following 4 hours and was compared to a pre-consumption sample. Antioxidant activity reached a peak after 90 minutes. In another study, Seigneur *et al.* (1990) administered three alcoholic beverages to 16 healthy subjects over a period of 15 days. The white wine led to an increase in total and LDL cholesterol and a marked increase in HDL cholesterol. Red wine led to a decrease in ADP induced platelet aggregation and an increase in HDL cholesterol. Demrow *et al.* (1994) administered red wine, red grape juice and white wine to anesthetised dogs. Platelet-inhibiting properties were found with the red wine and red juice but not with the white wine.

2.3.5 MOTIVATION FOR FURTHER INVESTIGATIONS

To account for the antioxidant activities of the individual phenolic compounds found in wines, we may consider structural factors such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl and keto groups, free carboxyl acid groups, and other structural features. The dihydroxylation in both rings and in the 3-position in catechin, myricetin, quercetin, and epicatechin is required for antioxidant activity. There is a need to consider possible effects of synergism and antagonism in complex mixtures of these phenolic compounds in different wines. More research is needed to evaluate individual components from different wines and from different vintages to gain a better understanding of the interaction of phenolic compounds and their changes on aging. All phenolic compounds that are absorbed could have a health impact. Previous work has shown that large doses of catechin are absorbed from the gut, but the catechin found in blood is largely transformed into glucuronides. When catechin was administered orally in human subjects, over half the amount given was absorbed and the rest excreted in the urine. It was reported that quercetin is not absorbed by humans (Frankel *et al.*, 1995). According to Pace-Asciak, Rounova, Hahn, Diamandis & Goldberg (1996) the differential effects of alcoholic beverages upon the reduction of platelet aggregation and thromboxane production should be investigated in human subjects.

Chapter 3

MATERIALS AND METHODS

3.1 INTRODUCTION

The total phenolic concentration in wine is dependent on the cultivar of the wine and is further enhanced through the winemaking process. Traditional fermentation following crushing and destemming leads to a maximum extraction of up to 60% (Zoecklein *et al.*, 1995: 115-116). Microbial activity may lead to increases in the concentrations of certain phenols. Fermentation and storage in oak provides additional sources of phenolics. This leads to a great variation of phenolic concentrations in wines.

There is also a great variation in the biological activity between the different phenolic components in the wines. It was therefore necessary not only to determine phenolic concentrations, but also the biological activity of the phenolics present in the different wines. In order to evaluate the inhibiting effect of the phenolics, the oxidation of isolated LDL was monitored.

Test procedures were performed at the Department of Haematology, University of the Orange Free State (UOFS) and Department Paramedical Sciences, Technikon Free State.

MATERIALS

3.2.1 APPARATUS

The apparatus used during the procedures were in good working condition and calibrated to give accurate results.

TABLE 3.1 The apparatus used during the procedures.

Apparatus	Description	Brand Name	Suppliers in South Africa
Automatic pipettes	10-100 µl 20-200 µl 200-1000 µl	Gilson	Laboratory and Scientific Equipment Co (Pty) Ltd
	5-40 µl 40-200 µl 200-1000µl 1-5 ml 2-10 ml	Finnpipette	AEC Amersham (Pty) Ltd
Balances	MC1 and Excellence	Sartorius	Zeiss
Centrifuges	Beckman Model TJ-S	Beckman	Beckman Coulter (Pty) Ltd
	Beckman Model J2-21	Beckman	Beckman Coulter (Pty) Ltd
	Heraeus Biofuge 13	Heraeus	Premier Technologies
Cell counter	Coulter MD18	Coulter	Beckman Coulter (Pty) Ltd
pH-meter	Metrohm 654 pH-meter	Metrohm	Swiss Lab Technologies
Rotary evaporator	Birby	Birby	Laboratory and Scientific Equipment Co (Pty) Ltd
Spectrophotometers	Shimadzu UV-1201	Shimadzu	The Scientific Group
	Spectro 22	Spectro Photometer	Optolabor (Pty) Ltd
	Pye Unicam 8700 UV/VIS (Philips)	Pye Unicam	Philcam

3.2.2 STANDARDS AND CONTROLS

TABLE 3.2 Standards and controls used during cholesterol determinations.

Cholesterol standards	Catalogue number	Brand Name	Suppliers in South Africa
Sigma (50 mg/dl)	C 9908	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Sigma (200 mg/dl)	C 0284	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Cholesterol controls			
Accutrol normal	C 2034	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Accutrol abnormal	C 3034	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd

REAGENTS, BUFFERS AND CARTRIDGES

Reagents, buffers and cartridges were obtained from the main suppliers of high-grade chemicals in South Africa.

TABLE 3.3 Reagents, buffers and cartridges used during the procedures.

Reagent	Catalogue Number	Brand Name	Suppliers in South Africa
Ammonium Ferrous Sulphate	Prod 10022	BDH	Merck NT Laboratories Supplies (Pty) Ltd
Ascorbic Acid	Prod 10030	BDH	Merck NT Laboratories Supplies (Pty) Ltd
Butylated Hydroxytolene	48F05045	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
(±)-Catechin	C1788	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Copper Sulphate	Art 2790	Merck	Merck NT Laboratories Supplies (Pty) Ltd
(-)-Epicatechin	E 1753	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Ethanol Absolute GR	Prod AB000983	Merck	Merck NT Laboratories Supplies (Pty) Ltd
Folin-Ciocalteu's phenol reagent	40023444	Merck	Merck NT Laboratories Supplies (Pty) Ltd
Hydrochloric acid	Prod 10307	BDH	Merck NT Laboratories Supplies (Pty) Ltd
Methanol Absolute GR	Art 6009	Merck	Merck NT Laboratories Supplies (Pty) Ltd
Plasmalyte B	TFA 3716	SABAX	Adcock Ingram Critical Care Ltd
Polyvinylpolypyrrolidone	P 6755	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Quercetin	Q 0125	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Resveratrol	R 5010	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Sodium Chloride	582 23 00	Saarchem	Saarchem Holpro Analytic
Sodium Hydroxide	Prod 30167	BDH	Merck NT Laboratories Supplies (Pty) Ltd
Xylenol Orange	7228000	Saarchem	Saarchem Holpro Analytic
Phosphate buffered saline 10mM	P 3813	Sigma (USA)	Sigma-Alrich S.A. (Pty) Ltd
Sep-Pak Cartridges (C18)	Part No WAT020515	Waters	Microsep

3.2.3 KITS

TABLE 3.4 Commercial kits used for the determination of cholesterol and the isolation of LDL, and the determination of L-ascorbic acid levels.

Kit	Catalogue number	Brand Name	Suppliers in South Africa
LDL-Direct Plus (Cholesterol Ratio System)	QS-8650	Isolab	IEPSA Diagnostics
L-Ascorbic Acid, Test-Combination	0409677	Boehringer Mannheim	Boehringer Mannheim

3.2.4 GLASSWARE AND PLASTICS

Disposable plastics and high quality glassware were used during the procedures.

TABLE 3.5 Disposable plastics and high quality glassware used.

Product	Description	Brand Name	Suppliers in South Africa
Bottles	40 ml	Schotts	Laboratory and Scientific Equipment Co (Pty) Ltd
EDTA-tubes	4,5 ml	Vacutainer Systems	MacMed Health Care Ltd
Microcentrifuge vials	1,5 ml transparent and amber Eppendorf tubes	Kartell	Laboratory and Scientific Equipment Co (Pty) Ltd
Plastic tubes	5 ml sterile 10 ml sterile	Kartell	Laboratory and Scientific Equipment Co (Pty) Ltd
Quartz cuvettes	Semi-micro	Lightpath Optical	Laboratory and Scientific Equipment Co (Pty) Ltd

3.2.6 WINES AND FRUIT JUICES

Distillers Corporation, Stellenbosch supplied six red and six white wines. The red wine selection consisted of 2 x Cabernet Sauvignon, 2 x Merlot, 1 x Johannisberger Red and 1 x Claret Select. The white wine selection consisted of 2 x Chardonnay, 2 x Sauvignon Blanc, 1 x Riesling and a 50/50 blend of Sémillon/Chardonnay. The harvest years ranged between 1990-1997. Distillers Corporation completed questionnaires, requesting information of the conditions under which the grapes were grown and harvested, the winemaking processes and other relevant information.

Red Grapetiser and White Grapetiser, both products from Appletiser, were obtained from a local supermarket. Appletiser completed a questionnaire on the composition of the grape juices. (All questionnaires are included in the Appendix.)

TABLE 3.6 Wines and grape juices included in the project.

No	Wine /Juice	Producer	Cultivar / Blend	Harvest Year
1	Red wine	Fleur du Cap	Cabernet Sauvignon	1990
2	Red wine	Fleur du Cap	Merlot	1995
3	Red wine	Drosdy-Hof	Merlot	1995
4	Red wine	Cellar Cask	Johannisberger Red*	N/A***
5	Red wine	Drosdy-Hof	Claret Select**	N/A***
6	Red wine	Stellenryck	Cabernet Sauvignon	1992
7	Red grape juice	Appletiser	Red Grapetiser	N/A***
8	White grape juice	Appletiser	White Grapetiser	N/A***
9	White wine	Theuniskraal	Sémillon/Chardonnay	1996
10	White wine	Fleur du Cap	Riesling	1997
11	White wine	Fleur du Cap	Chardonnay	1996
12	White wine	L'Ormarins	Sauvignon Blanc	1997
13	White wine	Drosdy-Hof	Chardonnay	1997
14	White wine	Le Bonheur	Sauvignon Blanc	1996

*Johannisberger Red a blend of Cabernet Sauvignon, Shiraz and Tinta Barocca

** Claret Select a blend of Cabernet Sauvignon, Pinotage and Tinta Barocca *** Not available

3.2.7 SUBJECTS

Healthy male personnel employed by the Technikon Free State within the age group 20-45 years gave consent to participate in this study.

Inclusion criteria:

They had to have a normal full blood count and a total cholesterol value of 5,2 mmol/l or less. The HDL levels should be greater than 20% of the total cholesterol value according to the formula: HDL divided by total cholesterol x 100 ≥ 20%. Body Mass

Index (BMI) had to be less than 28 kg/m^2. Blood specimens were collected after a 10 hour fast period.

Exclusion criteria:

Smokers were not included. Subjects on medication and supplementary vitamins were excluded. They had to be total abstainers or moderate alcohol consumers. No alcohol consumption during the last 10 hours before phlebotomy was permitted. Voluntary subjects completed and signed an indemnity form.

TABLE 3.7 Personal data on fifteen volunteer subjects.

No	Age	Race	Weight in Kg	Length in meter	BMI	Comply to criteria
1	30	W	82	1,86	23,7	Yes
2	44	W	83	1,78	25,9	Yes
3	31	W	85	1,78	26,5	Yes
4	23	W	75	1,90	20,8	Yes
5	29	W	75	1,74	24,8	Yes
6	25	W	75	1,75	24,5	Yes
7	36	W	85	1,62	32,7	No (sinusitus and low blood sugar)
8	28	W	85	1,82	25,7	Yes
9	30	B	70	1,70	24,2	Yes
10	23	W	92	1,89	25,7	Yes
11	32	W	90	1,83	26,9	No (smoker)
12	37	W	90	1,76	29	No (smoker)
13	23	W	85	1,66	30	No
14	29	W	65	1,68	23	Yes
15	35	W	75	1,90	20,8	Yes

3.3 METHODS

3.3.1 TOTAL PHENOLIC DETERMINATION

Grapes and wines contain a large diversity of phenolic compounds, derived from the basic structure of phenol. A variation of the original method for the determination of the total phenolic concentration, as described by Singleton & Rossi (1965), was used during this study (Zoecklein *et al.*, 1995: 455-458).

Many authors used gallic acid as a reference and results were expressed as mg gallic acid equivalent (GAE)/l (Zoecklein *et al.*, 1995: 457; Frankel *et al.*, 1995). During this study catechin was used as a reference, as described by Vinson & Hontz (1995). All the phenolic concentrations are expressed as mg catechin equivalent/l. In this study the phenolic concentrations are reported as mg/l, although they are actually mg catechin equivalent/l.

3.3.1.1 Principle

Phenolic substances are reduced by the Folin-Ciocalteu reagent which consists of a mixture of phosphotungstic acid and phosphomolybdic acid. As a result of this redox reaction, the reagent changes from yellow to a blue colour. The intensity of the blue colour produced is proportional to the phenol concentration. Folin-Ciocalteu promotes complete oxidation and thus also ensures the measurement of the more slowly reacting phenolics (Singleton & Rossi, 1965).

3.3.1.2 Preparation of reagents

A commercial Folin-Ciocalteu reagent was used. One liter of sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate into 700 ml deionised water. The volume was brought to 1 liter and boiled until all the crystals were dissolved. The solution was allowed to cool and another 2-3 g of sodium carbonate was added. After 24 hours the solution was filtered and was ready for use. A new calibration standard was prepared each time, by dissolving 10 mg catechin into 5 ml of ethanol. This was followed by the addition of 35 ml of deionised water to give a catechin concentration of 250 mg/l.

3.3.1.3 Experimental procedure

According to Zoecklein *et al.* (1995) a 1:10 dilution of a red wine should be used for the determination of total phenolic concentrations. During this study red wines were analysed in an undiluted and 1:10 dilution. For each determination a blank and five standards were done in duplicate. The catechin standards were prepared by diluting 250 mg/l of catechin with deionised water to give final concentrations of 50, 100, 150, 250 and 500 mg/l (refer to Table 3.8).

The phenol determinations were performed in 40 ml screw cap bottles. Two hundred µl of wine or fruit juice was added to 1800 µl of deionised water. This was followed by the addition of 14 ml deionised water. One ml of Folin-Ciocalteu reagent was added and vortexed for 30 seconds. Three ml of saturated sodium carbonate was added within the next 8 minutes (see Table 3.8). The samples were mixed and allowed to stand at 22°C for 2 hours. A Pye Unicam 8700 spectrophotometer, set at 765 nm, was used to determine the absorbance readings. The spectrophotometer was calibrated in the concentration mode and phenolic concentrations were obtained directly from the printout.

TABLE 3.8 Preparation of calibration standards and test samples for the Folin-Ciocalteu procedure.

	Blank	Standard 50 mg/l	Standard 100 mg/l	Standard 150 mg/l	Standard 250 mg/l	Standard 500 mg/l	Test Sample
Catechin 250 mg/l concentration	-	200 µl	400 µl	600 µl	1000 µl	2000 µl	-
Deionised water	2000 µl	1800 µl	1600 µl	1400 µl	1000 µl	-	1800 µl
Wine, juice or fraction	-	-	-	-	-	-	200 µl
Deionised water	14 ml	14 ml	14 ml	14 ml	14 ml	14 ml	14 ml
Folin-Ciocalteu reagent	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
Saturated sodium carbonate solution	3 ml	3 ml	3 ml	3 ml	3 ml	3 ml	3 ml

The total phenolic concentration was calculated by multiplying the mean of the duplicate readings obtained from the spectrophotometer by 5, taking the dilution factor of the wines, fruit juices or fractions into consideration.

TABLE 3.9 Mean readings calculated into phenolic concentrations (Wine 4 as an example).

Fraction	First reading	Second reading	Average	Calculation factor	Phenolic concentration in mg/l
Concentrated wine ROH+*	427	455	441	(x5)	2205
Concentrated wine ROH-**	415	400	408	(x5)	2040
Neutral fraction	164	163	163,5	(x2) (x5)	1635
Acid fraction	4	3	3,5	(x2) (x5)	35
Waste fraction	0	0	0	(x2) (x5)	0
Wine 1:10 dilution ROH+	41	41	41	(x10) (x5)	2050
Wine 1:10 dilution ROH-	39	40	39,5	(x10) (x5)	1975

*ROH+ = Wine containing alcohol **ROH- = Wine without alcohol

3.3.2 FRACTIONATION OF PHENOLICS

Neutral and acid phenolics were isolated from the beverages through solid phase column chromatography. Proteins were first removed from the juices by precipitation with cold ethanol (Jaworski & Lee, 1987).

3.3.2.1 Principle

The C18 cartridges selectively retain phenolics from the beverages, which are then eluted from the columns with methanol. It is therefore essential to remove all the alcohol from the beverages before passing it though the cartridges. By changing the pH of the columns the phenolics are separated into a neutral and an acid fraction.

3.3.2.2 Preparation of reagents

A concentrated hydrochloric acid (cHCl) was diluted too a 1M HCl solution and a 0,01M HCl solution by diluting 100 ml of cHCl and 1 ml of cHCl in one liter of deionised

water respectively. A 1M sodium hydroxide (NaOH) solution was prepared by dissolving 4g of NaOH into 40 ml of deionised water.

3.3.2.3 Preparation of samples

The ethanol had to be removed from the wines. This was done by rotary evaporation on an evaporator at 50°C. It was essential to know how much ethanol was present in the wines. The alcohol volume percentage (vol%), obtained from the labels on the wine bottles, was a good indication on how much ethanol had to be removed. The procedure lasted between 7-25 minutes. The wines were reconstituted to the pre-evaporation volume with deionised water.

Proteins were removed from the grape juice by adding one part juice to two parts of cold (-20°C) ethanol. The juices were deproteinated during a 2 hours incubation period at 4°C. This was followed by centrifugation at 38000 g for 30 minutes at 4°C in a Beckman Model J2-21 centrifuge. The supernatant was removed from the protein debris. The ethanol added to the grape juice was removed through rotary evaporation at 50°C. The procedure lasted 30 minutes. The deproteinated fruit juice was then diluted to the original volume with distilled water.

3.3.2.4 Storage of opened wines, juices and fractionated samples

The unprocessed, processed wines and juices were aliquoted into 10 ml sterile tubes and 1,5 ml microcentrifuge tubes, filled to the top, stoppered, covered in tin foil and stored in the dark at 4°C until used. The maximum storage period for the opened wines and juices were 14 days (Abu-Amsha *et al.*, 1996; Goldberg, *et al.*, 1996).

3.3.2.5 Experimental procedure

Fractionation of the beverages into the different phenolic fractions was done in duplicate. Separate C18 SEP-PAK cartridges were preconditioned for the fractionation of neutral and acidic phenolics. The cartridges for the neutral phenolics, were preconditioned with 2 ml of methanol followed by 2 ml of distilled water. The cartridges for the acidic phenolics were preconditioned with 2 ml of methanol, followed by 2 ml of 0,01M HCl.

The alcohol-free wines and fruit juices were adjusted to pH 7 with 1M NaOH. One ml of the adjusted wine or fruit juice was layered onto the cartridge which was prepared for the neutral phenolics. Neutral phenolics were absorbed onto the cartridge while the rest of the wine or fruit juice passed through. The effluent was adjusted to pH 2,5 with 1M HCl. It was then layered onto the cartridges prepared for the acid phenolics. Neutral and acidic phenolics were eluted from the cartridges with 2 ml of methanol. The eluates containing the same fractions were combined. The respective combined fractions were adjusted to 4 ml with deionised water. All the fractions were in a 1:2 dilution. The different fractions were divided and stored in amber microcentrifuge tubes at 4°C.

3.3.2.6 Linearity and accuracy of phenolic determination method

In an attempt to verify the accuracy and linearity of the phenolic concentration determinations in wines, spiking of a wine with a known concentration of a commercial phenolic was done. Red and white wines were compared with one another in a volumetric dilution, but with a ten times difference in the dilution factor.

3.3.2.6.1 Spiking

Red wine in a 1:10 dilution was spiked with a sequence of catechin concentrations. The first sample, which served as a blank contained diluted red wine only. Samples 2-6 contained both diluted wine and different concentrations of catechin. The quantities of catechin added were; 25, 75, 125, 175 and 225 µg. Total phenolic concentrations were measured using the Folin-Ciocalteu method. The blank reading was subtracted from the other five readings.

TABLE 3.10 A diluted red wine spiked with catechin.

	Blank	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Red wine 1:10 dilution	200 µl	200 µl	200 µl	200 µl	200 µl	200 µl
Deionised water	1800 µl	1700 µl	1500 µl	1300 µl	1100 µl	900 µl
250 mg/l catechin concentration		100 µl	300 µl	500 µl	700 µl	900 µl
Catechin in µg	0	25	75	125	175	225

3.3.2.7 Stripping of phenolics

Polyvinylpolypyrrolidone (PVPP) binds to flavonoids and a variety of other phenolic compounds through hydrogen bonding (Vrhovsek *et al.,* 1997). Ninety percent of phenolics can be stripped from wines and juices by passing the beverage through a column packed with PVPP (Abu-Amsha *et al.,* 1996). In order to confirm that it was the phenolic compounds and not other components of the wine that reacted with the Folin-Ciocalteu reagent, the samples were stripped of its phenolic compounds.

3.3.2.7.1 Experimental procedure

A 2% PVPP was prepared in 12% ethanol, and washed three times with the same solvent. The solution was packed in polystyrene syringes forming a bed of 2,5 ml PVPP. One ml of each, red wine, white wine, white grape juice and a 250 mg/l catechin solution were layered onto different PVPP beds. Once the beverages and

control were absorbed onto the bed, 1 ml of 12% ethanol was layered on top. The effluents were collected in clean test tubes.

One ml of the pre-stripped wine, juice or control was adjusted with deionised water to equal the volume of the post-stripped effluent. Total phenol determinations using the Folin-Ciocalteu method, were done on the pre- and post-stripped specimens. Biological activities were measured against LDL (method to follow).

3.3.3 BIOLOGICAL ACTIVITY DETERMINATION OF ANTIOXIDANTS PRESENT IN GRAPE BEVERAGES

The oxidation of LDL leads to the formation of lipid peroxides and conjugated dienes, which include fatty acid hydroperoxides and certain oxysterols, Chait (1992). The basis of the biological activity determination was the formation of conjugated dienes during the oxidation of LDL. The results were confirmed by measuring hydroperoxides.

3.3.3.1 Conjugated diene determination

Esterbauer, Striegl, Puhl & Rotheneder (1989) described the conjugated diene method. When LDL is incubated in the presence of copper sulphate, a lag period elapsed before the LDL is oxidised. This is due to the presence of natural antioxidants in human serum. The lag period is prolonged with the addition of antioxidants present in wines and fruit juices.

It was recommended that the lag time runs should be incubated at 37°C. Due to the lack of a temperature-controlled spectrophotometer, samples were heated to 27°C and the heat generated within the spectrophotometer, retained the samples at 27°C throughout the running procedure. Kanner, Frankel, Granit, German & Kinsella (1994)

measured LDL oxidation with the addition of 8 µM $CuSO_4$ at room temperature. Frei & Gaziano (1993) incubated the samples at 37°C after the addition of 1,25 µM copper chloride ($CuCl_2$) under constant stirring.

3.3.3.1.1 Principle

Conjugated dienes are formed during the oxidation of polyunsaturated fatty acids. Conjugated dienes may diminish during the later stages of lipid peroxidation and this puts a limitation on the incubation period of the procedure (Chajès, Sattler, Stultsching & Kostner, 1995).

3.3.3.1.2 Preparation of reagents

Phosphate buffer was prepared by dissolving one sachet of phosphate buffered saline (PBS) into 1000 ml of deionised water. PBS was stored in the dark at 4°C. A 10 mM stock solution of copper sulphate was prepared by dissolving 0,25 g of copper sulphate crystals into 100 ml of distilled water. This solution was kept in the dark at 4°C. A working solution was prepared freshly before each run, by diluting 1 part of stock into 9 parts of PBS giving a 1 mM $CuSO_4$ concentration. During the final dilution the 1 mM concentration was diluted another 100 times to give a 10 µM concentration.

3.3.3.1.3 Dilutions made of wines and fruit juices

Wine without alcohol was used for the fractionation into neutral and acidic phenolic fractions. Therefore, the phenolic concentration of the wine without alcohol was used for all further calculations in respect to the neutral and acidic fractions. However, when reporting the phenolic concentration on a wine, the concentration obtained from the wine with alcohol was reported.

The wines and juices, as well as the different fractions of the beverages were diluted in PBS to a phenolic concentration of 5 mg/l to compare the biological activity of the phenolic compounds present in the beverages on a concentration basis. Wines and juices were also volumetrically diluted in PBS (Abu-Amshu *et al*, 1996). Red wines and red grape juice were diluted 1:1000 and white wines and white grape juice 1:100 to compare the biological activity of the phenolics in the different wines and juices on a glass to glass basis. See Appendix for calculations made.

3.3.3.1.4 LDL isolation

A commercial kit, LDL-Direct Plus (Code QS-8650) was used for the isolation of serum lipoproteins into an alpha and a beta fraction through affinity chromatography. The alpha fraction corresponded to the HDL obtained after ultracentrifugation assays. The LDL and VLDL were combined in the beta fraction. The same kit was used for the determination of total cholesterol, HDL and LDL values.

3.3.3.1.4.1. Preparation of EDTA plasma

EDTA blood samples were collected, after a 10 hour fast period, from each volunteer and immediately wrapped in aluminium foil to protect the cholesterol against oxidation by ultra violet (UV) light. The separation of plasma was done in an UV protected room. Plasma was separated and aliquots of 1 ml of plasma were frozen at -80°C. An 18 parameter full blood count was performed, using the Coulter MD18. Total cholesterol determinations were done on all the plasma samples. Plasmas from the subjects that complied with the criteria were pooled for LDL preparation.

3.3.3.1.4.2 Preparation of reagents

Each kit consisted of separation columns, an alpha fraction elution agent and a beta fraction elution agent. The cholesterol reagent was freshly prepared before each

procedure by adding equal volumes of enzyme reagent and activator together. Normal saline was prepared by dissolving 0,89 g of sodium chloride (NaCl) into 100 ml of deionised water. Lyophilised normal and abnormal controls were diluted to the desired volume. Standard cholesterol samples were supplied ready for use.

3.3.3.1.4.3 Experimental procedure

EDTA plasma was thawed at room temperature. Equal volumes of the plasma from the 8 selected subjects were pooled together and mixed gently. The separation columns were prepared as described in the manufacturer's protocol.

3.3.3.1.4.3.1 Alpha fraction elution

The column was positioned over a test tube and 200 µl of the pooled plasma was added onto the column. The plasma was allowed to flow into the column and the eluate was collected. Fifty µl of the alpha fraction elution agent was added to the column and left for five minutes. This was followed by adding 1 ml of the alpha fraction elution agent to the column. The effluent of 1,25 ml was collected in a test tube and mixed well.

3.3.3.1.4.3.2 Beta fraction elution

The same column used for the alpha fraction was transferred to a clean test tube. From the beta fraction elution agent 2,7* ml was added and the eluate collected in a test tube. Beta fraction (LDL and VLDL) was aliquoted into 500 µl and 1 ml volumes and frozen at -80°C.

*According to the method it should be 2,5 ml, but 2,7 ml was used in obtaining a larger volume LDL with a lower mg/dl LDL-concentraiton.

3.3.3.1.4.3.3 Determination of cholesterol values

Total cholesterol determinations were done on the plasmas of the subjects. A total cholesterol determination was also done on the pooled plasma before LDL fractionation. The HDL concentration was measured in the alpha fraction and the LDL was measured in the beta fraction. Normal and abnormal controls were done in parallel with the test. Two standard cholesterol samples, 200 and 50 mg/dl respectively, were included for the calculation of the cholesterol values.

The plasma for total cholesterol, normal and abnormal controls and 200 mg/dl cholesterol standard were diluted by adding 200 µl of the sample into 2,3 ml isotonic saline. The 50 mg/dl cholesterol standard was diluted by adding 200 µl of the sample into 1,05 ml isotonic saline.

From the working cholesterol reagent 1,8 ml was added into all the tubes. Two hundred µl of the diluted samples, diluted cholesterol standards and alpha and beta fractions were added to the corresponding tubes and deionised water was added to the reagent blank. Tubes were incubated for 5 minutes at 37°C and read at 505 nm on the Shimadzu UV-1201 spectrophotometer. The instrument was set to zero with the reagent blank.

3.3.3.1.4.3.4 Calculation of cholesterol values

Cholesterol values were calculated according to the following formulas:

$$\frac{\text{Absorbance reading of the sample x 50 mg/dl}}{\text{Absorbance reading of the 50 mg/dl standard}} = \text{Alpha cholesterol concentration}$$

$$\frac{\text{Absorbance reading of the sample x 200 mg/dl}}{\text{Absorbance reading of the 200 mg/dl standard}} = \text{Beta cholesterol concentration}$$

$$\frac{\text{Absorbance reading of the sample x 200 mg/dl}}{\text{Absorbance reading of the 200 mg/dl standard.}} = \text{Total cholesterol concentration}$$

3.3.3.1.5 Experimental procedure

Pooled LDL, protected from UV light throughout the procedure, was diluted 1:6 in PBS to give a concentration of 200 µg/ml. During the preliminary runs a 200 µg/ml concentration gave the best results. The Shimadzu UV 1201 spectrophotometer could take up to 6 cuvettes. A run included a LDL as a negative control, LDL plus catechin as a positive control and 4 x LDL plus beverage and / or beverage fraction.

Each cuvette contained the following:

1,2 ml of a 200 µg/ml (1 part LDL* plus 5 parts PBS)

100 µl PBS for the blank or 100 µl of the wine / juice/ fraction in dilution

13 µl $CuSO_4$ working solution

*The LDL was added last and followed by gentle mixing.

TABLE 3.11 Reagents and samples added.

Reagents	Tube 1 Blank	Tube 2 Positive control	Tube 3	Tube 4	Tube 5	Tube 6
LDL 200 µg/ml	1,2 ml	1,2 ml	1,2 ml	1,2 ml	1,2 ml	1,2 ml
PBS	100 µl					
Catechin dilution**		100 µl				
Diluted wines, juices or fractions			100 µl	100 µl	100 µl	100 µl
$CuSO_4$ working solution	13 µl	13 µl	13 µl	13 µl	13 µl	13 µl

** Varied from run to run.

The tubes were incubated at 27°C for 10 minutes. During the preliminary runs the temperature of the test samples in the cuvettes were determined on several occasions and it was then concluded that once it reached 27°C it remained there throughout the day. Absorbance readings were taken every 500 seconds on a kinetics program. Graphs were constructed from the absorbance readings and displayed on the screen of the spectrophotometer. Results were printed and read into Excel* and the necessary graphs drawn.

*Trade mark of the Microsoft Company

The Shimadzu UV 1201, spectrophotometer was run in a kinetics program.

1	γ	= 234 nm
2	Number of measurements	= 11 meas, Int: 500 sec
3	Lag time	= 10 sec
4	Rate time	= 3000
5	Factor	= 1.0000
6	Rec range	= -0.2 - 0.5 A

Lag periods varied between 6-11 hours.

The rate of LDL oxidation was determined from the propagation phase of the time course curve using a spline function. The lag phase was obtained by drawing a tangent to the slope of the propagation phase and extrapolating it to the horizontal axis (Jialal & Grundy, 1993). The exact lag time cut-off was obtained through the X–co-ordinate of the intersection of the two regression lines.

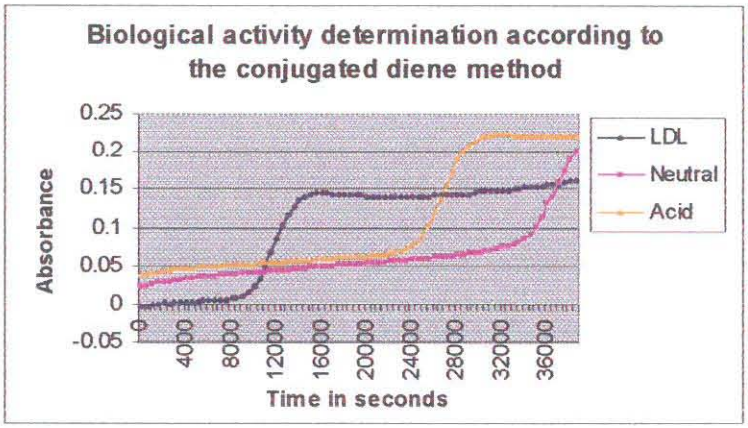


FIGURE 3.1 Biological activity determination according to the conjugated diene method.

3.3.3.1.6 Biological activities

The biological activities of the different fractions were calculated as follows:

$$\frac{\text{Mean lag time period of the fraction – the mean lag time period of the corresponding LDL}}{\text{Divided by the mean lag time period of the corresponding LDL}} \times 100 = \%\ \text{biological activity}$$

3.3.2.1.6.1 Linearity and accuracy of the biological activity method

Serial dilutions were made of a white and a red wine. The white wine was diluted 1:100, 1:125, 1:150, 1:175 and 1:200 and the red wine was diluted 1:1000, 1:1250, 1:1500, 1:1750 and 1:2000. Biological activity was then determined by the conjugated diene method.

3.3.3.2 Ferrous Xylenol Orange, Version II (FOX2) method

The FOX2 method was described for the measurement of hydroperoxides in plasma. An authentic plasma hydroperoxide can be determined by a strategy in which the hydroperoxide reductant, triphenylphosphine, is used to discriminate between the background signal generated by ferric ions present in plasma and those generated by hydroperoxide in plasma (Nourooz-Zadeh, Tajadinni-Sarmadi & Wolff, 1994).

3.3.3.2.1 Principle

The FOX2 assay is based upon the ability of hydroperoxides to oxidise ferrous ions to ferric ions under acidic conditions where the auto-oxidation of the ferrous ions is very slow (Nourooz-Zadeh, Tajaddini-Sarmadi, Ling & Wolff, 1996).

3.3.3.2.2 Preparation of reagents

Sulphuric acid (H_2SO_4) was diluted to a concentration of 250 mM by diluting 13,8 ml H_2SO_4 in 1000 ml deionised water. A stock solution of 1 mM xylenol orange and 2,5 mM ammonium ferrous sulphate was prepared by adding 76 mg of xylenol orange and 98 mg of ammonium ferrous sulphate into 100 ml of the 250 mM H_2SO_4 solution. A 4,4 mM Butylated hydroxytoluene (BHT) solution was prepared by adding 969,32 mg BHT into 1000 ml methanol. All the reagents were kept in the dark at 4°C. The working FOX2 reagent was prepared just before use, by adding 1 part of the stock solution to 9 parts of the BHT solution. The working reagent comprised of 250 µM

ammonium ferrous sulphate, 100 µM xylenol orange, 25 mM H_2SO_4 and 4 mM BHT in methanol.

A solution of 10 mM triphenylphosphine (TPP) was prepared by dissolving 262,29 mg triphenylphosphine into 1000 ml methanol. This solution was kept between 2-6 °C in the dark. A stock solution of 20 µM hydrogen peroxide (H_2O_2) was prepared by diluting 113,37 µl H_2O_2 (30%) into 500 ml distilled water. The working solution was prepared by diluting 0,5 ml of the stock reagent with 49,5 ml deionised water to give a 20 µM H_2O_2 solution.

3.3.3.2.3 Construction of the calibration curve

The FOX2 working reagent was routinely calibrated against solutions of H_2O_2 of known concentrations. A calibration curve was constructed which is included in the Appendix.

TABLE 3.12 Dilutions for the calibration curve.

Concentration	Stock solution	Deionised water
20 µM	10 ml	0
16 µM	8 ml	2 ml
12 µM	6 ml	4 ml
10 µM	5 ml	5 ml
8 µM	4 ml	6 ml
4 µM	2 ml	8 ml
2 µM	1 ml	9 ml
1 µM	500 µl	9,5 ml
0,5 µM	250 µl	9,75 ml

Plasmalyte B was used as a blank. Ninety µl of each calibration standard was added to 10 µl methanol in 1,5 ml microcentrifuge tubes, in duplicate. The tubes were incubated for 30 minutes at 22°C and 900 µl of working FOX2 reagent was added. This was followed by another 30 minutes incubation at 22°C. The vails were spun at 12000 g for 5 minutes in a Heraeus Biofuge 13 Eppendorf centrifuge. The

absorbance of the supernatant was measured by means of a Spectro 22 spectrophotometer at 560 nm.

3.3.3.2.4 Preparation of the LDL sample

A 200 µg/ml concentration of LDL in PBS was prepared. Working $CuSO_4$ solution was added. LDL plus copper sulphate was run as a blank. An aliquot of a 5 mg/l phenolic concentration of the wine or juice to be measured was added, see Table 3.13. These solutions were incubated in a 37°C waterbath in the dark. Aliquots from the solution were taken at definite time intervals and hydroperoxide concentrations were measured using the FOX2-reagent.

TABLE 3.13 LDL, wine or juice and copper sulphate solution for the FOX2 method.

Ingredients	Negative control LDL without the addition of an antioxidant	Test LDL with the addition of wine or juice in a 5 mg/l phenolic concentration	Positive control LDL with the addition of 5 mg/l catechin
PBS	2500 µl	5000 µl	5000 µl
LDL	500 µl	1000 µl	1000 µl
PBS	250 µl		
Wine or juice		500 µl	
Catechin			500 µl
$CuSO_4$ solution	32,5 µl	65 µl	65 µl

3.3.3.2.5 Experimental procedure

The blank and samples were done in duplicate. Four microcentrifuge tubes were prepared for each test procedure. To two of the tubes 10 µl of methanol was added and to two of the tubes 10 µl of the 10 mM TPP was added. From the LDL sample, 90 µl was pipetted into the 4 microcentrifuge tubes. The same was done to the negative and positive controls. The samples were incubated for 30 minutes at 22°C. To each of the 4 microcentrifuge tubes, 900 µl of the working FOX2 reagent was added and

incubated for another 30 minutes at 22°C. The tubes were then spun down in a Eppendorf centrifgue at 12000 g for 5 minutes.

The absorbance of the supernatants was determined at 560 nm. The reading of the sample containing TPP was subtracted from the reading of the sample containing methanol. The mean difference was calculated and converted to µM hydroperoxide by means of a standard curve, included in the Appendix.

TABLE 3.14 The conversion of absorbance readings into µM hydroperoxide from the standard curve.

Absorbance*	1,05-1,5 µM	Absorbance*	1,55-2 µM	Absorbance*	2,05-2,5 µM	Absorbance*	2,55-3 µM
0,014-0,015	1,05	0,031-0,032	1,55	0,048-0,049	2,05	0,066-0,067	2,55
0,016-0,017	1,1	0,033-0,034	1,6	0,05	2,1	0,068-0,069	2,6
0,018-0,019	1,15	0,035	1,65	0,051-0,052	2,15	0,07-0,071	2,65
0,02	1,2	0,036-0,037	1,7	0,053-0,054	2,2	0,072-0,073	2,7
0,021-0,022	1,25	0,038-0,039	1,75	0,055	2,25	0,074-0,075	2,75
0,023	1,3	0,04	1,8	0,056-0,057	2,3	0,076-0,077	2,8
0,024-0,025	1,35	0,041-0,042	1,85	0,058-0,059	2,35	0,078-0,079	2,85
0,026-0,027	1,4	0,043-0,044	1,9	0,06-0,061	2,4	0,08-0,081	2,9
0,028	1,45	0,045	1,95	0,062-0,063	2,45	0,082	2,95
0,029-0,03	1,5	0,046-0,047	2	0,064-0,065	2,5	0,083	3

*The difference between the absorbance reading from the TPP and the absorbance reading of the methanol samples.

3.3.3.2.6 Calculation of the biological activity according to the FOX2 method

The differences between the lag time of the LDL plus wine and the LDL without wine was divided by the LDL without wine and expressed as a percentage.

$$\frac{\text{Lag time (minutes) of LDL plus wine} - \text{lag time (minutes) of LDL (negative control)}}{\text{Lag time (minutes) of LDL}} = \%\ \text{activity}$$

3.3.4 THE INFLUENCE OF ASCORBIC ACID ON THE BIOLOGICAL ACTIVITY OF WINES AND JUICES

The concentration of vitamin C in grapes ranges from 5 to 150 mg/kg in fresh fruit (Zoecklein *et al.*, 1995: 190). Distillers Corporation confirmed they had added 25 mg/l to the white wines supplied for the project, but none was added to the red wines. Ascorbic acid is a monobasic acid with lactone ring formation. Vitamin C acts as an antioxidant either alone or in conjunction with sulphur dioxide. All wines from Distillers Corporation were also dosed with sulphur dioxide to an average concentration of 40 mg/l.

3.3.4.1 Ascorbic acid levels in wines and juices

Ascorbic acid levels were measured using the L-Ascorbic Acid kit from Boehringer Mannheim. The kit is based on a colorimetric method. Two readings are taken during the procedure. L-Ascorbic acid (L-ascorbate) and a reducing substance ($X\text{-}H_2$) reduce the tetrazolium salt 3-(4,5-dimethylthiazolyl-2)–2,5-diphenyltetrazolium bromide (MTT) in the presence of the electron carrier 5-methylphenazinium (PMS) at pH 3,5 to a formazan. The total of all reducing substances present in each sample was measured. Reagents supplied with the kit were ready for use.

Ascorbic acid measurements were performed on all 14 beverages. Two samples were prepared for each test determination. Into these samples the following was added; 1 ml of solution one, 1,5 ml of deionised water and 100 µl beverage. One ascorbate oxidase spatula was added to the sample blank only. Both samples were mixed and incubated for 6 minutes at 37°C. During the incubation period the sample blank was mixed every two minutes. The spatula was removed from the sample and both samples were read at 578 nm on the Shimadzu UV-1201 spectrophotometer. Solution

3 was added to both samples, mixed well and incubated for another 15 minutes at 37°C, whilst protected from UV light. Both samples were then read at 578 nm.

The calculation for each sample was done according to:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/L]}$$

V = final volume (ml)

V = sample volume (ml)

MW = molecular weight of the substance to be assayed [g/mol]

D = light path (cm)

ε = extinction coefficient for MTT-formazan at 578 nm = 16,9 [1 x $mmol^{-1}$ x cm^{-1}]

3.3.4.2 Ascorbic acid subjected to column chromatography

A 250 mg/l concentration of ascorbic acid was prepared by dissolving 10 mg of ascorbic acid in 40 ml of deionised water. The concentration was further diluted 1:10 to give a 25 mg/l concentration. One ml of the solution was exposed to solid phase extraction by running it through the neutral and acidic prepared C18 cartridges. The neutral, acid and waste effluents were collected. The biological activity of these fractions plus a 1 mg/l ascorbic acid dilution were determined by the conjugated diene method.

3.3.4.3 Biological activity of ascorbic acid in parallel with commercial phenolic activities

Four commercial phenolics plus ascorbic acid at a concentrations of 250 mg/l each were prepared in deionised water, ethanol or a combination of ethanol and deionised water (see Table 3.15 for details). These dilutions were diluted to 1 mg/l concentrations and measured for conjugated dienes against LDL in the presence of the copper sulphate working reagent.

TABLE 3.15 Dilutions made on commercial phenolics and ascorbic acid.

Substance	Catechin	Epicatechin	Resveratrol	Quercetin	Ascorbic acid
Amount in milligrams	10 mg	10 mg	1 mg	10 mg	10 mg
Ethanol	5 ml	5 ml	4 ml	40 ml	
Distilled water	35 ml	35 ml			40 ml
Concentration	250 mg/l	250 mg/l	250 mg/l	250 mg/l	250 mg/l

3.3.4.4 Contribution of ascorbic acid to the antioxidant activity of wine

According to Boulton *et al.* (1996: 83) ascorbic acid can be used as an antioxidant either alone or in conjunction with sulfur dioxide. It affects the enzyme activity only by its competition for and depletion of the available oxygen. It can reverse the first step of phenol oxidation, that is, converting the quinone back to its corresponding phenol. This leads to a delay in the onset of browning of wines. Taking the concentration of ascorbic acid present in some of the wines and the juices into account a mathematical calculation could be made on the influence of ascorbic acid towards the total biological activity of these beverages.

The percentage ascorbic acid biological activities were calculated according to the following formula:

$$\frac{\text{AA* in mg/l X 46\% (Percentage biological activity of 1 mg/l AA)}}{\text{Dilution factor of wine of juice (phenolic or volumetric)}} = \text{\% biological activity of AA in beverage}$$

*Ascorbic acid

3.3.5 STATISTICS

Statistical analysis was performed using the Student t-test.

CHAPTER 4

RESULTS

4.1 INTRODUCTION

The *in vitro* antioxidant activities of wines on isolated LDL are confirmed in Europe, California in the United States of America and Australia. No data was found on the antioxidant activity of South African wines and juices. The aim of the study was to compare the phenolic concentration of the beverage and correlate this concentration with their biological activity. In order to compare the wines, a volumetric dilution and phenolic concentration dilution were analysed.

4.2 WINES AND FRUIT JUICES

The information on the wines, as obtained from Distillers Corporation, and on the grape juices obtained from Appletiser, are included in the Appendix. The skin contact period and fermentation period occurs simultaneously at most wineries, but it could vary from winery to winery and also from batch to batch. The Stellenryck Cabernet Sauvignon had skin contact for 8 days. It was fermented for 8 days at 28°C. Wood contact was for 18 months in 300 liter barrels. Fleur du Cap Cabernet Sauvignon was made under similar conditions as the previous one with the exception that it had wood contact for 24 months. The Fleur du Cap Merlot had 9 days skin contact with 9 days fermentation at 28°C. The wine was matured for 12 months in 300 liter wood barrels. The Drosdy-Hof Merlot had skin contact for 8 days with fermentation for 8 days at 28°C. It was then matured for 18 months in 300 liter wood barrels. The Cellar Cask Johannisberger Red, with 8 days on the skin and 8 days fermentation at 28°C, had no wood contact. The Drosdy-Hof Claret had 4 days skin contact and was fermented for 8 days at 28°C. The wine was matured in 500 liter old barrels for 12 months.

The white wines had no skin contact. The 2 Chardonnay wines had 4 months each wood contact in 300 liter barrels. The Fleur du Cap Chardonnay had 14 days fermentation at 15°C and the Drosdy-Hof Chardonnay had 14 days fermentation at 14°C. The rest of the white wines had no wood contact. Le Bonheur Sauvignon Blanc was fermented for 15 days at 13°C. L'Ormarins Sauvignon Blanc was fermented at 16°C for 15 days. The Sémmilon / Chardonnay was fermented for 15 days at 14°C and the Fleur du Cap Riesling for 16 days at 14°C.

The information from Appletiser was not as informative. Red Grapetiser was prepared from 100% Muscat Grape. The White Grapetiser, was prepared from Neutral Grapes (75%) and Muscat Grapes (25%). Neither the Red nor the White Grapetiser had any wood contact.

4.3 SUBJECTS

TABLE 4.1 Laboratory information and data of the eight selected subjects included in the pooled plasma.

No	Period*in min	Full Blood Count	Total Cholesterol value in mmol/l	Comply with criteria
1	40	N	4,4	Yes
4	60	Plt ↓ 104	4,7	Yes
5	75	N	4,6	Yes
6	70	N	3,5	Yes
8	60	N	4,1	Yes
10	35	N	4,5	Yes
14	70	N	4,1	Yes
15	45	WCC↓ 3,8	5,1	Yes
Mean	54		4,4	
Highest	75		5,1	
Lowest	30		3,5	

* Period between withdrawl of blood and plasma frozen at -80°C

N = Normal, Plt = Platelet count (normal reference range 150-450 x 10^9/l), WCC = White cell count (normal reference range 4,5-10,5 x 10^9/l)

Cholesterol reference ranges as obtained from the LDL-Direct Plus kit:

Total cholesterol for both male and female

Age 20-29	3,12 – 6,24 mmol/l
Age 30-39	3,64 – 7,02 mmol/l

Eight men complied with the criteria and their plasma was included in the plasma pool for LDL separation. The full blood counts of the 8 subjects were within the normal reference ranges except for subject 4 with a platelet count lower than normal and

subject 15 with a white cell count lower than normal. These two parameters had no effect on LDL oxidation and the subjects were included in the study.

4.4 TOTAL PHENOLIC DETERMINATION

The wines and juices were fractionated into neutral and acidic fractions, with pH adjustments made before running the samples through the appropriate cartridges. The phenolic concentrations were determined in fractionated and unfractionated wines and juices.

4.4.1 EVAPORATION OF ETHANOL AND VOLUME REPLACEMENT

The alcohol vol% as specified on the bottles, was used as an indication for volume replacement. It took between 7-25 minutes to evaporate the ethanol from the wines. During this period the phenolics in the wines were exposed to 50°C. The volume replaced by deionised water in 50 ml wine, varied between 7 to 11 ml.

TABLE 4.2 Evaporation and deproteinisation of the wines and juices.

Number	Alcohol vol% Bottle	Alcohol vol% Distillers Corporation	Deproteinization with ethanol, time period	Ethanol evaporation period	Volume replaced by deionised water (total of 50 ml)
Wine 1 (red)	12,5	12,3		20 minutes	7 ml
Wine 2 (red)	13	13		18 minutes	7 ml
Wine 3 (red)	13	12,99		15 minutes	11 ml
Wine 4 (red)	11,5	11,5		10 minutes	7 ml
Wine 5 (red)	12	12,1		20 minutes	8 ml
Wine 6 (red)	12,5	12,56		15 minutes	7 ml
Mean on 1-6					**7,8 ml**
Juice 7 (red)			3 hours	30 minutes	0 ml
Juice 8 (white)			3 hours	30 minutes	2 ml
Wine 9 (white)	12	12		25 minutes	10 ml
Wine 10 (white)	11	11,87		7 minutes	7 ml
Wine 11 (white)	12,5	12,54		10 minutes	10 ml
Wine 12 (white)	12,5	12,84		10 minutes	11 ml
Wine 13 (white)	13	12,89		10 minutes	11 ml
Wine 14 (white)	12,5	12,5		7 minutes	7 ml
Mean (9-14)					**9,3 ml**
Mean (1-14)				**16,2 minutes**	**7,5 ml**

In both the juices it took 30 minutes to evaporate double the volume of ethanol from the juice. Phenolics in the juices were exposed to 50°C for 30 minutes. The replacement volume with deionised water was 0 ml in the red juice, and 2 ml in the white juice.

4.4.2 THE pH VALUES

The pH values as determined in the laboratory corresponded well to those obtained from Distillers Corporation. The mean pH value of 3,5 of the 12 wines was the same before removal of ethanol and deproteination of the 2 juices, as well as after. The pH 7 adjusted beverages, once drained through the cartridges, constantly gave higher pH values. The effluent was adjusted to pH 2,5 and drained through the cartridge and collected as the waste fraction. During the last fractionation step the mean pH reading decreased from 2,6 to 2,4.

TABLE 4.3 The pH of the beverages and the fractions.

No	Wine ROH+ / Unprocessed juice	Wine ROH- / DP* juice	Adjusted wines / juices Pre-neutral cartridge	Post-neutral cartridge	Acidified wines / juices	Eluted neutral fraction	Eluted acid fraction	Waste fraction
Wine 1	3,5	3,3	**7,0**	7,3	**2,5**	7,5	3,0	-
Wine 2	3,5	3,3	**7,0**	7,3	**2,5**	7,7	3,6	2,4
Wine 3	3,6	3,3	**7,0**	7,3	**2,4**	7,7	3,7	2,6
Wine 4	3,8	3,5	**7,3**	7,4	**2,5**	7,9	3,8	2,6
Wine 5	3,8	3,6	**7,0**	7,3	**2,3**	7,7	2,8	2,2
Wine 6	3,5	3,4	**7,0**	7,3	**2,8**	7,7	3,9	2,4
Juice 7*	3,3	3,9	**6,9**	7,2	**2,8**	7,9	3,8	2,4
Juice 8*	3,2	3,7	**6,8**	7,1	**2,9**	8,0	4,0	2,5
Wine 9	3,4	3,2	**7,0**	7,3	**2,7**	8,1	3,7	2,4
Wine 10	3,3	3,3	**7,1**	7,4	**2,4**	8,5	3,4	2,3
Wine 11	3,7	3,6	**7,3**	7,4	**2,8**	8,1	3,8	2,6
Wine 12	3,5	3,4	**6,9**	7,3	**2,8**	8,1	3,8	2,6
Wine 13	3,5	3,4	**6,8**	7,2	**2,2**	7,9	3,1	2,2
Wine 14	3,5	3,5	**6,8**	7,3	**2,3**	8,0	3,3	2,2
Mean	**3,5**	**3,5**	**7,0**	**7,3**	**2,6**	**7,9**	**3,6**	**2,4**
SD	± 0,177	± 0,191	**± 0,159**	± 0,082	**± 0,23**	± 0,25	± 0,369	± 0,157
CV			**2,27%**		**8,85%**			

* Deproteinated Juice

4.4.3 PHENOLIC CONCENTRATIONS

The total phenolic concentration of the red wines varied between 1590 and 2750 mg/l, with a mean value of 2162 mg/l. The undiluted sample measurements on wine 6 exceeded the calibration concentration and could therefore not be used.

TABLE 4.4 Phenolic concentrations in mg/l of the red wines.

Red wine	Date opened	ROH+ total	ROH+ 1:10 dilution	ROH- total	ROH- 1:10 dilution	Neutral fraction	Acid fraction	Waste fraction	% Neutral, acid and waste recovery
1	26/11/97	2430	2550	2230	2400	1970	120	30	88%
2	3/12/97	2335	2325	2390	2425	1955	80	20	85%
3	8/12/97	2205	2050	2040	1975	1635	35	0	85%
4	8/12/97	1590	1650	1720	1725	1250	60	10	77%
5	17/12/97	1685	1645	1775	1750	1330	90	40	83%
6	17/12/97	>CC*	2750	>CC*	2750	2385	130	60	94%
Mean of 1-6		**2166**	**2162**	**2151**	**2171**	**1754 81%**	**86 4%**	**27 1%**	**86%**

>CC = Exceeded the calibration concentration

*For calculation purposes 2750 was used

The total phenolic concentration of the unprocessed white juice gave a concentration of 100 mg/l and on repeating the sample, 80 mg/l. The deproteinated white juice gave a phenolic concentration of 95 mg/l and on repeating the sample it gave a value of 75 mg/l. The sum of the three fractions, neutral, acid and waste gave a phenolic concentration of 175 mg/l, which was used as the phenolic concentration of the white juice.

TABLE 4.5 Phenolic concentrations in mg/l of the grape juices.

Grape juice	Date opened	Unprocessed total	Deproteinated total	Neutral fraction	Acid fraction	Waste fraction	% Neutral, acid and waste recovery
7	30/1/98	410	383	175 45,5%	140 37%	60 15,5%	98%
8	30/1/98	100 (180) 80	95 (175) 75	70 40%	55 31%	50 29%	180% 100%

The total phenolic levels in the white wines were in general very high and it ranged between 225 – 315 mg/l. The percentage recovery of the three fractions, neutral, acid and waste, was very good.

TABLE 4.6 Phenolic concentrations in mg/l of the white wines.

White wine	Date opened	ROH+ total	ROH- total	Neutral fraction	Acid fraction	Waste fraction	% Neutral, acid and waste recovery
9	20/2/98	230	200	160	30	10	100%
10	20/2/98	225	215	150	40	10	93%
11	6/3/98	315	290	220	30	10	90%
12	6/3/98	240	213	160	35	15	99%
13	14/3/98	290	285	210	40	30	98%
14	14/3/98	250	240	190	60	30	117%
Mean of 9-14		**258**	**240**	**182** **76%**	**39** **17%**	**18** **7%**	**99,5%**

4.4.4 LINEARITY AND ACCURACY OF THE METHOD

A 1:10 dilution of red wine was spiked with a 250 mg/l catechin concentration to prove linearity and accuracy of the phenolic determination method. The quantity of catechin added to each of the wines, in the third column of Table 4.7, compared well to the difference between the sample and blank reading in the sixth column.

TABLE 4.7 Red wine spiked with catechin.

	Volume added	Catechin added	Duplicate readings on spectrophotometer	Mean reading	Mean reading minus blank
Blank, 1:10 dilution of red wine	0	0	52 52	52,0	0
Diluted red wine plus catechin	100 µl	25 µg	79 78	78,5	26,5
Diluted red wine plus catechin	300 µl	75 µg	125 127	126,0	74,0
Diluted red wine plus catechin	500 µl	125 µg	186 185	185,5	133,5
Diluted red wine plus catechin	700 µl	175 µg	239 232	235,5	183,5
Diluted red wine plus catechin	900 µl	225 µg	279 281	280,0	228,0

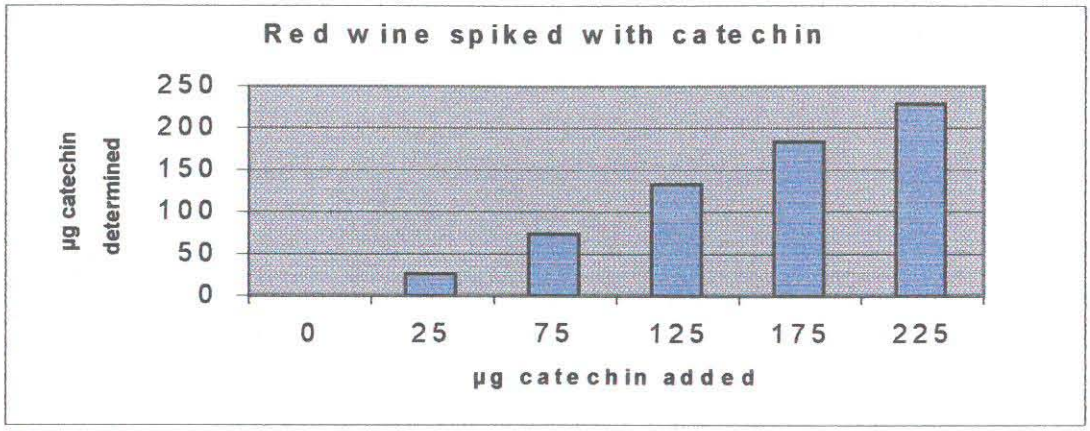


FIGURE 4.1 A 1:10 red wine dilution spiked with 25, 75, 125, 175 and 225 µg catechin.

4.4.5 STRIPPING OF THE PHENOLICS.

Red wine, white wine, white grape juice and catechin solution were stripped from phenolics through a bed of packed PVPP. The percentage phenolics left in the post-PVPP stripped columns were 1% in the red wine, 0% in the white wine, 7% in the juice and 8% percent in the catechin.

TABLE 4.8 Pre- and post-PVPP stripped phenolic content of the beverages and control.

	Phenolic concentration in mg/l	% Phenolic concentration left
Red wine pre	750	
Red wine post	10	1%
White wine pre	95	
White wine post	0	0%
Juice pre	75	
Juice post	5	7%
Catechin pre	125	
Catechin post	10	8%

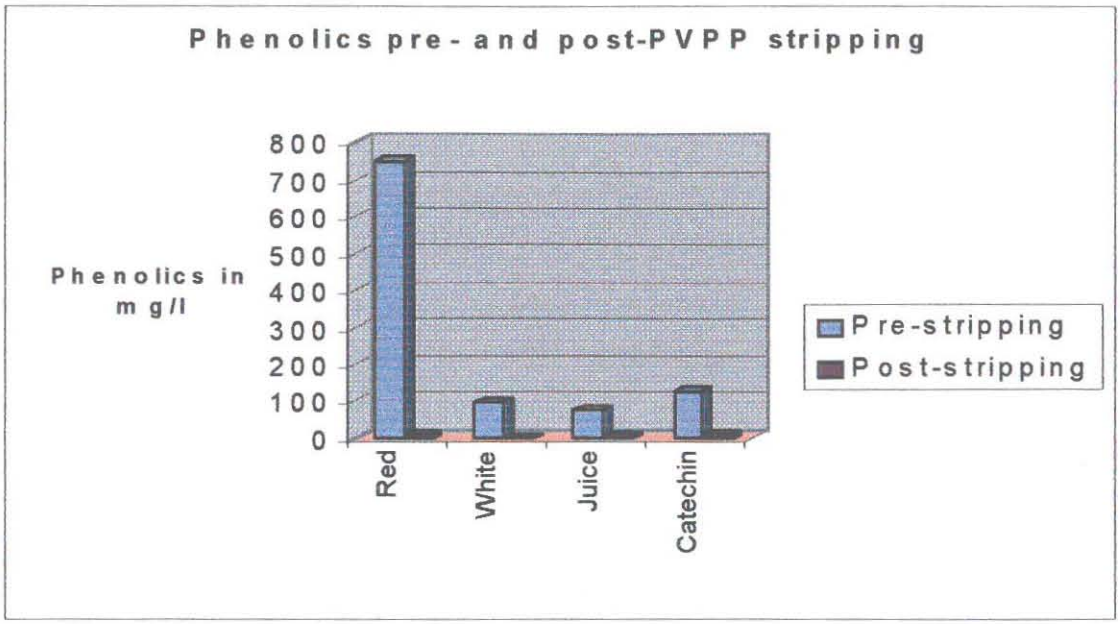


FIGURE 4.2 The phenolic concentrations in wines, juice and catechin of the pre- and post-stripped samples.

4.5 BIOLOGICAL ACTIVITIES

The higher the phenolics in the wine, the longer expected lag period and therewith the higher percentage biological activity was expected. In diluting all the wines and juices to the same phenolic concentration, a phenolic comparison between wines and juices was possible.

4.5.1 CHOLESTEROL VALUES

The pooled plasma was fractionated on two different occasions. The second fractionation showed a slight decrease in cholesterol values, as seen in Table 4.9. All the values were within normal reference ranges.

TABLE 4.9 Cholesterol and control values of the pooled plasma.

Date	Fraction	Cholesterol mg/dl*	Cholesterol mmol/l*	Cholesterol µg/ml	1:6 Dilution** µg/ml	Reference ranges
24 November 1997	Total cholesterol	183	**4,8**			120-270 mg/dl (3,12-7,02 mmol/l)
	α	51	**1,3**			29-61 mg/dl (0,75-1,59 mmol/l)
	β	117	**3,04**	1170	195	No references
	Normal control	159	**4,13**			122-164 mg/dl (3,2-4,2 mmol/l)
	Abnormal control	245	**6,37**			196-266 mg/dl (5,1-6,9 mmol/l)
14 February 1998	Total cholesterol	Not done				
	α	48	**1,25**			29-61 mg/dl (0,75-1,59 mmol/l)
	β	115	**2,99**	1150	192	No references
	Normal control	149	**3,87**			122-164 mg/dl (3,2-4,2 mmol/l)
	Abnormal control	250	**6,5**			196-266 mg/dl (5,1-6,9 mmol/l)

*mmol/l = mg/dl x 0,026

**200 µl LDL solution + 1000 µl PBS

4.5.2 LAG TIME

The absorbance readings obtained from the spectrophotometer were read into Excel* with the lag time on the X-axis and the absorbance readings on the Y-axis. The exact cut-off point from the lag time was calculated from the X co-ordinate intersection of the regression line. The lag periods were recalculated into mean values in seconds, minutes and hours. In the phenolic concentration dilutions the lag periods were prolonged in comparison to those of the volumetric dilutions. The results were tabulated in Tables 4.10, 4.11 and 4.12.

*Trade mark of the Microsoft Company

TABLE 4.10 Mean lag periods of the red wines in seconds, minutes and hours

Red wine	ROH + 1:1000	ROH – 1:1000	Neutral 1:1000	Acid 1:1000	ROH+ 5 mg/l	ROH- 5 mg/l	Neutral 5 mg/l	Acid 5 mg/l	N+A 5 mg/l
Wine 1	19629 s 327 m 5h27m	19858 s 331 m 5h31m	18581 s 310 m 5h10m	6826 s 114 m 1h54m	33938 s 566 m 9h26m	34375 s 573 m 9h33m	26574 s 443 m 7h23m	26696 s 445 m 7h25m	22870 s 381 m 6h21m
Wine 2	16433 s 281 m 4h41m	17680 s 295 m 4h55m	17826 s 297 m 4h57m	10825 s 180 m 3h	28751 s 479 m 7h59m	30414 s 507 m 8h27m	25475 s 425 m 7h5m	24261 s 404 m 6h44m	18091 s 302 m 5h2m
Wine 3	15925 s 265 m 4h25	15827 s 264 m 4h24m	14246 s 237 m 3h58m	12336 s 206 m 3h26m	29948 s 499 m 8h19m	30492 s 508 m 8h28m	30285 s 505 m 8h25m	31888 s 531 m 8h52m	17530 s 292 m 4h52m
Wine 4	19403 s 323 m 5h23m	17255 s 288 m 4h48m	21584 s 360 m 6h	7442 s 124 m 2h4m	30277 s 505 m 8h25m	29193 s 487 m 8h7m	31500 s 525 m 8h45m	30442 s 507 m 8h27m	18960 s 316 m 5h16m
Wine 5	22473 s 375 m 6h15m	22917 s 382 m 6h22m	21865 s 364 m 6h4m	12335 s 206 m 3h26m	26988 s 450 m 7h30m	27015 s 450 m 7h30m	26520 s 442 m 7h22m	23899 s 398 m 6h38m	20178 s 336 m 5h36m
Wine 6	26516 s 442 m 7h22m	25549 s 426 m 7h6m	24126 s 402 m 6h42m	16002 s 267 m 4h27m	27801 s 463 m 7h43m	27132 s 452 m 7h32m	26512 s 442 m 7h22m	23575 s 393 m 6h33m	19824 s 330 m 5h30m

TABLE 4.11 Mean lag periods of the grape juices in seconds, minutes and hours.

Grape juices	ROH+ 1:1000	ROH- 1:1000	Neutral 1:1000	Acid 1:1000	ROH+ 5 mg/l	ROH- 5 mg/l	Neutral 5 mg/l	Acid 5 mg/l	N+A 5 mg/l
Juice 7	18823 s 314 m 5h14m	18301 s 305 m 5h5m	16827 s 280 m 4h40m	14384 s 240 m 4h	30996 s 517 m 8h37m	33722 s 562 m 9h22m	29825 s 497 m 8h17m	31073 s 518 m 8h38m	23792 s 397 m 6h37m
	ROH+ 1:100	**ROH- 1:100**	**Neutral 1:100**	**Acid 1:100**	**ROH+ 5 mg/l**	**ROH- 5 mg/l**	**Neutral 5 mg/l**	**Acid 5 mg/l**	**N+A 5 mg/l**
Juice 8	19833 s 331 m 5h31m	19828 s 330 m 5h30m	13783 s 228 m 3h48m	13816 s 230 m 3h50m	35268 s 588 m 9h48m	31393 s 523 m 8h43m	32646 s 544 m 9h4m	23229 s 387 m 6h27m	19945 s 332 m 5h32m

TABLE 4.12 Mean lag periods of the white wines in seconds, minutes and hours.

White wine	ROH+ 1:100	ROH- 1:100	Neutral 1:100	Acid 1:100	ROH+ 5 mg/l	ROH- 5 mg/l	Neutral 5 mg/l	Acid 5 mg/l	N+A 5 mg/l
Wine 9	18215 s 304 m 5h4m	18639 s 311 m 5h11m	16713 s 279 m 4h38m	11186 s 186 m 3h6m	23828 s 397 m 6h37m	25608 s 427 m 7h7m	24976 s 416 m 6h56m	33377 s 556 m 9h16m	21587 s 360 m 6h
Wine 10	17131 s 286 m 4h46m	18903 s 315 m 5h15m	16588 s 276 m 4h36m	9196 s 153 m 2h33m	31728 s 529 m 8h49m	27673 s 461 m 7h41m	29011 s 484 m 8h4m	28299 s 472 m 7h52m	20906 s 348 m 5h48m
Wine 11	22386 s 373 m 6h13m	24484 s 408 m 6h48m	18799 s 313 m 5h13m	10521 s 175 m 2h55m	28067 s 468 m 7h48m	26354 s 439 m 7h19m	27899 s 465 m 7h45m	29578 s 493 m 8h13m	19870 s 331 m 5h31m
Wine 12	21637 s 361 m 6h1m	22514 s 375 m 6h15m	16886 s 281 m 4h41m	10172 s 170 m 2h50m	26122 s 435 m 7h15m	32013 s 534 m 8h54m	27621 s 460 m 7h40m	27357 s 456 m 7h36m	20322 s 339 m 5h39m
Wine 13	22126 s 369 m 6h9m	22433 s 374 m 6h14m	19964 s 333 m 5h33m	9674 s 161 m 2h41m	26567 s 443 m 7h23m	31119 s 519 m 8h39m	25139 s 419 m 6h59m	28727 s 480 m 8h	17799 s 297 m 4h57m
Wine 14	19037 s 317 m 5h17m	19492 s 325 m 5h25m	17020 s 284 m 4h44m	12699 s 212 m 3h32m	22679 s 378 m 6h18m	23873 s 398 m 6h38	24537 s 409 m 6h49m	28321 s 472 m 7h52m	19117 s 319 m 5h19m

4.5.3 PERCENTAGE BIOLOGICAL ACTIVITY

The mean percentage biological activities of the white wines in the phenolic concentration dilutions were significantly higher in comparison to the red wines.

TABLE 4.13 Biological activity of the different beverages and corresponding fractions.

Wine / Juice	ROH + 1:1000	ROH – 1:1000	Neutral 1:1000	Acid 1:1000	ROH+ 5 mg/l	ROH- 5 mg/l	Neutral 5 mg/l	Acid 5 mg/l	N+A 5 mg/l
Wine 1	97	98	87	0	169	185	177	178	130
Wine 2	162	181	184	0	286	220	242	226	188
Wine 3	119	119	96	6	115	119	118	129	141
Wine 4	66	66	44	0	145	136	154	146	95
Wine 5	82	86	77	0	144	145	140	116	83
Wine 6	88	81	71	13	174	167	161	132	95
Mean 1-6	**102**	**105**	**93**	**3**	**172**	**162**	**165**	**155**	**122**
SD 1-6	± 34	± 41	± 48	± 5	± 60	± 37	± 43	± 41	± 39
Juice 7	17	14	5	0	149	171	140	150	91
	ROH+ 1:100	**ROH- 1:100**	**Neutral 1:100**	**Acid 1:100**	**ROH+ 5 mg/l**	**ROH- 5 mg/l**	**Neutral 5 mg/l**	**Acid 5 mg/l**	**N+A 5 mg/l**
Juice 8	74	55	31	31	231	195	231	135	102
Wine 9	155	161	134	56	261	288	278	406	227
Wine 10	171	198	162	45	364	304	324	314	206
Wine 11	226	257	174	53	429	397	426	457	274
Wine 12	306	323	217	91	288	375	310	306	202
Wine 13	169	173	143	18	238	296	220	265	126
Wine 14	162	168	134	75	211	227	237	289	159
Mean 9-14	**198**	**213**	**161**	**56**	**299**	**315**	**299**	**340**	**199**
SD 9-14	± 59	± 64	± 32	± 25	± 83	± 62	± 74	± 75	± 52

4.5.3.1 Linearity and accuracy of biological activity

Serial dilutions of a red wine and a white wine indicated a constant decline in biological activity as tabulated in Table 4.14. These results were plotted in histograms, as Figures 4.3 and 4.4.

TABLE 4.14 Lag periods and biological activities of serial dilutions of a red and a white wine.

Red wine	LDL	1:1000 dil	1:1250 dil	1:1500 dil	1:1750 dil	1:2000 dil
Run 1	8387	18396,3	18009,8	16601,2	15812,3	15085
Run 2	9423,8	22247	19156,9	17498,4	18302	16258,1
Mean	8905,4	20321,7	18583,4	17049,8	15812,3	15671,6
% Biological activity		128%	109%	91%	91%	76%
White wine	**LDL**	**1:100 dil**	**1:125 dil**	**1:150 dil**	**1:175 dil**	**1:200 dil**
Run 1	8881,4	19782,3	18034,5	16561	16085,8	16565,6
Run 2	8638,6	19681,4	18293,5	16885,2	16028	15005,2
Mean	8760	19731,9	18164	16723,1	16056,9	15785,4
% Biological activity		125%	107%	91%	83%	80%

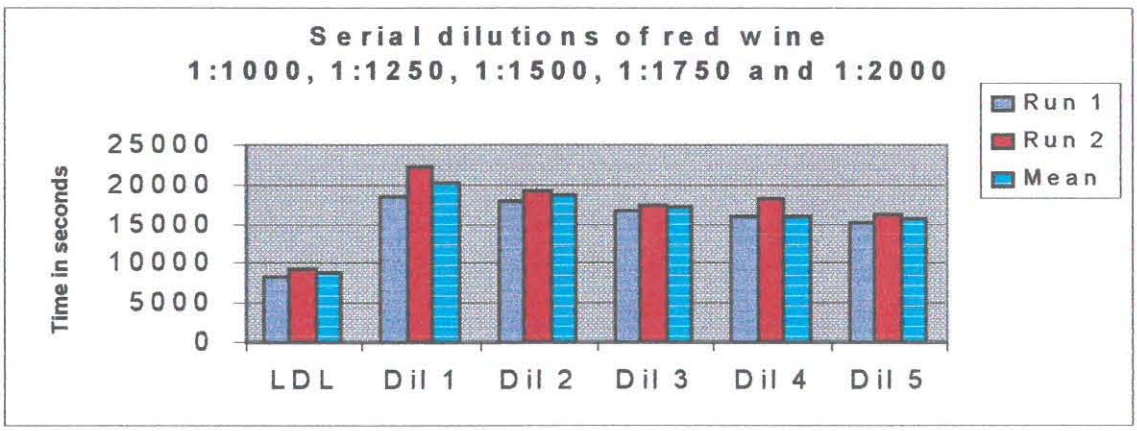


FIGURE 4.3 Lag time of red wine in five serial dilutions and their mean, with LDL as a control.

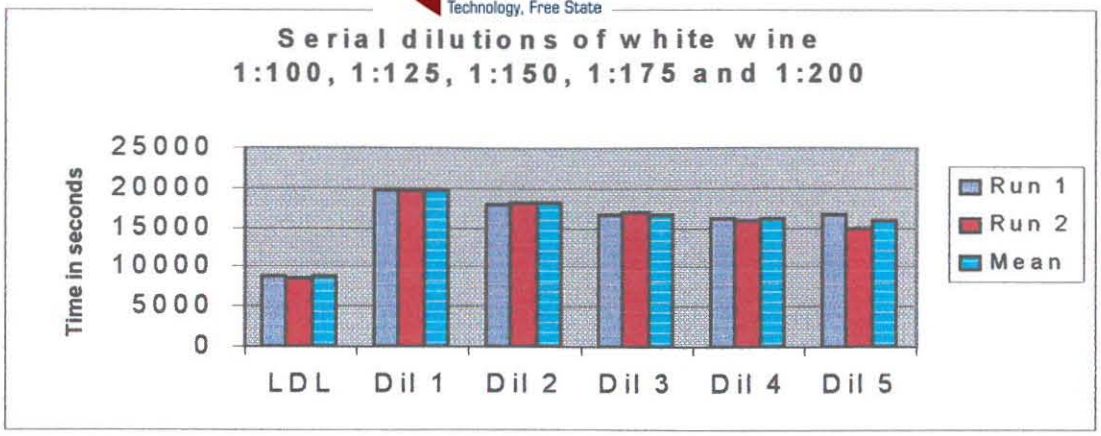


FIGURE 4.4 Lag time of white wine in five serial dilutions and their mean, with LDL as a control.

4.5.3.2. Biological activity on pre- and post-phenolic stripped beverages.

A significant decline was observed in the post-stripped samples in comparison to the pre-stripped samples. The post-stripped percentage biological activity were less when compared to the control level.

TABLE 4.15 Pre- and post-stripped percentage biological activity.

	Mean lag period in seconds	Percentage biological activity
LDL pre-run	8356	
LDL post-run	7717	
Red wine pre	31250	274%
Red wine post	7471	0%
White wine pre	11511	38%
White wine post	7156	0%
Juice pre	11404	36%
Juice post	7105	0%
Catechin pre	19463	133%
Catechin post	7003	0%

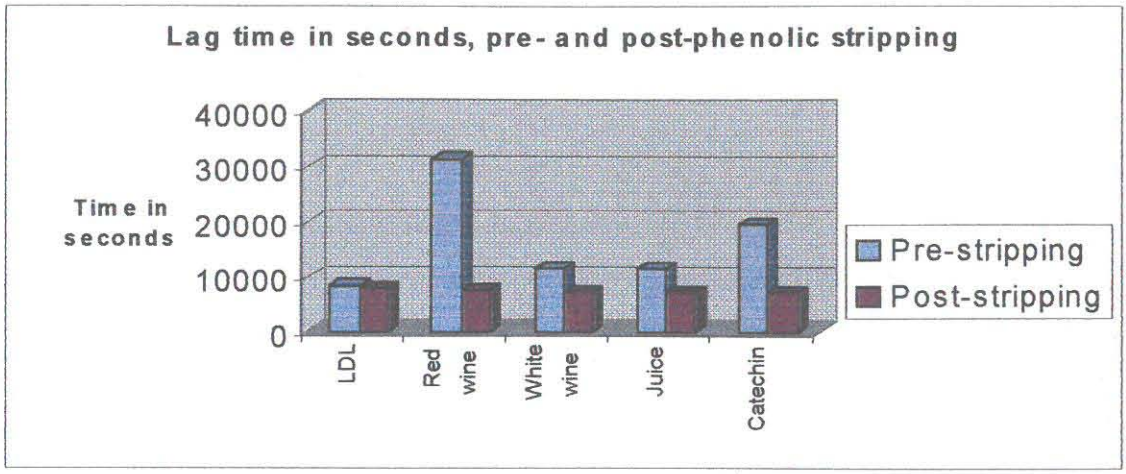


FIGURE 4.5 Lag periods of the pre- and post-stripped wines, juice and control samples.

4.5.3.3 Comparing red wine with white wine in 5 mg/l phenolic concentration and 1:100 dilution

The mean biological activity of the red wines versus the white wines was compared in a 5 mg/l phenolic concentration. The acid fraction of the white wine gave the highest biological activity of 340%.

TABLE 4.16 The mean biological activity of red wine versus white wine.

	Wine ROH+ 5 mg/l phenolics	Wine ROH- 5 mg/l phenolics	Neutral fraction 5 mg/l phenolics	Acid fraction 5 mg/l phenolics	Neutral plus acid fraction 5 mg/l phenolics
Red wine mean % biological activity	172%	162%	165%	155%	122%
White wine mean % biological activity	299%	315%	299%	340%	199%
White wine divided by red wine	1,74	1,94	1,81	2,19	1,63

The mean biological activity of the red wines and the white wines was also compared per volume, in a 1:100 dilution. Simultaneously, the mean phenolic concentration of the red wines was compared with those of the white wines.

TABLE 4.17 The mean percentage biological activity of red wine and white wine, in a 1:100 dilution and the mean phenolic concentration of red wine and white wine.

	Wine plus alcohol	Wine without alcohol	Neutral fraction	Acid fraction
Red wine 1:1000 dilution, mean % biological activity	102%	105%	93%	3%
White wine 1:100 dilution, mean % biological activity	198%	213%	161%	56%
Red wine (x 10)* divided by white wine	**5,2**	**4,9**	**5,8**	**0.5**
Red wine mean phenolics in mg/l	2162	2171	1754	86
White wine mean phenolics in mg/l	258	240	182	39
Phenols of red wine divided by phenolics of white wine	**8,4**	**9,0**	**9,6**	**2,2**

*To convert the red wine to a 1:100 dilution.

4.5.3.4 Comparing red juice with white juice in 5 mg/l phenolic concentration and volumetric dilution

The percentage biological activity of red juice was compared with white juice in a 5 mg/l phenolic concentration.

TABLE 4.18 Comparison of the biological activity of the red juice versus the white juice in a 5 mg/l phenolic concentration.

	Unprocessed juice , 5 mg/l phenolics	Deproteinated juice, 5 mg/l phenolics	Neutral fraction 5 mg/l phenolics	Acid fraction 5 mg/l phenolics	Neutral plus acid fraction 5 mg/l phenolics
Red juice % biological activity	149%	171%	140%	150%	91%
White juice % biological activity	231%	195%	231%	135%	102%
White juice divided by red juice	**1,6**	**1,1**	**1,7**	**0,9**	**1,1**

The percentage biological activity of the red juice and the white juice was compared in a 1:100 dilution. Simultaneously the phenolic concentration of the red juice was compared to that of the white juice.

TABLE 4.19 Biological activity in a 1:100 dilution, as well as the phenolic concentration of the red and white juice.

	Unprocessed juice	Deproteinated juice	Neutral fraction	Acid fraction
Red juice 1:1000 dilution, biological activity	17%	14%	5%	0%
White juice 1:100 dilution, biological activity	74%	55%	31%	31%
Red juice (x 10)* divided by white juice	**2,3**	**2,5**	**1,6**	**0**
Red juice phenols in mg/l	383 mg/l	175 mg/l	140 mg/l	60 mg/l
White juice phenols in mg/l	175 mg/l	70 mg/l	55 mg/l	50 mg/l
Phenolic concentration of red juice divided by phenolic concentration of white juice	**2,2**	**2,5**	**2,5**	**1,2**

*Converting the dilution to 1:100

4.5.3.5 The influence of alcohol on phenolic biological activity

The mean biological activity of wine with alcohol and wines without alcohol, both in a phenolic concentration dilution and in a volumetric dilution, was compared, as tabulated in Table 4.20.

TABLE 4.20 Influence of ethanol on percentage biological activity (wines only).

Wine	Wine ROH+ 1:1000	Wine ROH+ 1:100	Wine ROH- 1:1000	Wine ROH- 1:100	Wine ROH+ 5 mg/l	Wine ROH- 5 mg/l
1	97%	970%	98%	980%	169%	185%
2	162%	1620%	181%	1810%	286%	220%
3	119%	1190%	119%	1190%	115%	119%
4	66%	660%	66%	660%	145%	136%
5	82%	820%	86%	860%	144%	145%
6	88%	880%	81%	810%	174%	167%
Mean 1-6		**1023%**		**1052%**	**172%**	**162%**
9		155%		161%	261%	288%
10		171%		198%	364%	304%
11		226%		257%	429%	397%
12		306%		323%	288%	375%
13		169%		173%	238%	296%
14		162%		168%	211%	227%
Mean 9-14		**198%**		**213%**	**299%**	**315%**
Mean (1-6) + (9-14)		**611%**		**633%**	**235%**	**238%**
SD		± 490		± 520	± 95	± 93

4.5.3.6 A comparison between the neutral and acidic phenolic fractions in wines and juices

The biological activities between the neutral fractions and the acid fractions of the wines and juices were compared with one another both in a phenolic concentration dilution and a volumetric dilution.

TABLE 4.21 Biological activity of neutral and acidic phenolics in wines and juices.

Wine and/or juice	Neutral fraction 1:1000	Neutral fraction 1:100	Acidic fraction 1:1000	Acidic fraction 1:100	Neutral fraction 5 mg/l phenolics	Acidic fraction 5 mg/l phenolics	Neutral + acidic fraction 5 mg/l phenolics
Wine 1	87%	870%	0%	0%	177%	178%	130%
Wine 2	184%	1840%	0%	0%	242%	226%	188%
Wine 3	96%	960%	6%	60%	118%	129%	141%
Wine 4	44%	440%	0%	0%	154%	146%	95%
Wine 5	77%	770%	0%	0%	140%	116%	83%
Wine 6	71%	710%	13%	130%	161%	132%	95%
Juice 7	5%	50%	0%	0%	140%	150%	91%
Juice 8		31%		31%	231%	135%	102%
Wine 9		134%		56%	278%	406%	227%
Wine 10		162%		45%	324%	314%	206%
Wine 11		174%		53%	426%	457%	274%
Wine 12		217%		91%	310%	306%	202%
Wine 13		143%		18%	220%	265%	126%
Wine 14		134%		75%	237%	289%	159%
Mean		**474%**		**40%**	**226%**	**232%**	**151%**
Mean phenolic concentra -tion		847 mg/l		67,5 mg/l			

4.5.3.7 The influence of protein on the biological activity of the juices

Proteins were removed from the juices before fractionation into neutral and acid fractions. The unprocessed red juice and the deproteinated red juice were converted to a 1:100 dilution for volumetric comparison. Phenolic concentrations were also tabulated.

TABLE 4.22 Influence of protein on percentage biological activity (juices only).

Percentage biological activity	Red grape juice	White grape juice
Unprocessed juice 1:1000 dilution	17%	Not determined
Unprocessed juice 1:100 dilution	170%	74%
Deproteinated juice 1:1000 dilution	14%	Not determined
Deproteinated juice 1:100 dilution	140%	55%
Unprocessed juice 5 mg/l concentration	149%	231%
Deproteinated juice 5 mg/l concentration	171%	195%
Phenol concentration		
Unprocessed juice	410 mg/l	180 mg/l
Deproteinated juice	383 mg/l	175 mg/l

4.5.4 LIPID PEROXIDATION BY MEANS OF FERROUS XYLENOL ORANGE, VERSION II

The determinations were performed four times. During the first, second and third procedure, aliquots were taken every half an hour. During the last procedure aliquots were taken every fifteen minutes.

TABLE 4.23 FOX2 results expressed in hours, percentage biological activity and µM hydroperoxide concentration.

Date	2 December 1997			12 December 1997				20 December 1997				3 April 1998	
	LDL	Wine 1	Cat	LDL	Wine 2	Wine 3	Wine 4	LDL	Wine 5	Wine 6	Cat	LDL	Wine 12
Lag time in hours	3½	7	4½	2½	6½	7	7	2½	7	6½	4½	2½	6
% BA*		100%			160%	180%	180%		180%	160%			140%
µM** values reached	2,75	2,4	3,2	2,6	2,65	2,95	2,9	2,8	3,15	3,4	3	2,2	2,25

*Biological activity

**Normal reference ranges for serum/plasma 0,22 – 7,8 µM (Nourooz-Zadela)

Samples were incubated and measured at different time intervals until the exponential phase was completed. The absorbance readings on the FOX2 were recalculated from a standard curve (included in the Appendix) to µM hydroperoxide concentrations.

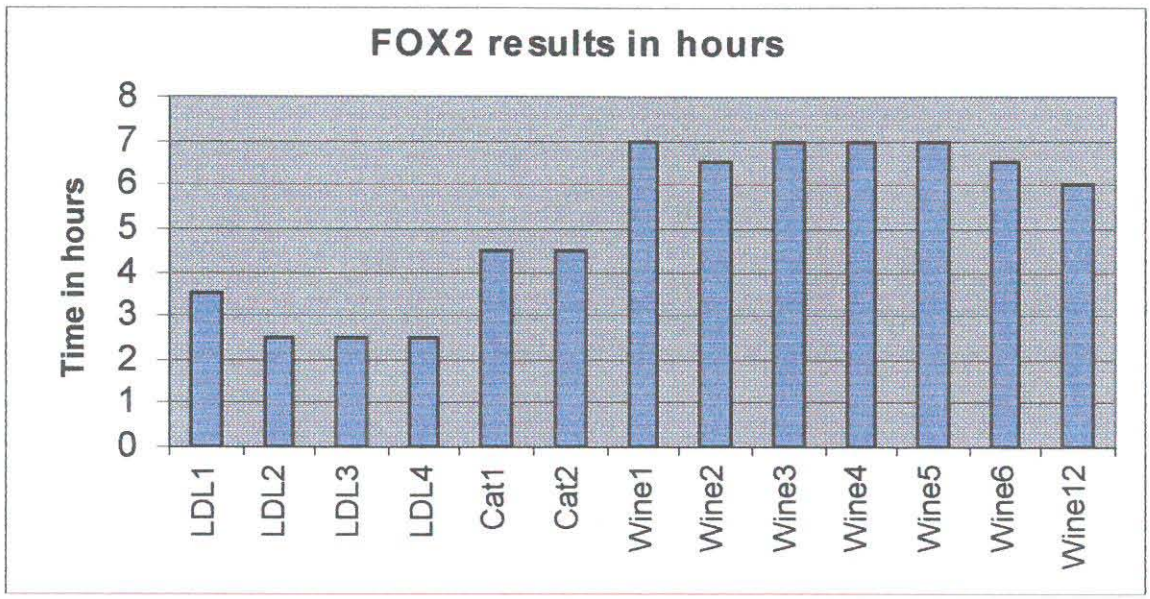


FIGURE 4.6 Lag time of the different samples as determined by the FOX2 method.

4.6 ASCORBIC ACID

The possibility was that natural ascorbic acid present in the grapes and those added during the wine making process, might influence the total biological activity.

4.6.1 ASCORBIC ACID LEVELS IN WINES AND FRUIT JUICES

Trace amounts of ascorbic acid were present in wines 2 and 6. The red grape juice presented with 30 mg/l and the white juice with 5 mg/l ascorbic acid. All the white wines, except for wine 11, contained ascorbic acid.

TABLE 4.24 Ascorbic acid and phenolics present in the 14 beverages.

Number	Ascorbic acid in mg/l	Phenolic concentration in mg/l
Wine 1	0	2430
Wine 2	<1	2335
Wine 3	0	2205
Wine 4	0	1590
Wine 5	0	1685
Wine 6	<1	2750
Juice 7	30	410
Juice 8	5	180
Wine 9	4	230
Wine 10	7	225
Wine 11	0	315
Wine 12	10	240
Wine 13	6	290
Wine 14	3	250

4.6.2 ASCORBIC ACID SUBJECTED TO COLUMN CHROMATOGRAPHY

Ascorbic acid was subjected to column chromatography under similar conditions as the wines and juices. The procedure was repeated twice.

TABLE 4.25 Lag time and biological activity of ascorbic acid, before and after column chromatography.

	Time in seconds	Time in Seconds	Time in seconds	Time in seconds	Time in seconds	Mean time in seconds	% Biological activity
LDL	6475	4751	5554	5424		5551	
Ascorbic acid (before)	5110	7605	6370	9178		7066	39%
Neutral	3436	7643	6767	8145		6498	17%
Acid	12179	7765	4570	8413	8500	8285	49%
Waste	5207	4889	5602	7669		5842	5%

4.6.3 BIOLOGICAL ACTIVITY OF ASCORBIC ACID IN PARALLEL TO COMMERCIAL PHENOLIC ACTIVITIES

Lag time was compared between catechin, epicatechin, resveratrol, quercetin and ascorbic acid in a 1 mg/l concentration. Ascorbic acid gave a biological activity of 52%.

TABLE 4.26 Lag times of commercial phenolics and ascorbic acid in 1 mg/l concentrations.

	LDL	Catechin	Epicatechin	Resveratrol	Quercetin	Ascorbic acid
Run 1	7499,5	18326	20109,8	19364	20820,4	13363,8
Run 2	8572	21054	22009,1	19371,7	23463,5	13004,5
Mean	8035,8	19690	21059,5	19367,9	22142	13184,2
Percentage biological activity		145%	162%	141%	176%	52%

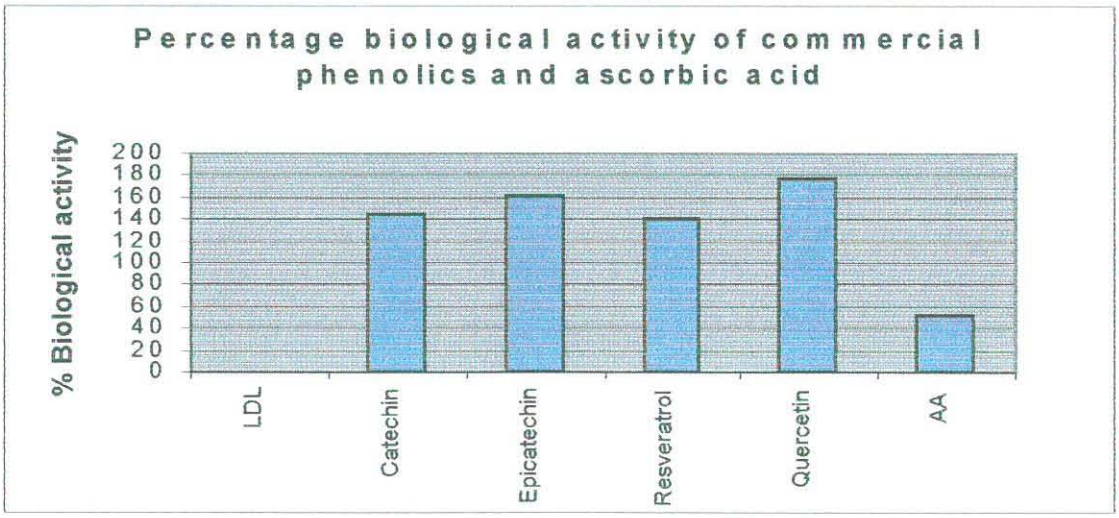


FIGURE 4.7 Different biological activities of commercial phenolics and ascorbic acid at 1 mg/l concentrations.

4.6.4 CONTRIBUTION OF ASCORBIC ACID TO THE ANTIOXIDANT ACTIVITY OF WINE

The percentage biological activity of ascorbic acid in 1 mg/l concentration was 39% and 52% respectively in 4.6.2 and 4.6.3. The average of 46% was used for the calculation of percentage biological activity of ascorbic acid in wines and juices. Wines and juices were in phenolic concentration and volumetric dilutions, as indicated in Table 4.27.

TABLE 4.27 Influence of ascorbic acid on phenolic biological activity.

No	Ascorbic acid in mg/l	Dilutions on beverages		% Phenolic biological activity		*% Ascorbic acid biological activity	
		Phenolic concentration dilutions	Volumetric dilutions	Phenolic concentration activity	Volumetric dilution activity	Phenolic concentration activity	Volumetirc dilution activity
1	0	1:486	1:1000	169%	97%	0%	0%
2	>1	1:466	1:1000	286%	162%	0%	0%
3	0	1:440	1:1000	115%	119%	0%	0%
4	0	1:318	1:1000	145%	66%	0%	0%
5	0	1:337	1:1000	144%	82%	0%	0%
6	>1	1:505	1:1000	174%	88%	0%	0%
7	30	1:82	1:1000	149%	17%	17%	1%
8	5	1:36	1:100	231%	74%	6%	2%
9	4	1:46	1:100	261%	155%	4%	2%
10	7	1:45	1:100	364%	171%	7%	3%
11	0	1:63	1:100	429%	226%	0%	0%
12	10	1:48	1:100	288%	306%	10%	5%
13	6	1:58	1:100	238%	169%	5%	3%
14	3	1:50	1:100	211%	162%	3%	1%

*Ascorbic acid in mg/l x 46%

Dilution factor of wine or juice (phenolic or volumetric) = % biological activity of ascorbic acid

4.7 STATISTICS

Where necessary appropriate data was selected from the preceding results and analysed for statistical significant differences by means of the Student t-test and accepted or rejected according to the null hypothesis. The mean phenolic concentration of undiluted red wine, containing alcohol, was compared to a 1:10 dilution. Wine with alcohol was compared with wine without alcohol to determine the influence of alcohol on phenolic determination. The phenolic concentration of the acid fractions of white and red wines was compared to one another. The biological activities of the neutral, acid and combination of neutral and acid fractions of the wines were compared with each other. The influence of the biological activity in wines with and without alcohol was also captured. The biological activity of the neutral phenolic fraction in a 5 mg/l concentration was compared with the acid phenolic fraction in the same concentration.

TABLE 4.28 Selected results obtained with the Student t-test.

Group 1	Group 2	Average 1	Average 2	t-Test	Null hypothesis
PC* Red wine ROH+ undiluted	PC* Red wine ROH+ 1:10 dilution	2166 mg/l	2162 mg/l	0,987653	Accept
PC* Red wine ROH- undiluted	PC* Red wine ROH- 1:10 dilution	2151 mg/l	2171 mg/l	0,933285	Accept
PC* Red wine ROH+ 1:10 dilution	PC* Red wine ROH- 1:10 dilution	2162 mg/l	2171 mg/l	0,971893	Accept
PC* White wine ROH+	PC* White wine ROH-	258 mg/l	241 mg/l	0,428502	Accept
PC* Acid fraction white	PC* Acid fraction red	39 mg/l	86 mg/l	0,012336	Reject
BA** Red wine ROH+ 5 mg/l phenolics	BA** White wine ROH+ 5 mg/l phenolics	172%	299%	0,012534	Reject
BA** Red wine ROH- 5 mg/l phenolics	BA** White wine ROH- 5 mg/l phenolics	162%	315%	0,000414	Reject
BA** Neutral fraction red	BA** Neutral fraction white	165%	299%	0,003266	Reject
BA** Acid fraction red	BA** Acid fraction White	155%	340%	0,000345	Reject
BA** N+A red	BA** N+A white	122%	199%	0,015845	Reject
BA** Wine ROH+ 1:100	BA** Wine ROH- 1:100	611%	633%	0,916986	Accept
BA** Wine ROH+ 5 mg/l phenolics	BA** Wine ROH- 5 mg/l phenolics	235%	238%	0,940285	Accept
BA** 14 beverages neutral fraction 5 mg/l phenolics	BA** 14 beverages acid fraction 5 mg/l phenolics	226%	232%	0,863762	Accept

*PC = phenolic concentration in mg/l
**BA = Biological activity in percentage

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

The structure and colour of wine is dependent on the phenolics present in the skins, seeds and pulp of the grape. Up to 60% of phenolics can be extracted from the fruit into the wine depending on the wine making process. Fermentation and storage in oak provides additional sources of phenolics. The temperature of fermentation enhances the extraction of specific phenolics (Zoecklein *et al.*, 1995:129-130). Abu-Amsha *et al.* (1996), concluded that the higher the phenolic concentration in a beverage, the greater the antioxidant activity.

5.1.1 PHENOLIC COMPOUNDS

Phenolics in the juice of grapes are mostly non-flavonoid and account for a small percentage of the phenolics in red wines. Flavonoids are extracted from the skin, seeds and pulp of the grape and account for 80 to 90% of the phenolics in red wine, in contrast with 25% of the phenolics in white wine. According to Zoecklein *et al.* (1995: 117), aged white wine should present with total phenolic concentrations between 190 – 290 mg GAE/l and aged red wines with concentrations between 955 – 1215 mg GAE/l. Great variations in phenolic concentrations exist amongst different red wine cultivars. No reference ranges were obtained for phenolic concentrations in grape juices.

5.1.1.1 Phenolic concentrations in wine

With reference to Tables 4.4 the highest phenolic concentrations were found in the Stellenryck and Fleur du Cap Cabernet Sauvignon wines with 2750 mg/l and 2550 mg/l respectively. Total phenolic concentrations reported by Frankel *et al.* (1995) in

Cabernet Sauvignon ranged between 2164 and 3340 mg GAE/l and between 1800 and 2133 mg GAE/l in the Merlot wines. The Fleur du Cap Merlot presented with a phenolic concentration of 2325 mg/l and the Drosdy-Hof with a concentration of 2050 mg/l. The lowest levels in the red wines were found in the blended wines. The average phenolic concentration in the red wines analysed was 2162 mg/l. The high phenolic concentrations in the Cabernet Sauvignon and Merlot wines could be attributed to the cultivars, but also to the winemaking process. These four wines had skin contact from 8 to 9 days, and matured in 300 liter barrels for 12 to 24 months.

Phenolic concentrations in the white wines, as tabulated in Table 4.6, ranged from 315 mg/l and 290 mg/l in the Chardonnay wines to 225 mg/l in the Riesling wine. The Sauvignon Blanc and blended wines gave levels of 250, 240 and 230 mg/l respectively. Frankel *et al.* (1995) reported concentrations of 240 and 259 mg GAE/l for Chardonnay and 165 and 193 mg GAE/l for Sauvignon Blanc. The average phenolic concentration in the white wines analysed was 258 mg/l. The higher phenolic concentrations obtained in the Chardonnay wines could be attributed to the cultivar and also to the fact that these two wines spent 4 months each in wood.

5.1.1.2 Phenolic concentrations in juice

The Red Grapetiser gave a phenolic concentration of 410 mg/l (Table 4.5). The total phenolic concentration determined in the White Grapetiser was 100 mg/l and 80 mg/l on duplicate determinations. The sum of the neutral, acid and waste phenolic fractions was used as total phenolic concentration for the White Grapetiser. The Red and White Grapetiser gave low phenolic concentrations, as was expected, due of the juice extraction process. Both the juices had no wood and no skin contact.

5.1.1.3 Fractionation of phenolics

Teissedre *et al.* (1995) found high levels of catechin, gallic acid, epicatechin, rutin, myricetin, quercetin and caffeic acid in red wines. In the white wines they reported high levels of catechin, epicatechin, gallic acid and caffeic acid. Most of these phenolics are classified as neutral phenolics and therefore the majority of phenolics present in wines and juices belong to the neutral class.

5.1.1.3.1 Neutral and acidic phenolic fractions in wines

Both the red and white wine, as summarised in Table 4.4, presented with higher levels of neutral phenolics in comparison with the acidic phenolics. The mean acidic phenolic concentration in the white wines was 17% in comparison with the 4% in the red wines. Therefore, a greater percentage of the phenolics present in white wines could be classified as acidic phenolics. The recovery of phenolics in the neutral, acid and waste fractions of the white wines was 99,5% in comparison with 86% of the red wines. A higher percentage of phenolics in red wines were not recovered and this could be attributed to the better binding of certain specific phenolics in the red wines making them more difficult to elute from the column. Another possibility was the binding capacity of the cartridge for specific phenolics present in the red wines.

5.1.1.3.2 Neutral and acidic phenolic fractions in juices

The difference in phenolic concentrations between the neutral and acidic phenolic fractions in the juices, as depicted in Table 4.5, was not as pronounced as those found in the wines. In the red juice the values between neutral and acidic phenolics constituted 45,5% and 37% whereas in the white juice it was 40% and 31% respectively. From these results it was clear that acidic phenolics were present in higher concentrations in the juices in comparison with the wines.

5.1.1.4 Factors influencing the phenolic concentration

Factors and conditions that could influence the phenolic concentration in the wines and juices were investigated. The possibility that non-phenolic substances in wines and juices were measured as phenolics had to be ruled out.

5.1.1.4.1 Linearity of the phenolic method

Originally there was doubt about the linearity of the Folin-Ciocalteu method. The same red wine diluted to different concentrations gave different total phenolic levels after the dilutions were accounted for in the calculation. The more diluted the wine the higher the phenolic concentration became. This phenomenon was not observed in white wines. The results in Table 4.7 confirmed the linearity and accuracy of serial dilutions on the same wine for phenolic determination. The lower phenolic concentration obtained in the more concentrated red wines could be attributed to the red colour of the wine. The principle of the Folin-Ciocalteu method is based on a change in colour and could have been influenced by the dark red colour. The more diluted the red wine, the smaller the influence of the red colour and the more accurate the phenolic determination became.

No significant difference ($p > 0,05$)* was found, as reflected in Table 4.4, between the mean phenolic concentration in red wine in the undiluted and the 1:10 dilution samples, with and without alcohol. In wines 1-5 both the diluted and undiluted samples gave phenolic concentrations but in wine 6, the reading of the concentrated samples was so high that results were only obtained from the diluted samples. Therefore in wine six, 2750 mg/l was used as the phenolic concentration of the undiluted samples, with and without alcohol. This example confirmed our theory about the influence of the wine colour.

*Student t-test (p values in Table 4.28).

5.1.1.4.2 Alcohol

No statistical significant difference ($p > 0,05$) was observed between mean phenolic concentrations in the red wine (as tabulated in Table 4.4) with alcohol in a 1:10 dilution in comparison to the red wine without alcohol in a 1:10 dilution. Similar results were observed ($p > 0,05$) in the white wine, as summarised in Table 4.6. In conclusion, the determination of phenolic content was not influenced by alcohol.

5.1.1.4.3 Temperature

The phenolics were exposed to 50°C for between 7-25 minutes during the evaporation of alcohol from the wines, as indicated in Table 4.2. According to Table 4.20, in which the mean phenolic concentrations of wines exposed to heat and those not exposed to heat were compared, it could be concluded that exposure to heat for a short period had no influence on the determination of the phenolic concentration in wines.

As summarised in Table 4.2, both the juices were exposed to 50°C for 30 minutes and in both cases a reduction in phenolic concentration was noticed, as depicted in Table 4.5. These results indicate to the influence of temperature on the phenolic concentrations, but this finding needs to be confirmed with a larger number of specimens before a conclusion could be made.

5.1.1.4.4 Proteins

According to Table 4.5, the phenolic concentration in the red juice decreased from 410 mg/l to 383 mg/l after removal of the proteins. The removal of proteins from the white juice had a similar effect on the total phenolic concentration. Even though the number of samples analysed was very small the tendency to remove phenolics with the proteins existed. One possibility is that phenolics conjugate onto proteins and precipitate with

them. Another possibility is that proteins contain phenolic-like amino acids such as tyrosine and phenylanaline that will precipitate with them.

5.1.1.4.5 Non-phenolic compounds

Flavonoids and a variety of other phenolic compounds form hydrogen bonding with PVPP and 90% of the phenolics could be removed from wines (Vrhovsek, *et al.*, 1997) by this mechanism. The phenolic concentration of the pre-PVPP red wine decreased to 1% in the post-PVPP sample, as tabulated in Table 4.8. The white wine decreased to 0%, the white juice to 7% and the catechin to 8%. In all the samples there was a decline of more than 90% in the phenolic concentration as was described by Abu-Amsha *et al.* (1996). This finding excluded the possibility of measuring non-phenolic compounds as phenolics.

5.1.1.4.6 Influence of pH

Phenolics in wine are stable at a pH of 3,5 or less (Boulton *et al.*, 1996; 410). This is the reason why pH has such an impact on the oxidation of wines. The neutral fraction obtained after the fractionation of wines had a mean pH of 7,9 (Table 4.3). The separate fractions were analysed for phenolic concentrations within 2 – 3 hours after fractionation. The high pH could therefore not have influenced the phenolic content in the wines.

5.1.2 BIOLOGICAL ACTIVITY

Biological activity of the phenolics in wines and juices is the ability to prolong the lag phase which preceeds LDL oxidation. The red wines presented with a 9 times higher phenolic concentration in comparison to the white wines. A much higher antioxidant biological activity was therefore expected in the red wines. With reference to Table

4.13, the volumetric biological activity of red wine was mathematically converted from a 1:1000 dilution to a 1:100 dilution for comparison with white wines.

5.1.2.1 Biological activity in wines

The wine with the highest biological activity was the Fleur du Cap Merlot followed by the Drosdy-Hof Merlot, Fleur du Cap Cabernet Sauvignon, Stellenryck Cabernet Sauvignon, Drosdy-Hof Claret, while the lowest activity in the red wines was Cellar Cask Johannisberger Red. Biological activity in the red wines ranged from 1620% to 660%.

The biological activity of the L'Ormarins Sauvignon Blanc was 306%. The biological activity decreased in the order of Fleur du Cap Chardonnay, Fleur du Cap Riesling, Drosdy-Hof Chardonnay, Le Bonheur Sauvignon Blanc to the Theuniskraal Sémillon / Chardonnay with 155%.

The total phenolic concentration and the biological activity of the wines were not directly proportional to one another, however the phenolic content usually was indicative of the biological activity of a particular wine. The Merlot wines presented with the highest biological activity, although their phenolic concentrations were lower than those in the Cabernet Sauvignon wines, as indicated in Table 4.4. The Chardonnay wines gave the highest phenolic concentration in the white wines, as summarised in Table 4.6, while they were placed 2nd and 4th in biological activity.

Biological activity of the wine is depended on the cultivar as concluded by Teissedre *et al.* (1995). The Merlot followed by Cabernet Sauvignon cultivars were more pronounced in their inhibition of LDL oxidation. The authors concluded that the Sauvignon Blanc followed by the Chardonnay were the best LDL oxidation inhibitors of the white wines. The results obtained from this study were in agreement with those

reported on the red wines, but do not fully support those results reported on the white wines. If larger numbers of wines were analysed more conclusive evidence could have been obtained.

According to Table 4.16, a significant difference ($p < 0,05$) was found in comparing the mean biological activity of red wine diluted to a specifc phenolic concentration with white wine in the same phenolic concentration dilution, containing alcohol. An even bigger difference ($p < 0,0005$) was found between the red and white wine without alcohol. The percentage biological activity of white wine with and without alcohol was divided by the percentage biological activity of red wine with and without alcohol, with factors 1,74 and 2,19 respectively. This meant that for every one part of white wine used to demonstrate biological activity, 1,74 or 2,19 parts of red wine should be used. Vinson & Hontz (1995) compared red and white wine in a 3 µM phenolic concentration and concluded that white wines contained stronger reacting antioxidants. It was evident that the white wines analysed contained a phenolic or phenolic combination with better antioxidant activities in comparison to the red wines. From these results it can be concluded that should red and white wine have had the same phenolic levels, white wine would have had antioxidant activity twice as potent as the activity in red wines.

The mean biological activity of red wine in a volumetric dilution, with reference to Table 4.17, was compared with that of white wine, with and without alcohol. In dividing the biological activity of the red wine with that of the white wine, factors of 5,2 and 8,4 was obtained. In dividing the mean phenolic concentration of these two groups with each other, factors of 8,4 and 9,0 were obtained. The phenolic factor indicated that for every 1 part of red wine 8,4 parts of white wine would give the same phenolic concentration. The volumetric factor suggested that one part of red wine equaled 5,2 parts of white wine in giving the same biological activity. The expected 8,4 parts as deduced from the phenolic concentration was therefore diminished to 5,2 parts in terms of biological

activity. A similar pattern was followed for the wines without alcohol where an expected factor of 9,0 being diminished to 4,9.

In conclusion, it is evident that even though white wine contained lower levels of phenolics, the phenolics present in white wines possessed stronger antioxidant activity in comparison to those in red wine. The expected biological activity of a wine as derived from the phenolic concentration is not directly proportional to the observed biological activity.

5.1.2.2 Biological activity in juices

The Red Grapetiser prolonged the lag phase before LDL oxidation by 170% and the White Grapetiser by 74%, according to Table 4.13. The biological activity was proportional to the phenolic concentration. Though when comparing the Red Grapetiser, with a phenolic concentration of 410 mg/l with the white wines, a much higher biological activity was expected.

The biological activity of the white juice in a phenolic concentration dilution, as indicated in Table 4.18, was higher in comparison with the red juice. The factor of 1,6 for unprocessed and 1,1 for deproteinated juice were much lower in comparison to the factors obtained with the wines. More conclusive evidence could have been made if more samples were analysed.

5.1.2.3 Biological activity of neutral and acidic phenolic fractions

The highest percentage of phenolics was present in the neutral phenolic fraction. This explained the reason why the biological activity of the neutral phenolic fraction in a volumetric dilution gave the highest biological activity. Though when the neutral and acidic phenolic fractions were compared with one another in a phenolic concentration dilution the acidic phenolics gave the highest biological activity.

5.1.2.3.1 In wines

As tabulated in Table 4.16, the mean biological activities of the neutral and acidic phenolic fractions of the white wines in comparison to those of the red wines in a phenolic concentration dilution, significant differences ($p < 0,005$ and $p < 0,0005$) were found. The neutral and acidic fractions in the white wines gave much higher biological activities. A significant difference ($p < 0,05$) was found in the combination of neutral and acidic phenolic fractions, between the red and white wines with a factor of 1,63 in favour of the white wine. According to the Wynboer (1997) no quercetin was present in the 7 South African white wines analysed. In the same study catechin and epicatechin were present in much higher concentrations in the red wines in comparison to those in the white wines. Resveratrol is mainly present in red wines with very low concentrations in white wines (Jeandet *et al.*, 1993).

The volumetric dilution comparison, as depicted in Table 4.17, between the mean biological activity of the neutral and acidic phenolic fractions, indicated a much higher biological activity of neutral phenolic fractions in both the red and white wines. When comparing the neutral fraction of the white wines to those of the red wines a factor of 5,8 was found, but when comparing the mean phenolic concentrations of these two fractions the factor was 9,6. This meant that when concentrating on only the phenolic concentration, 9,6 parts of white wine equaled 1 part red wine, but with the biological activity 1 part of red wine equaled 5,8 parts of white wine. The higher phenolic concentration in the neutral fractions resulted in the higher percentage biological activity.

5.1.2.3.2 In juices

As summarised in Table 4.18, the neutral phenolic fraction of the white juice gave a higher biological activity in comparison to those in the red juice. The opposite was observed in the acidic phenolic fraction, where the red juice had a biological activity of 150% versus the 135% of the white juice. The neutral and acidic phenolic combination was also in favour of the white juice. The Red Grapetiser contained 30 mg/l ascorbic acid that contributed to the higher biological activity in the acidic fraction of the red juice, with reference to 5.1.2.4.5.5.

5.1.2.3.3 In wines and juices

The neutral phenolic fraction of all 14 beverages (as indicated in Table 4.21) in a 1:100 dilution gave a mean biological activity of 474% versus 40% in the mean acidic phenolic fraction. A higher mean phenolic concentration was found in the neutral fraction. When comparing the neutral and acidic phenolic fractions in a phenolic concentration dilution, no significant difference ($p > 0,05$) was found.

It can be concluded that when comparing the neutral and acidic phenolic fractions to one another in the same phenolic concentration dilution, the mean percentage biological activity differed by 6%. This confirmed the proposition, that even though acidic phenolics were low in number, they possessed strong biological activity.

5.1.2.4 Factors influencing the biological activities

Temperature and the protection of LDL and beverages against UV light were crucial in the determination of the lag periods.

5.1.2.4.1 Linearity of method

Five serial dilutions of a red and a white wine showed a gradual decrease in lag periods as shown in the histograms, Figure 4.3 and Figure 4.4. The percentage biological activity in the white wine decreased gradually, as depicted in Table 4.14. These results demonstrated the linearity of the method. It was therefore permissible to mathematically convert a 1:1000 dilution of red wine to a 1:100 dilution.

5.1.2.4.2 Alcohol

The mean percentage biological activity, with reference to Table 4.20, of wine with and without alcohol in a volumetric dilution and a phenolic dilution were compared and no significant difference ($p > 0,05$) was found. Abu-Amsha *et al.* (1996) confirmed that ethanol at concentrations of 0,1 – 0,05% had no effect on copper induced oxidation. The results obtained from this study concluded that *in vitro* measurements were not influenced by alcohol.

5.1.2.4.3 Temperature

The results depicted in Table 4.20 were also applicable to the influence of temperature. The wines without alcohol were exposed to 50°C to evaporate the alcohol. It could thus be concluded that phenolics exposed to 50°C for a short period had no effect on the biological activity of the phenolics in wines.

The biological activity of the deproteinated red juice decreased during the volumetric dilution analysis, as seen in Table 4.22, but increased during the phenolic concentration dilution analysis in comparison with the unprocessed juice. During the analysis of the deproteinated white juice there was a decline in both the volumetric and the phenolic concentration analyses in comparison with the unprocessed juice. This decrease in

biological activity could rather be attributed to the removal of proteins, as discussed in 5.1.2.4.4.

5.1.2.4.4 Proteins

In both the red and white juice, as indicated in Table 4.22, there was a decrease in the biological activity of the deproteinated juice as compared to the unprocessed juice during the volumetric dilution analysis. During the phenolic dilution analysis red juice increased from 149% to 171% and the white juice decreased from 231% to 195%. Three out of the four analyses resulted in a decrease in biological activity after the removal of proteins. A larger number of samples should be analysed before the influence of proteins on the biological activity of juices can be concluded.

5.1.2.4.5 Non-phenolic compounds

The post-PVPP lag period, as tabulated in Table 4.15, of the stripped samples almost equaled the lag period on the LDL control sample, which indicate to no biological activity of the stripped samples analysed. It was evident that all the biological active phenolics were stripped from the beverages.

5.1.2.4.5.1 Influence of pH

The mean pH on the neutral phenolic fractions was 7,9 as indicated in Table 4.3. Phenolics in wine oxidise more easily at a neutral pH, resulting in a decline in their antioxidant activity. Each run was repeated at least once, but sometimes two to three times. During this period the neutral phenolics were stored in a pH of 7,9 for five days and sometimes even for 12 days. The separate lag periods are included in the Appendix, in Table form and also as Graphs. However during the analyses there was no decline in biological activity on the stored samples analysed. From these results it is clear that the pH did not influence the antioxidant activity to any extend. For future

analysis it would, however, be advisable to adjust the pH of the neutral phenolic fraction to 3,5.

5.1.2.4.5.2 *Ascorbic acid*

The antioxidant activity of ascorbic acid is well documented. *In vivo* studies done by Whitehead *et al.* (1995) concluded that after the administration of 300 ml of wine and 1000 mg ascorbic acid to subjects, the highest antioxidant activity levels were measured in the subjects who consumed ascorbic acid. Lower levels of antioxidant activity were detected in those who consumed the red wines, with even lower levels in those who consumed the white wines. Measurements were done at 1 and 2 hours after consumption.

5.1.2.4.5.3 *Subjection of ascorbic acid to C18 cartridges*

Aliquots of a 1 mg/l ascorbic acid was subjected to similar fractionation procedures as the beverages. The acid fraction gave a biological activity of 49% in comparison to the 17% in the neutral fraction and 5% in the waste fraction (Table 4.25). The neat 1 mg/l ascorbic acid concentration presented with a biological activity of 39%. From these results it is evident that whenever ascorbic acid is present in a wine, more potent biological activity will be present in the acid fraction.

5.1.2.4.5.4 *Biological activities of ascorbic acid and some commercial phenolics*

Comparing the biological activity of ascorbic acid with commercial phenolics, as indicated in Table 4.26, the highest biological activity was obtained with the quercetin and the lowest with ascorbic acid. Vinson and Hontz (1995) compared wines versus 3 µM concentrations of ascorbic acid and found the wines to exceed the ascorbic acid in biological activity by more than 8-9 times. The results obtained during this study confirmed a 2-3 times stronger biological activity of the commercial phenolics in

comparison to the ascorbic acid. The *in vitro* antioxidant activity of ascorbic acid was not as effective as commercial phenolics on a gram to gram basis.

5.1.2.4.5.5 *Influence of ascorbic acid on biological activity*

Five of the white wines, as tabulated in Table 4.24, plus the 2 juices contained ascorbic acid. As summarised in Table 4.27, the synergistic effect of ascorbic acid on the biological activity of these beverages was mathematically calculated for the phenolic concentration dilution as well as the volumetric dilution. Seventeen % of the 149% biological activity in the 5 mg/l phenolic concentration in the red juice, could be attributed to ascorbic acid. One % of the 17% in the volumetric dilution could be attributed to ascorbic acid. Whether the phenolics and the ascorbic acid in the beverages functioned separately or whether the biological activity of the phenolics in the wines was enhanced by the ascorbic acid, needs to be investigated in a follow-up study.

The acid fraction of the red juice in the 5 mg/l phenolic concentration dilution gave a higher biological activity in comparison to the acid fraction of the white juice. The concentration of ascorbic acid in the red juice, as indicated in Table 4.28, was 30 mg/l versus the 5 mg/l in the white juice. As concluded in 5.1.2.4.5.3 a greater percentage of biological activity of commercial ascorbic acid resides in the acid fraction (Table 4.25). The high level of ascorbic acid in the Red Grapetiser can explain the higher biological activity observed in the acidic fraction of the red juice.

5.1.2.5 FOX2 results

The results obtained with the FOX2 method, as shown in Table 4.23, confirmed the antioxidant activity of phenolics in 7 of the wines. Maximum µM hydroperoxide values ranged between 2,2 to 3,4 µM. Lag periods of the wines varied between 6, 6,5 and 7 hours. Biological activity ranged between 100% - 180%. The catechin solution included as a positive control in both instances gave a lag period of 4,5 hours. The lag

periods were lower in comparison to those found in the conjugated diene method. Chait *et al.* (1996) concluded that with the correlation of different oxidation parameters, different results were obtained. Even though the results from the conjugated diene method and the FOX2 method differed in lag periods both could be used for the analysis of antioxidant activity of phenolics in wines and juices.

5.2 CONCLUSIONS

The Stellenryck Cabernet Sauvignon presented with the highest phenolic concentration. The Fleur du Cap Merlot was by far the wine with the most potent antioxidant activity.

5.2.1 PHENOLIC LEVELS IN WINES AND JUICES

The Cabernet Sauvignon wines had the highest phenolic concentrations, followed by the Merlot wines. The blended red wines had much lower phenolic concentrations. The Red Grapetiser showed a higher phenolic concentration in comparison to the white wines, followed by Chardonnay, Sauvignon Blanc and lastly was Sémillon / Chardonnay and the Riesling. The lowest phenolic concentration was obtained in the White Grapetiser. The phenolic levels in the beverages were dependent on the cultivar and were influenced by the winemaking process. The phenolic concentrations corresponded well to those reported in the literature (Frankel *et al.* 1995; Zoecklein *et al.* 1995: 117).

5.2.1.1 Neutral and acidic phenolics in wines and juices

The greater percentage of the phenolics in the beverages was present in the neutral fraction after fractionation. It can thus be stated that the majority of phenolics in wines belong to the neutral group.

5.2.1.2 Factors influencing the phenolic concentration determination

The linearity and accuracy of the phenolic determination was confirmed through spiking. Red wines with very high levels of phenolics, measured as concentrated wine samples, gave lower concentrations of phenolics in comparison to the diluted wines. This can be ascribed to the intensity of the colour in the wine that can interfere with the colour change, on which principle the Folin-Ciocalteu method is based.

Neither alcohol nor temperature had an influence on the phenolic determination in wines. The reduction in the phenolic concentration after the removal of proteins from the juices and the exposure to 50°C could be attributed to the loss of phenolics that may co-precipitated with the proteins.

During the stripping of phenolics from the wines and juices more than 90% of the phenolics were removed from the beverages. The high pH of the neutral phenolic fraction did not influence the phenolic determination.

5.2.2 BIOLOGICAL ACTIVITY IN WINES AND JUICES

The two Merlot wines had the highest biological activities. The two Cabernet Sauvignon wines were next in line and the two blended red wines gave the poorest biological activities of the red wines. The influence of cultivar was not as prominent in the white wines. The biological activities decreased from a Sauvignon Blanc with 306%, Chardonnay, Riesling, Chardonnay, Sauvignon Blanc and last was the Sémillon / Chardonnay with 155%. The red juice and the Riesling had biological activities of 170% and 171% respectively. The white juice gave the poorest percentage biological activity.

Even though white wines possessed lower concentrations of phenolics, the phenolics in the white wines presented with more potent antioxidant activities when comparing red and white wine in phenolic concentration dilution to one another. The phenolic

concentration in a wine or juice was not directly proportional to the biological activity of that wine or juice.

5.2.2.1 Neutral and acidic phenolic fractions in wines and juices

Neutral phenolic fractions possessed higher phenolic concentrations and therefore presented with higher antioxidant activity in comparison to that of the acid fraction. Neutral and acidic phenolic fractions in the white wines in the same phenolic concentration dilution to that of red wine reacted as stronger antioxidants when compared to the red wines.

5.2.2.2 Factors influencing the biological activity

Linearity and accuracy of the conjugated diene method were confirmed by serial dilutions of the same wine. Alcohol and temperature did not influence the biological activity of the wines. The removal of proteins from the juices and exposure to higher temperatures could have influenced the biological activity of the juices.

Stripping the wines and juices of phenolics resulted in no antioxidant activity. Neutral phenolic fractions with a high pH showed no deterioration of the phenolic biological activity on repeating the same sample.

The higher antioxidant activity observed in the acid fractions of the white wines and juices in comparison to those in red wines could be attributed to the presence of ascorbic acid in five of the white wines and both the juices. Mathematical calculations confirmed a minor influence of ascorbic acid on the biological activity of those wines and juices.

Commercial ascorbic acid was measured in parallel to four commercial phenolics at the same concentration. The results confirmed that phenolics possessed a stronger antioxidant activity in comparison with ascorbic acid on a gram to gram basis.

5.2.2.3 General factors influencing the biological activity of white wines

According to Teissedre *et al.* (1995) the Chardonnay and the Sauvignon Blanc wines were the white wines with more prominent antioxidant activity. The results obtained from this study were contradictory to their observation. The Fleur du Cap wines were among the first 3 wines in line for the highest biological activities.

L'Ormarins Sauvignon Blanc was the white wine with the highest biological activity (306%). It contained a phenolic concentration of 240 mg/l and an ascorbic acid concentration of 10 mg/l. During the wine making process the wine had no wood contact. The Fleur du Cap Chardonnay gave a biological activity of 226%. This wine had the highest phenolic concentration of 315 mg/l, with no ascorbic acid. The wine had four months wood contact. Three factors were in favour for biological activity, the cultivar, high phenolic concentration and wood contact, however the biological activity did not perform according to expectation.

There was no noticeable difference between the 171% biological activity of the Fleur du Cap Riesling and the 169% biological activity of the Drosdy-Hof Chardonnay. The Riesling had a phenolic concentration of 225 mg/l and ascorbic acid concentration of 7 mg/l. This wine had no wood contact. The Chardonnay had all the factors in favour for biological activity, namely a phenolic concentration of 290 mg/l, ascorbic acid of 6 mg/l, 4 months wood contact, plus the cultivar. These factors did not contribute positively to the biological activity.

Le Bonheur Sauvignon Blanc gave a biological activity of 162%, with a phenolic concentration of 250 mg/l and ascorbic acid content of 3 mg/l. The wine had no wood contact. The Theuniskraal Sémillon / Chardonnay had a biological activity of 155%, phenolic concentration of 230 mg/l, ascorbic acid of 4 mg/l with no wood contact. Although both wines contained relatively high phenolic concentrations their biological activities were fairly low.

The white wines analysed during this study showed moderately high phenolic concentrations. The biological activity of these wines varied between 155% and 306%.

5.2.2.4 General factors influencing the biological activity of red wines

According to Teissedre *et al.* (1995) Merlot wines have the highest biological activity followed by the Carbernet Sauvignon wines. The results obtained from this study support their observation. The Fleur du Cap wines presented with very high biological activity, as was the case with the white wines.

The Fleur du Cap Merlot gave a biological activity of 1620%, a phenolic concentration of 2325 mg/l, it had skin contact and fermentation for 9 days, and was on wood for 12 months in 300 liter barrels. The Drosdy-Hof Merlot had a biological activity of 1190%, a phenolic concentration of 2050 mg/l, spent 8 days on the skin, with 8 days fermentation, and had wood contact for 24 months in 300 liter barrels. Both the Merlot wines had skin and wood contact, with 3rd and 4th highest phenolic concentrations, all in favour of powerful antioxidant activity.

The Fleur du Cap Cabernet Sauvignon gave a biological activity of 970%, a phenolic concentration of 2550 mg/l, 8 days skin contact and fermentation with wood contact for 18 months in 300 liter barrels. The Stellenryck Cabernet Sauvignon gave 880% biological activity, a phenolic concentration of 2750 mg/l, 8 days on the skin and 8 days

fermentation and was on wood for 18 months in 300 liter barrels. The Drosdy-Hof Claret with a biological activity of 820%, a phenolic concentration of 1645 mg/l, spent 4 days on the skin with 8 days fermentation and was on wood for 12 months in 500 liter barrels. The Cellar Cask Johannisberger Red had a biological activity of 660%, a phenolic concentration of 1650 mg/l with skin contact and fermentation for 8 days, but had no wood contact. The phenolic concentrations were lower in the last two wines in comparison to the other red wines. The last wine had no wood contact, which could explain its lower antioxidant activity.

One outstanding phenomenon was the small difference (60%) of the biological activity, of the Stellenryck Carbernet Sauvignon (phenolic concentration of 2750 mg/l) and the Drosdy-Hof Claret (phenolic concentration of 1645 mg/l). In contrast the biological activity of the red wines varied between 1620% and 660% with a difference of 960%.

5.2.3 PROSPECTIVE STUDIES

This study confirmed antioxidant activity in South African wines and grape juices. Other questions that need to be addressed are: whether the French Paradox is dependant on the consumption of red wine only, or whether wine plus the Mediterranean diet contribute to the lower incidence of CHD? What additional influences do the genetic factor of the French people and their life style have in preventing CHD?

5.2.3.1 *In vitro* studies

Future investigations must entail the separation and the fractionation of phenolics from wines and their identification. The synergism and/or antagonism of these phenolics with one another and also with vitamins e.g. ascorbic acid, contributing to influencing their biological activities, must be investigated.

5.2.3.2 *In vivo* studies

Wine, red and white, as well as some enriched with vitamins, should be given to voluntary subjects for consumption. Pre- and post-blood samples should be taken from these subjects and analysed for antioxidant activity. It will be interesting to follow the absorbtion, excretion and half-life of the antioxidants in the human body.

5.2.4 POSITIVE CONTRIBUTION

Wine makers should be encouraged to produce wines, especially white wines with higher concentrations of antioxidants that would enhance their biological activities. The South African population should be encouraged to consume these wines in moderate amounts to prevent or diminish heart diseases.

ACKNOWLEDGEMENT

The author would hereby like to express her sincere thanks to the following institutions for making it possible to complete this research project:

The Department Paramedical Sciences of the Technikon Free State, for supplying the necessary laboratory equipment and reagents as well as the computer facilities.

The Department Haematology of the University of the Orange Free State, for supplying the necessary equipment and reagents for the analyses not done at the Technikon.

Distillers Corporation, Stellenbosch for supplying the wines analysed.

The following people are thanked for their assistance, counseling, patients and kindness:

Mr Francois Cloete, the supervisor, without whose assistance this project would not have materialised.

Mrs Mareli Kelderman, for laboratory assistance.

Mr Hennie Smit and Mrs Naomi van der Merwe for computer assistance.

Prof JG Barnard as co-supervisor.

Mr Erhard Wolf and personnel from Distillers Corporation and Bergkelder, Stellenbosch for support and answering questions asked.

Ms Lindi Stewart, Appletiser, Grabouw, for answering questions asked on the juices analysed.

REFERENCES

Abu-Amsha, R., Croft, K.D., Puddey, I.B., Proudfoot, J.M. & Beilin, J.B., 1996. Phenolic content of various beverages determines the extent of inhibition of human serum and low-density lipoprotein oxidation *in vitro*: identification and mechanism of action of some cinnamic acid derivatives from red wine. *Clinical Sciences,* **91**, 449-458.

Bolton, E.J., Jessup, W., Stanley, K.K. & Dean, R.T., 1994. Enhanced LDL oxidation by murine macrophage foam cells and their failure to secrete nitric oxide. *Atherosclerosis,* **106**, 213-223.

Bombardelli, E. & Morazzoni, P., 1995. *Vitis vinifera* L. *Fitoterapia,* **LXVI** (4), 291-317.

Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E., 1996. Principles and Practices of Winemaking. New York: Chapman & Hall.

Buettner, G.R., 1993. The pecking order of free radicals and antioxidants: Lipid peroxidaton, α-Tocopherol, and Ascorbate. *Archives of Biochemistry and Biophysics,* **300** (2), 535-543.

Buist, R.A., 1996. Cholesterol de-emphasised. *Cardiovascular Journal of Southern Africa (SAMJ Supplement)*, **5**, 246-248.

Chait, A., 1992. Methods for assessing lipid and lipoprotein oxidation. *Clinical Chemistry,* **3,** 389-394.

Chajès, V., Wolfgang, S., Stultschnig, M. & Kostner, G.M., 1996. Photometric evaluation of lipid peroxidation products in human plasma and copper oxidized low-

density lipoproteins: correlation of different oxidation parameters. *Atherosclerosis,* **121,** 193-203.

Chang, W.C. & Hsu, F.L., 1992. Inhibition of Platelet Activation and Endothelial Cell Injury by Polyphenolic Compounds Isolated from *Lonicera japonica* Thunb. *Prostaglandins Leukotrienes and Essential Fatty Acids,* **42,** 307-312.

Demrow, H.S., Slane, P.R. & Folts, J.D., 1995. Administration of Wine and Grape Juice Inhibits *In Vivo* Platelet Activity and Thrombosis in Stenosed Canine Coronary Arteries. *Circulation,* **91**, 1182-1188.

De Rijke, Y.B., Demacker, P.N.M., Assen, N.A., Sloots, L.M., Katan, M.B. & Stalenhoef, A.F.H., 1996. Red wine consumption does not affect oxidizability of low-density lipoproteins in volunteers. *Am. J. Clin. Nutr.,* **63,** 329-334.

De Whalley, C.V., Rankin, S.M., Hoult, R.S., Jessup, W. & Leake, D.S., 1990. Flavonoids inhibit the oxidative modification of low-density lipoproteins by macrophages. *Biochemical Pharmacology,* **39** (11), 1743-1750.

Esterbauer, H., Striegl, G., Puhl, H. & Rotheneder, M., 1989. Continuous monitoring of *in vitro* oxidation of human low-density lipoproteins. *Free Radical Res. Commun.,* **6**, 67-75.

Frankel, E.N., Kanner, J., German, J.B., Parks, E. & Kinsella, J.E., 1993. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *The Lancet*, **341**, 454-457.

Frankel, E.N., Waterhouse, A.L. & Teissedre, P.L., 1995. Principal Phenolic Phytochemicals in Selected California Wines and Their Antioxidant Activity in Inhibiting Oxidation of Human Low-Density Lipoproteins. *J. Agric. Food Chem.,* **43**, 890-894.

Frei, B. & Gaziano, J.M., 1993. Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependant and –independent oxidation. *Journal of Lipid Research,* **34,** 2135-2143.

Goldberg, D.M., 1995a. Does Wine Work? *Clin Chem.,* **41** (1), 14-16.

Goldberg, D.M., Karumanchiri, A., Ng, E., Yan, J., Diamandis, E.P. & Soleas, G.J., 1995b. Direct Gas Chromatographic-Mass Spectrometric Method To Assay *cis*-Resveratrol in Wines: Preliminary Survey of Its Concentration in Commercial Wines. *J. Agric. Food Chem.,* **43**, 1245-1250.

Goldberg, D.M., Yan, J., Ng, E., Diamandis, E.P., Karumanchiri, A., Soleas, G. & Waterhouse, A.L., 1995c. A Global Survey of *Trans*-Resveratrol concentrations in Commercial Wines. *Am. J. Enol. Vitic.,* **46** (2), 159-165.

Goldberg, D.M., Hahn, S.E. & Parkes, J.G., 1995d. Beyond alcohol: Beverage consumption and cardiovascular mortality. *Clinica Chimica Acta,* **237**, 155-187.

Goldberg, D.M., Tsang, E., Karumanchiri, A., Diamandis, E.P., Soleas, G. & Ng, E., 1996. Method to Assay the Concentration of Phenolic Constituents of Biological Interest in Wines. *Analytical Chemistry,* **68**, 1688-1694.

Gryglewski, R.J., Korbut, R., Robak J. & Swies, J., 1987. On the mechanism of antithrombotic action of flavonoids. *Biochemical Pharmacology,* **36** (3), 317-322.

Jaworski, W.J. & Lee, C.Y., 1987. Fractionation and HPLC Determination of Grape Phenolics. *J. Agric. Food Chem.,* **35**, 257-259.

Jeandet, P., Bessis, R. & Gautheron, B., 1991. The production of Resveratrol (3,5,4'-trihydroxystilbene) by Grape Berries in Different Developmental Stages. *Am. J. Enol. Vitic.,* **42**, 41-46.

Jeandett, P., Bessis, R., Maume, B.F. & Sbaghi, M., 1993. Analysis of Resveratrol in Burgundy Wines. *Journal of Wine Research,* **4** (2), 79-85.

Jeandet, P., Bessis, R., Bernard, F.M., Maume, B.F., Meunier, P., Peyron, D. & Trollat, P., 1995. Effect of Enological Practices on the Resveratrol Isomer Content of Wine. *J. Agric. Food Chem.,* **43**, 316-319.

Jialal, I. & Grundy, S.M., 1993. Effect of dietary supplementation with alpha-tocopherol on the oxidative modificaion of low density lipoprotein. *Journal of Lipid Research*, **33**, 899-906.

Kanner, J., Frankel, E., Granit, R., German, G. & Kinsella, J.E., 1994. Natural Antioxidants in Grapes and Wines. *J. Agric. Food. Chem.,* **42,** 64-69.

Kaplan, A., Szabo, L.L. & Opheim, K.E., 1988. Clinical Chemistry: Interpretation and Techniques, 3rd edition. Baltimore: Williams & Wilkins.

Kinsella, J.E., Frankel, E., German, B. & Kanner, J., 1993. Possible Mechanisms for the Protective Role of Antioxidants in Wine and Plant Foods. *Food Technology*, **April,** 85-89.

Klatsky, A.L. & Armstrong, M.A., 1993. Alcohol Beverages Choice and Risk of Coronary Artery Disease Mortality: Do Red Wine Drinkers Fare Best? *The American Journal of Cardiology,* **71**, 467-469.

Lamuela-Raventós, R.M., Romero-Pérez, A.I., Waterhouse, A.L. & de la Torre-Boronat, M.C., 1995. Direct HPLC Analysis of *cis*- and *trans*-Resveratrol and Piceid Isomers in Spanish Red *Vitis vinifera* Wines. *J. Agric. Food Chem.,* **43**, 281-283.

Larrauri, J.A., Ruperez, P. & Calixto, S., 1996. Antioxidant Activity of Wine Pomace. *Am. J. Enol. Vitic.,* **47**(4), 369-372.

Levy, R.I., 1981. Cholesterol, Lipoproteins, Apoproteins, and Heart Disease: Present Status and Future Prospects. *Clinical Chemistry,* **27** (5), 653-662.

Maxwell, S., Cruickshank, A. & Thorpe, G., 1994. Red wine and antioxidant activity in serum. *The Lancet,* **344,** 193-194.

Mera, S.L., 1994. Diet and Disease. *British Journal of Biomedical Science,* **51**, 189-206.

Nourooz-Zadeh, J., Tajaddini-Sarmadi, J. & Wolff, S.P., 1994. Measurement of Plasma Hydroperoxide Concentrations by the Ferrous Oxidation-Xylenol Orange Assay in Conjunction with Triphenylphosphine. *Analytical Biochemistry*, **220,** 403-409.

Nourooz-Zadeh, J., Tajaddini-Sarmadi, J., Ling K.L.E. & Wolff, S.P., 1996. Low-density lipoprotein is the major carrier of lipid hydroperoxides in plasma. *Biochem. J.,* **313,** 781-786.

Pace-Asciak, C.R., Rounova, O., Hahn, S.E., Diamandis, E.P. & Goldberg, D.M., 1996. Wines and grape juices as modulators of platelet aggregation in healthy human subjects. *Clinical Chimica Acta,* **246**, 163-182.

Pattichis, K., Louca, L.L., Jarman, J., Sandler, M. & Glover. V., 1993. Phenolic substances in red wine and release of platelet 5-hydroxytryptamine. *The Lancet,* **341,** 1104.

Pestana, J.A.X., Steyn, K., Leiman, A. & Hartzenberg, G.M., 1996. The direct and indirect costs of cardiovascular disease in South Africa in 1991. *SAMJ*, **86**, 679-684.

Renaud, S. & de Lorgeril, M., 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *The Lancet,* **339**, 1523-1526.

Renaud, S.C., Beswick, A.D., Fehily, A.M., Sharp, D.S. & Elwood, P.C., 1992. Alcohol and platelet aggregation: the Caerphilly Prospective Heart Disease study. *Am. J. Clin. Nutr.,* **55**, 1012-1017.

Rossouw, J.E., Steyn, K., Berger, G.M.B., Vermaak, W.J.H., Kock, J., Seftel, H.C. & Gevers, W., 1988. Action limits for serum total cholesterol. *SA Medical Journal,* **73**, 693-700.

Sanders, A.B., 1991. Symposium on 'Mediterranean food and health'. The Mediterranean diet: fish and olives, oil on troubled waters. *Proceedings of the Nutrition Society,* **50**, 513-517.

Seigneur, M., Bonnet, J., Dorain, B., Benchimol, D., Drouillet, F., Gourverneur, G., Larrue, J., Crockett, R., Boisseau, M., Ribereau-Gayon, P. & Bricaud, H., 1990. Effect of the consumption of alcohol, white wine, and red wine on platelet function and serum lipids. *Journal of Applied Cardiology,* **5,** 215-222.

Siemann, E.H. & Creasy, L.L., 1992. Concentration of the Phytoalexin Resveratrol in Wine. *Am. J. Enol. Vitic.,* **43**(1) 49-52.

Sies. H., 1993. Strategies of antioxidant defense. *Eur. J. Biochem.,* **215**, 213-219.

Singleton, V.L. & Rossi, J.A., 1965. Colorimetry of total phenolic with phosphomolybdic-phosphotungstic acid reagent. *Am. J. Enol. Vitic.,* **16,** 144-158.

Teissedre, P.L., Waterhouse, A.L. & Frankel, E.N., 1995. Principal phenolic phytochemicals in French Syrah and Grenache Rhone wines and their antioxidant activity in inhibiting oxidation of human low density lipoproteins. *Journal International des Sciences de la Vigne et du Vin,* **29** (4), 205-212.

Trela, B.C. & Waterhouse, A.L., 1996. Resveratrol: Isomeric Molar Absorptivities and Stability. *J. Agric. Food Chem.,* **44**, 1253-1257.

Van Velden, D., 1997. Wine as health protector. Wynboer, January, 14-20.

Vinson, J.A. & Hontz, B.A., 1995. Phenol Antioxidant Index: Comparative Antioxidan Effectiveness of Red and White Wines. *J. Agric. Food Chem.*, **43**, 401-403.

Vrhovsek, U., Wendelin, S. & Eder, R., 1997. Effects of Various Vinification Techniques on the Concentration of *cis*- and *trans*-Resveratrol and Resveratrol Glucoside Isomers in Wine. *Am. J. Enol. Vitic.*, **48** (2), 214-219.

Whitehead, T.P., Robinson, D., Sharon, A., Syms, J. & Hale, A., 1995. Effect of Red Wine Ingestion on the Antioxidant Capacity of Serum. *Clin. Chem.*, **41**(1), 32-35.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nury, F.S., 1995. Wine Analysis and Production. New York: Chapman & Hall.

TABLE APPENDIX A.1 INFORMATION FROM DISTILLERS CORPORATION ON RED WINES.

Verskaffer / Landgoed	Fleur du Cap	Fleur du Cap	Drosdy-Hof	Cellar Cask	Drosdy-Hof	Stellenryk
Kultivar / versnit	Cabernet Sauvignon	Merlot	Merlot	Johannisberger Red	Claret	Cabernet Sauvignon
Oesjaar	1990	1995	1995			1992
Tipe grond	Geel verweerde graniet en diep rooi grond	Diep rooi	Diep rooi tot verweerde graniet	Verweerde graniet. Sand	Diep rooi tot verweerde graniet.	Geel verweerde graniet, diep rooi hutton
Helling	S en O	SO en NO	S, SO, NO en SW	S, SO, NW en NO	S, SO, NO en SW	N en SW
Temperatuur skommeling / Bestendige jaar	12°C	12°C	11°C	11°C	11°C	12,5°C
Teen hoeveel grade balling is die druiwe geoes?	21,57°B	22,9°B	22,7°B	20,1°B	21,2°B	22°B
Hoe lank het die wyn op die doppe gegis?	8 dae	9 dae	8 dae	8 dae	4 dae	8 dae
Hoe lank het die totale gistingsproses geduur?	8 dae	9 dae	8 dae	8 dae	8 dae	8 dae
Teen watter gemiddelde temperatuur het die gistingsproses plaasgevind?	28°C	28°C	28°C	28°C	28°C	28°C
Hoeveel oorblywende suiker is in die wyn?	1,9 g/l	3,27 g/l	2,87 g/l	5,8 g/l	3,8 g/l	2,4 g/l
Wat is die suurinhoud van die wyn?	6,2 g/l	5,7 g/l	5,4 g/l	6,4 g/l	4,8 g/l	6,0 g/l
Wat is die alkoholinhoud van die wyn? Vol %	12,3	13	12,99	11,5	12,1	12,56
Wat is die pH van die wyn?	3,47	3,4	3,56	3,5	3,63	3,45
Is hierdie wyn in hout verouder?	Ja	Ja	Ja	Nee	Ja	Ja
Indien wel, hoe lank is die wyn in die hout verouder?	24 maande	12 maande	18 maande	NVT	12 maande	18 maande
In watter soort hout (nuwe hout of ou hout) en hoe groot was die vate?	50% nuut 50% 2de vul 300 liter	30% nuut 70% 2de vul 300 liter	100% oud 300 liter	NVT	100% oud 5000 liter	100% nuut 300 liter
Is hierdie wyn met enige ander wyn versny?	Nee	Nee	Nee	Nee	Nee	Nee

TABLE APPENDIX A.2 INFORMATION FROM DISTILLERS CORPORATION ON WHITE WINES.

Verskaffer / Landgoed	Theuniskraal	Fleur du Cap	Fleur du Cap	L' Ormarins	Drosdy-Hof	Le Bonheur
Kultivar / versnit	Sémillon/Chardonnay	Riesling	Chardonnay	Sauvignon Blanc	Chardonnay	Sauvignon Blanc
Oesjaar	1996	1997	1996	1997	1997	1996
Tipe grond	Leem en graniet sand, leem klei.	Ligte sand, klei onderlaag.	Diep rooi tot geel skalie.	Duplex.	Diep rooi tot verweerde graniet.	Verweerde graniet, rooi leem, potklei en sand.
Helling	S en O	SO en NO	SO	S, NO en SW	S, SO, NO en SW	N, O en SO
Temperatuur skommeling / Bestendige jaar	10°C	12°C	12°C	14°C	11°C	10°C
Teen hoeveel grade balling is die druiwe geoes?	21,1°B	20°B	22°B	22°B	22,6°B	21,9°B
Hoe lank het die wyn op die doppe gegis?	NVT	NVT	NVT	NVT	NVT	NVT
Hoe lank het die totale gistingsproses geduur?	15 dae	16 dae	14 dae	15 dae	14 dae	15 dae
Teen watter gemiddelde temperatuur het die gistingsproses plaasgevind?	14°C	14°C	15°C	16°C	14°C	13°C
Hoeveel oorblywende suiker is in die wyn?	1,4 g/l	7,9 g/l	1,5 g/l	2,07 g/l	1,8 g/l	1,5 g/l
Wat is die suurinhoud van die wyn?	6,0 g/l	6,6 g/l	6,1 g/l	6,4 g/l	6,3 g/l	6,0 g/l
Wat is die alkoholinhoud van die wyn? Vol%	12,0	11,87	12,54	12,84	12,89	12,5
Wat is die pH van die wyn?	3,24	3,42	3,83	3,48	3,54	3,66
Is hierdie wyn in hout verouder?	Nee	Nee	Ja	Nee	Ja	Nee
Indien wel, hoe lank is die wyn in die hout verouder?	NVT	NVT	4 maande	NVT	4 maande	NVT
In watter soort hout (nuwe hout of ou hout) en hoe groot was die vate?	NVT	NVT	30% nuwe 70% oud 300 liter	NVT	100% oud 300 liter	NVT
Is hierdie wyn met enige ander wyn versny?	Nee	Nee	Nee	Nee	Nee	Nee

TABLE APPENDIX A.3 INFORMATION FROM APPLETISER ON GRAPE JUICES.

Information on the labels	Red Grapetiser 22:25 LA57/1, 200 ml	White Grapetiser 12:10 LA55/0, 200 ml
Cultivars used in the production of the Grapetiser (%)	Muscat grapes 100%	Neutral grapes 75% Muscat grapes 25%
The acid contents of the Grapetiser?	4,8 ± 0.1 g/l	4.6 ± 0.1 g/l
The pH of the Grapetiser?	3.2 - 3.5	3.1 – 3.3
How long has the Grapetiser had skin contact?	Natural red grape skin extract used for colour at manufacturing	N/A
Has the Grapetiser had wood contact?	No	No
If yes, for how long has the Grapetiser been on wood?	N/A	N/A
Do you use a special kind of wood?	N/A	N/A
Does Grapetiser contain any vitamins that could act as antioxidants?	Natural content of grape juice. Will vary with season and source.	Same as for red Grapetiser.
If yes, the concentrations?	Analyse specific batches.	Analyse specific batches.

TABLE APPENDIX A.4 ASCORBIC ACID ADDED TO WINES.

AANDAG: Marina Brand, Paramediese Wetenskappe, Technikon Vrystaat

Faksnommer: 051 5073355

Wyn	Natuurlike askorbiensuur aanwesig Ja / nee	Asorbiensuur bygevoeg Ja / nee	Konsentrasie askorbiensuur aanwesig
Fleur du Cap Cabernet Sauvignon '90	Toets nie vir natuurlike askorbiensuur nie.		
Fleur du Cap Merlot '95	Toets nie vir natuurlike askorbiensuur nie.		
Drosdy-Hof Merlot '95	Toets nie vir natuurlike askorbiensuur nie.		
Cellar Cask Johannisberger Red	Toets nie vir natuurlike askorbiensuur nie.		
Drosdy-Hof Claret	Toets nie vir natuurlike askorbiensuur nie.		
Stellenryk Cabernet Sauvignon '92	Toets nie vir natuurlike askorbiensuur nie.		
Theuniskraal Sémmilon/Chardonnay '96	Toets nie vir natuurlike askorbiensuur nie.	Ja 25 mg/l	
Fleur du Cap Riesling '97	Toets nie vir natuurlike askorbiensuur nie.	Ja 25 mg/l	
Fleur du Cap Chardonnay '96	Toets nie vir natuurlike askorbiensuur nie.	Ja 25 mg/l	
L'Ormarins Sauvignon Blanc '97	Toets nie vir natuurlike askorbiensuur nie.	Ja 25 mg/l	
Drosdy-Hof Chardonnay '97	Toets nie vir natuurlike askorbiensuur nie.	Ja 25 mg/l	
Le Bonheur Sauvignon Blanc '96	Toets nie vir natuurlike askorbiensuur nie.	Ja 25 mg/l	

TABLE APPENDIX B.1 DILUTIONS MADE ON WINES AND FRUIT JUICES (PAGE 1).

No	Wine / Juice / Neutral and / or Acid fraction / Catechin	Phenolic concentration mg/l Concentrated	Phenolic concentration mg/l 1:10 dilution	Phenolic concentration mg/l 1:2 dilution	Phenolic concentration used for dilutions	Dilution 1:100 1:1000 5 mg/l	First dilution Fraction in PBS	Final dilution Fraction in PBS
1	Wine ROH+	2430	2550		2430	1:1000	100 + 900	20 + 1980
	Wine ROH-	2230	2400		2230		100 + 900	20 + 1980
	Neutral fraction			1970	985		100 + 400	20 + 1980
	Acid fraction			120	60		100 + 400	20 + 1980
	Catechin	250			250		100 + 900	20 + 1980
	Wine ROH+		2550		2430	5 mg/l		20 + 9700
	Wine ROH-		2400		2230			20 + 8900
	Neutral fraction			1970	985			20 + 3920
	Acid fraction			120	60			100 + 1100
	N + A fraction			2090	1045			10 + 2080
2	Wine ROH+	2335	2325		2330	1:1000	100 + 900	20 + 1980
	Wine ROH-	2390	2425		2410		100 + 900	20 + 1980
	Neutral fraction			1955	978		100 + 400	20 + 1980
	Acid fraction			80	40		100 + 400	20 + 1980
	Catechin	250			250		100 + 900	20 + 1980
	Wine ROH+	2335	2325		2330	5 mg/l		20 + 9300
	Wine ROH-	2390	2425		2410			20 + 9620
	Neutral fraction			1955	977,5			20 + 3890
	Acid fraction			80	40			100 + 700
	N + A fraction			2035	1017,5			10 + 2025
3	Wine ROH+	2205	2050		2200	1:1000	100 + 900	20 + 1980
	Wine ROH-	2040	1975		2040		100 + 900	20 + 1980
	Neutral fraction			1635	820		100 + 400	20 + 1980
	Acid fraction			35	20		100 + 400	20 + 1980
	Catechin	250			250		100 + 900	20 + 1980
	Wine ROH+	2205	2050		2200	5 mg/l		20 + 8780
	Wine ROH-	2040	1975		2040			20 + 8140
	Neutral fraction			1635	820			20 + 3260
	Acid fraction			35	20			100 + 300
	N + A fraction			1670	835			20 + 3320

TABLE APPENDIX B.1 DILUTIONS MADE ON WINES AND JUICES (PAGE 2).

No	Wine / Juice / Neutral and / or acid fraction / Catechin	Phenolic concentration mg/l Concentrated	Phenolic concentration mg/l 1:10 dilution	Phenolic concentration mg/l 1:2 dilution	Phenolic concentration used for dilutions	Dilution 1:100 1:1000 5 mg/l	First dilution Fraction in PBS	Final dilution Fraction in PBS
4	Wine ROH+	1590	1650		1590	1:1000	100 + 900	20 + 1980
	Wine ROH-	1720	1725		1720		100 + 900	20 + 1980
	Neutral fraction			1250	625		100 + 400	20 + 1980
	Acid fraction			60	30		100 + 400	20 + 1980
	Catechin	250			250		100 + 900	20 + 1980
	Wine ROH+	1590	1650		1590	5 mg/l		20 + 6340
	Wine ROH-	1720	1725		1720			20 + 6860
	Neutral fraction			1250	625			20 + 2480
	Acid fraction			60	30			100 + 500
	N + A fraction			1310	655			20 + 2600
5	Wine ROH+	1685	1775		1685	1:1000	100 + 900	20 + 1980
	Wine ROH-	1645	1750		1645		100 + 900	20 + 1980
	Neutral fraction			1330	665		100 + 400	20 + 1980
	Acid fraction			90	45		100 + 400	20 + 1980
	Catechin	250			250		100 + 900	20 + 1980
	Wine ROH+	1685	1775		1685	5 mg/l		20 + 6720
	Wine ROH-	1645	1750		1645			20 + 6560
	Neutral fraction			1330	665			20 + 2640
	Acid fraction			90	45			20 + 160
	N + A fraction			1420	710			20 + 2820
6	Wine ROH+	-	2750		2750	1:1000	100 + 900	20 + 1980
	Wine ROH-	-	2750		2750		100 + 900	20 + 1980
	Neutral fraction			2385	1193		100 + 400	20 + 1980
	Acid fraction			130	65		100 + 400	20 + 1980
	Catechin	250			250		100 + 900	20 + 1980
	Wine ROH+	-	2750		2750	5 mg/l		20 + 10980
	Wine ROH-	-	2750		2750			20 + 10980
	Neutral fraction			2385	1193			20 + 4752
	Acid fraction			130	65			20 + 240
	N + A fraction			2515	1258			20 + 5012

TABLE APPENDIX B.1 DILUTIONS MADE ON WINES AND JUICES (PAGE 3).

No	Wine / Juice / Neutral and / or acid fraction / Catechin	Phenolic concentration mg/l Concentrated	Phenolic concentration mg/l 1:10 dilution	Phenolic concentration mg/l 1:2 dilution	Phenolic concentration used for dilutions	Dilution 1:100 1:1000 5 mg/l	First dilution Fraction in PBS	Final dilution Fraction in PBS
7	Juice	410			410	1:1000	100 + 900	20 + 1980
	Juice deproteinated	382,5			382,5		100 + 900	20 + 1980
	Neutral fraction			175	87,5		100 + 400	20 + 1980
	Acid fraction			140	70		100 + 400	20 + 1980
	Catechin	250			250	1:1000	100 + 900	20 + 1980
	Juice	410			410	5 mg/l		20 + 1620
	Juice deproteinated	382,5			382,5			20 + 1510
	Neutral fraction			175	87,5			100 + 1650
	Acid fraction			140	70			100 + 1300
	N and A fraction			315	157,5			100 + 3050
8	Juice	100			100 (180)	1:100	100 + 900	100 + 900
	Juice deproteinated	95			95 (175)		100 + 900	100 + 900
	Neutral fraction			70	35		100 + 400	100 + 900
	Acid fraction			55	27,5		100 + 400	100 + 900
	Catechin	250			250	1:1000	100 + 900	100 + 900
	Juice	100			100 (180)	5 mg/l		100 + 3500
	Juice deproteinated	95			95 (175)			100 + 3400
	Neutral fraction			70	35			100 + 600
	Acid fraction			55	27,5			100 + 450
	N + A fraction			125	62,5			100 + 1150
9	Wine ROH+	230			230	1:100	100 + 900	100 + 900
	Wine ROH-	200			200		100 + 900	100 + 900
	Neutral fraction			160	80		100 + 400	100 + 900
	Acid fraction			30	15		100 + 400	100 + 900
	Catechin	250			250	1:100	100 + 900	100 + 900
	Wine ROH+	230			230	5 mg/l		100 + 4500
	Wine ROH-	200			200			100 + 3900
	Neutral fraction			160	80			100 + 1500
	Acid fraction			30	15			100 + 200
	N + A fraction			190	95			100 + 1800

TABLE APPENDIX B.1 DILUTIONS MADE ON WINES AND FRUIT JUICES (PAGE 4).

No	Wine / Juice / Neutral and / or acid fraction / Catechin	Phenolic concentration mg/l Concentrated	Phenolic concentration mg/l 1:10 dilution	Phenolic concentration mg/l 1:2 dilution	Phenolic concentration used for dilutions	Dilution 1:100 1:1000 5 mg/l	First dilution Fraction in PBS	Final dilution Fraction in PBS
10	Wine ROH+	225			225	1:100	100 + 900	100 + 900
	Wine ROH-	215			215		100 + 900	100 + 900
	Neutral fraction			150	75		100 + 400	100 + 900
	Acid fraction			40	20		100 + 400	100 + 900
	Catechin	250			250	1:100	100 + 900	100 + 900
	Wine ROH+	225			225	5 mg/l		100 + 4400
	Wine ROH-	215			215			100 + 4200
	Neutral fraction			150	75			100 + 1400
	Acid fraction			40	20			100 + 300
	N + A fraction			190	95			100 + 1800
11	Wine ROH+	315			315	1:100	100 + 900	100 + 900
	Wine ROH-	290			290		100 + 900	100 + 900
	Neutral fraction			220	110		100 + 400	100 + 900
	Acid			30	15		100 + 400	100 + 900
	Catechin	250			250	1:100	100 + 900	100 + 900
	Wine ROH+	315			315	5 mg/l		100 + 6200
	Wine ROH-	290			290			100 + 5700
	Neutral fraction			220	110			100 + 2100
	Acid fraction			30	15			100 + 200
	N + A fraction			250	125			100 + 2400
12	Wine ROH+	240			240	1:100	100 + 900	100 + 900
	Wine ROH-	213			213		100 + 900	100 + 900
	Neutral fraction			160	80		100 + 400	100 + 900
	Acid fraction			35	18		100 + 400	100 + 900
	Catechin	250			250	1:100	100 + 900	100 + 900
	Wine ROH+	240			240	5 mg/l		100 + 4700
	Wine ROH-	213			213			100 + 4160
	Neutral fraction			160	80			100 + 1500
	Acid fraction			35	18			100 + 260
	N + A fraction			195	98			100 + 1860

TABLE APPENDIX B.1 DILUTIONS MADE ON WINES AND FRUIT JUICES (PAGE 5).

No	Wine / Juice / Neutral and / or acid fraction / Catechin	Phenolic concentration mg/l Concentrated	Phenolic concentration mg/l 1:10 dilution	Phenolic concentration mg/l 1:2 dilution	Phenolic concentration used for dilutions	Dilution 1:100 1:1000 5 mg/l	First dilution Fraction in PBS	Final dilution Fraction in PBS
13	Wine ROH+	290			290	1:100	100 + 900	100 + 900
	Wine ROH-	285			285		100 + 900	100 + 900
	Neutral fraction			210	105		100 + 400	100 + 900
	Acid fraction			40	20		100 + 400	100 + 900
	Catechin	250			250	1:1000	100 + 900	20 + 1980
	Wine ROH+	290			290	5 mg/l		100 + 5700
	Wine ROH-	285			285			100 + 5600
	Neutral fraction			210	105			100 + 2000
	Acid fraction			40	20			100 + 300
	N + A fraction			250	125			100 + 2400
14	Wine ROH+	250			250	1:100	100 + 900	100 + 900
	Wine ROH-	240			240		100 + 900	100 + 900
	Neutral fraction			190	95		100 + 400	100 + 900
	Acid fraction			60	30		100 + 400	100 + 900
	Catechin	250			250	1:1000	100 + 900	20 + 1980
	Wine ROH+	250			250	5 mg/l		100 + 4900
	Wine ROH-	240			240			100 + 4700
	Neutral fraction			190	95			100 + 1800
	Acid fraction			60	30			100 + 500
	N + A fraction			250	125			100 + 2400

```
Program Regressie;

{Pascal program om dubbel regressie passing mee te doen en }
{dan 'n x-as afsnit te gaan bereken waar twee regressielyne}
{mekaar sny.                                               }
{Geskryf deur Hennie Smit (M.Sc) Technikon Vrystaat        }

{$R-,S-}
uses Crt;

const
  Max = 1000;

type
  List = array[1..Max] of Integer;
var
  Datax : array[1..100] of real;
  Datay : array[1..19,1..100] of real;
  I,j,k,hh,kk,g,kry,q,m,her: Integer;
  gem,hel1,helt1,afsny1,hel2,helt2,afsny2:real;
  verskil1,verskil2,verskil3,afsnit,tal,dummy:real;
  F,GR : text;
  naaam,nnaa : string;

Begin
assign(GR,'Resasc1.txt');
 rewrite(GR);

{ for m :=1 to 50 do}   {Inlees van 50 lêers se name en data}
{ begin
  str(m,nnaa);}
{   naaam:='c:\tp\bin\marina\graf'+nnaa+'.txt';}
  naaam:='c:\tp\bin\marina\ascor1.txt';

  assign(F,naaam);
  reset(F);
  i:=1;
  her:=-1;
 readln(F,dummy);

While not eoln(F) do
 begin
  read(F,dummy);
  her:=her+1;
 end;

reset(F);

While not EOF(F) do
 begin
  Read(F,Datax[i]);
   for hh:=1 to her do
      begin
       Read(F,Datay[hh,i]);
      end;
      readln(F);
```

```
        i:=i+1;
 end;


k:=0;
kk:=0;
for hh:=1 to her do
begin
kry:=0;
j:=0;
while (j<i) and (kry=0) do  {Kies van eeste regressielyn se
punte}
  begin
   j:=j+1;
   Verskil1:=Datay[hh,j+1]-Datay[hh,j];
   Verskil2:=Datay[hh,j+2]-Datay[hh,j+1];
   Verskil3:=Datay[hh,j+3]-Datay[hh,j+2];
   Gem:=(Verskil1+Verskil2+Verskil3)/3;
   If gem>0.01 then
    begin
     k:=j-1;
     kry:=1;
    end;
   end;
kry:=0;
while (kry=0) and (j<i) do   {Kies van tweede regressielyn se
punte}
  begin
   j:=j+1;
   Verskil1:=Datay[hh,j+1]-Datay[hh,j];
   Verskil2:=Datay[hh,j+2]-Datay[hh,j+1];
   Verskil3:=Datay[hh,j+3]-Datay[hh,j+2];
   Gem:=(Verskil1+Verskil2+Verskil3)/3;
   If gem<0.005 then
    begin
     kk:=j-1;
     kry:=1;
    end;
   end;

{Berekening van helling van regressielyn 1}

helt1:=0;
afsny1:=0;
For j:= 2 to k-1 do
  begin
    hel1:=(Datay[hh,j+1]-Datay[hh,j])/(Datax[j+1]-Datax[j])/(k-
2);
    helt1:=helt1+hel1;
    afsny1:=afsny1+(Datay[hh,j]-(hel1*(k-2))*Datax[j])/(k-2);
  end;

{Berekening van helling van regressielyn 2}
helt2:=0;
afsny2:=0;
```

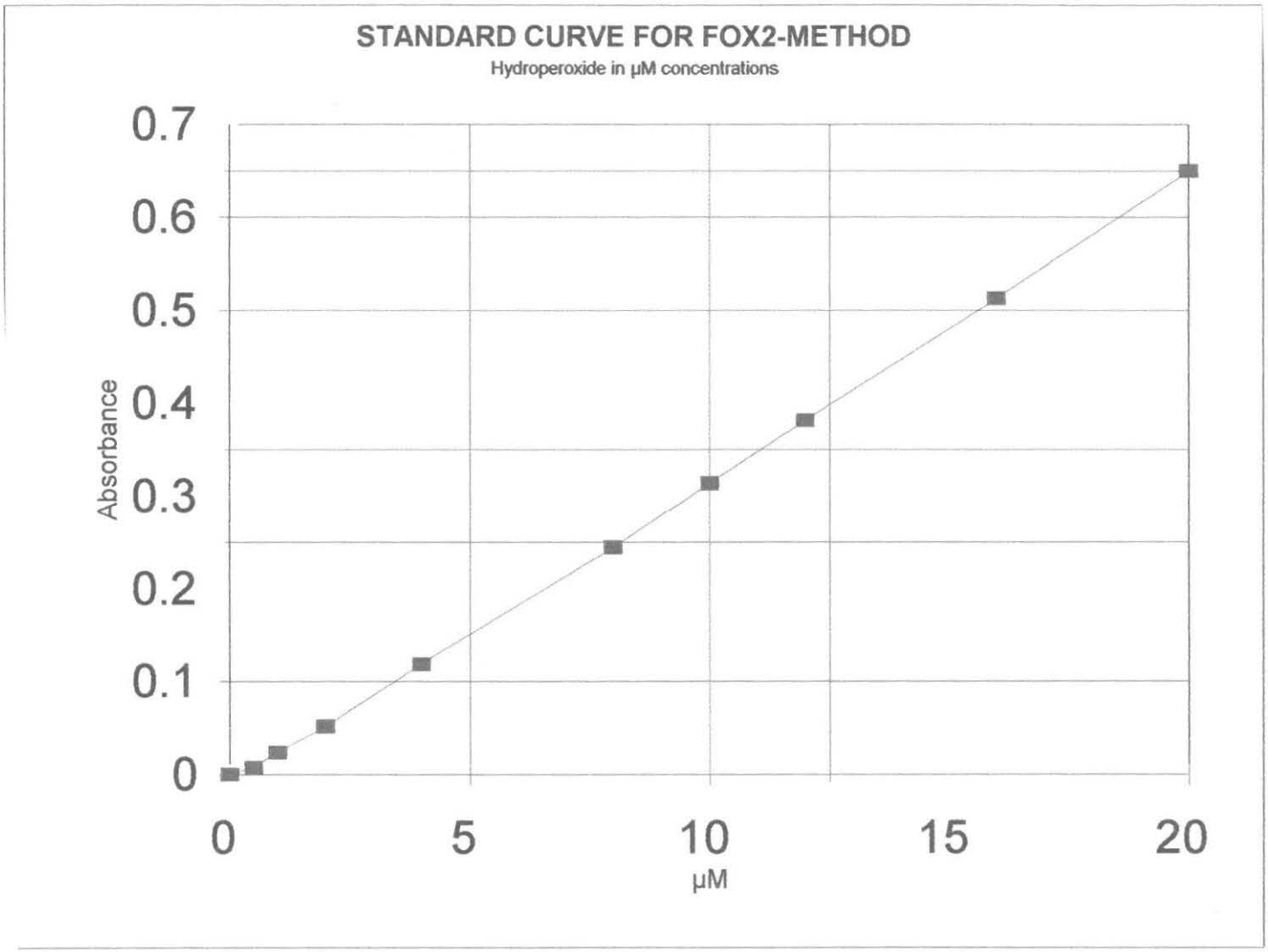
```
For j:= k+2 to kk-1 do
  begin
     hel2:=(Datay[hh,j+1]-Datay[hh,j])/(Datax[j+1]-
Datax[j])/(kk-(k+2));
     helt2:=helt2+hel2;
     afsny2:=afsny2+(Datay[hh,j]-(hel2*(kk-
(k+2))*Datax[j]))/(kk-(k+2));
  end;

{Berekening van x-as afsnit}

 Afsnit:=(afsny2-afsny1)/(helt1-helt2);

{ writeln(GR,m:5,'  ',hh:5,' Punt van oorgang
:=',afsnit:10:1,k:4,kk:4);}
 writeln(GR,afsnit:10:1);

end;
writeln(GR);
 close(F);
{end;}
close(GR);
end.
```



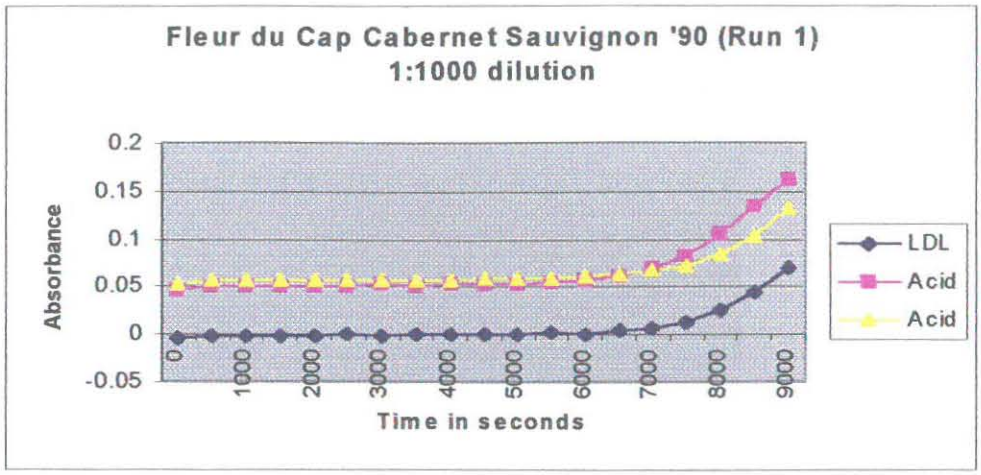


FIGURE APPENDIX F1. WINE 1. FLEUR DU CAP CABERNET SAUVIGNON '90

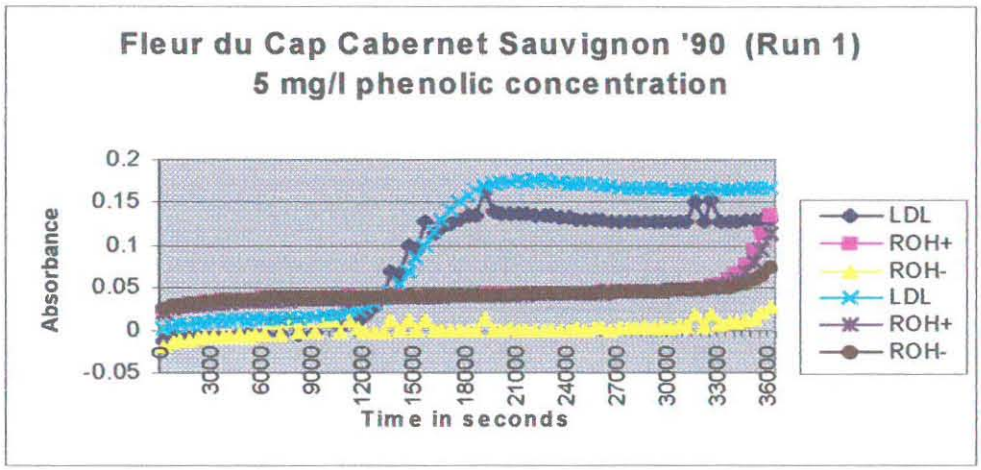


FIGURE APPENDIX F2. WINE 1. FLEUR DU CAP CABERNET SAUVIGNON '90

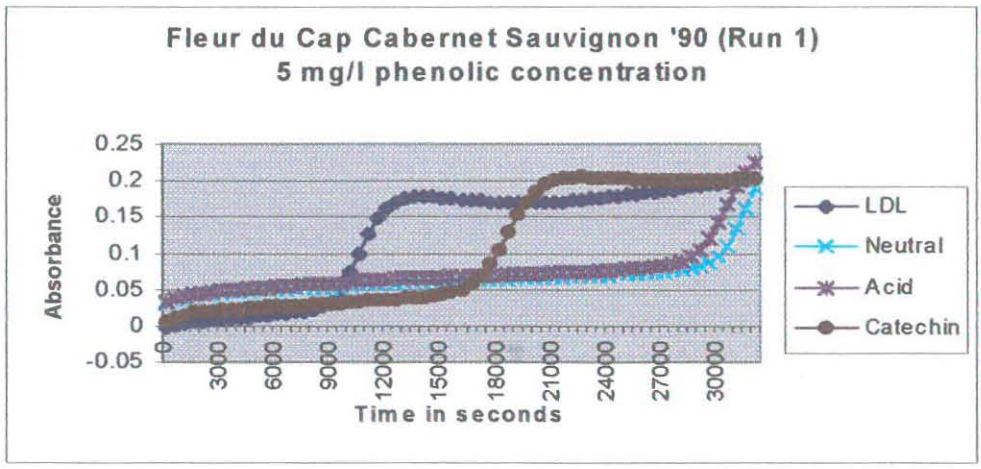


FIGURE APPENDIX F3. WINE 1. FLEUR DU CAP CABERNET SAUVIGNON '90

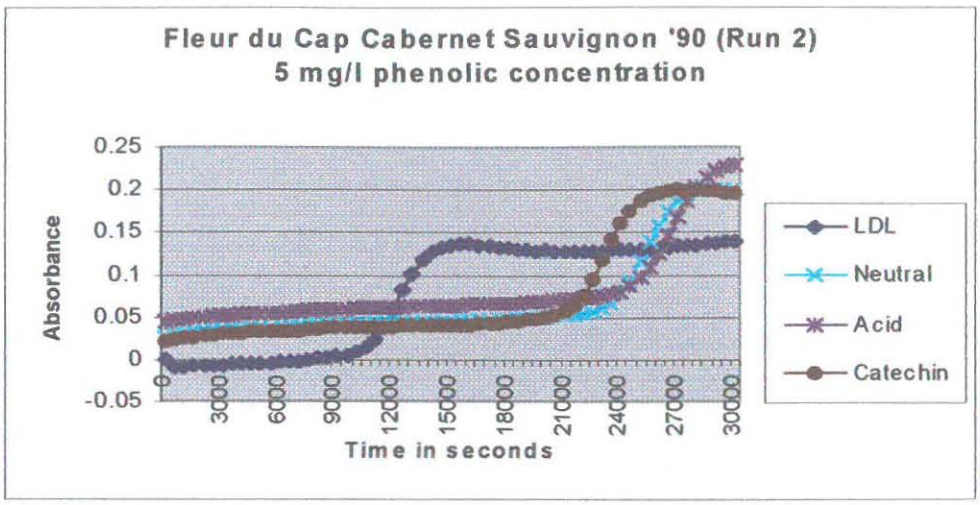


FIGURE APPENDIX F4. WINE 1. FLEUR DU CAP CABERNET SAUVIGNON '90

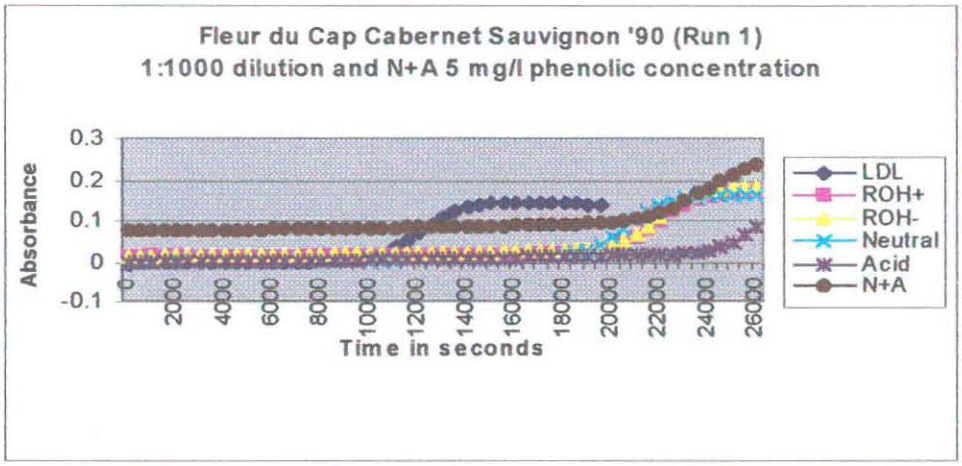


FIGURE APPENDIX F5. WINE 1. FLEUR DU CAP CABERNET SAUVIGNON '90

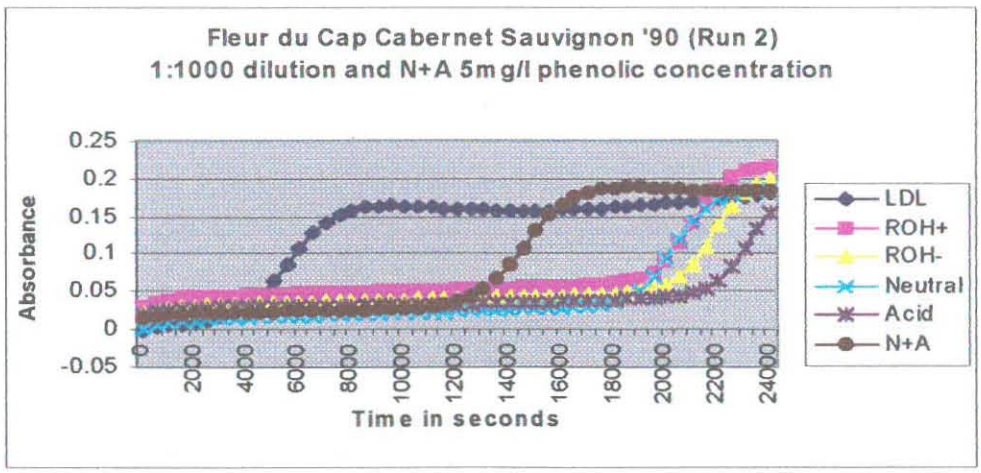


FIGURE APPENDIX F6. WINE 1. FLEUR DU CAP CABERNET SAUVIGNON '90

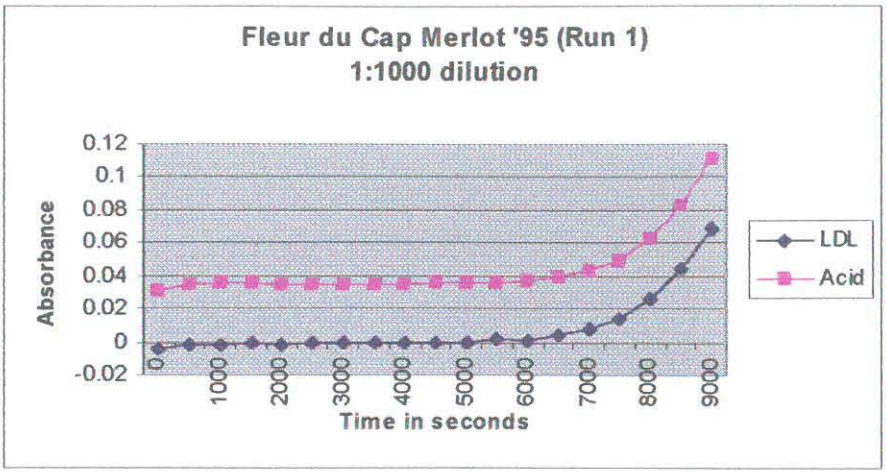


FIGURE APPENDIX F7. WINE 2. FLEUR DU CAP MERLOT '95

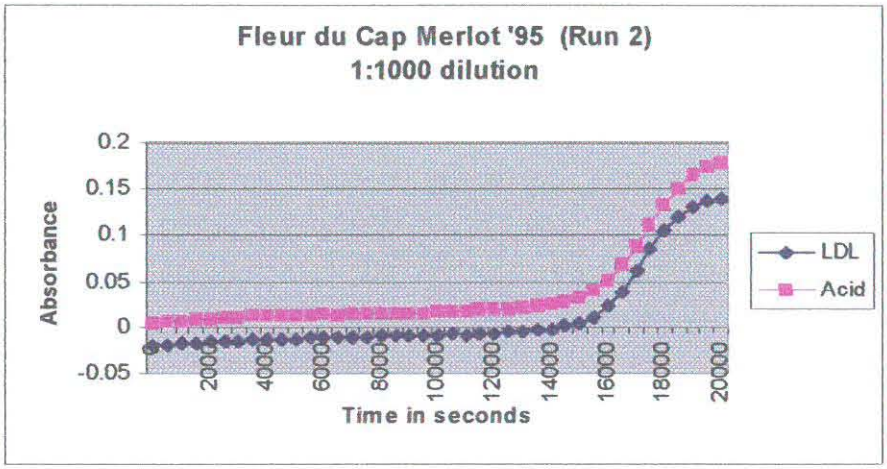


FIGURE APPENDIX F8. WINE 2. FLEUR DU CAP MERLOT '95

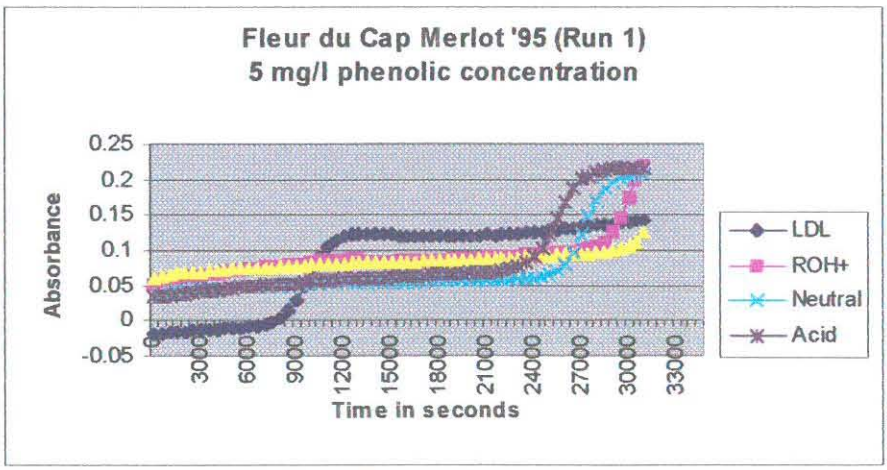


FIGURE APPENDIX F9. WINE 2. FLEUR DU CAP MERLOT '95

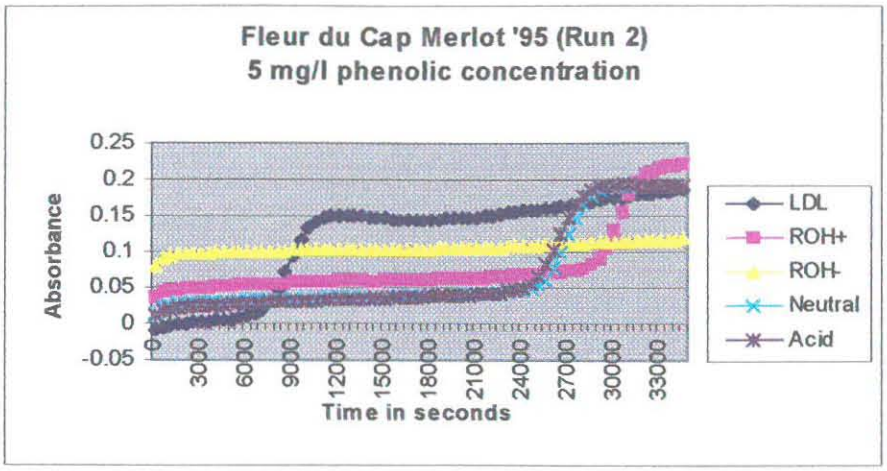


FIGURE APPENDIX F10. WINE 2. FLEUR DU CAP MERLOT '95

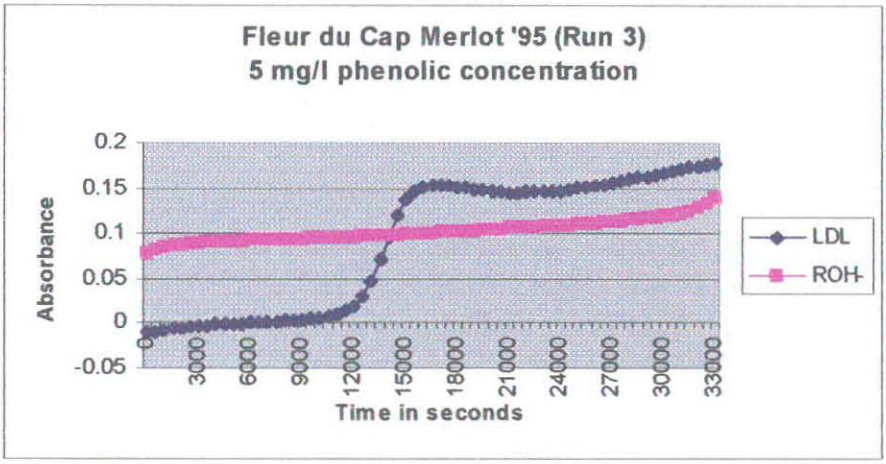


FIGURE APPENDIX F11. WINE 2. FLEUR DU CAP MERLOT '95

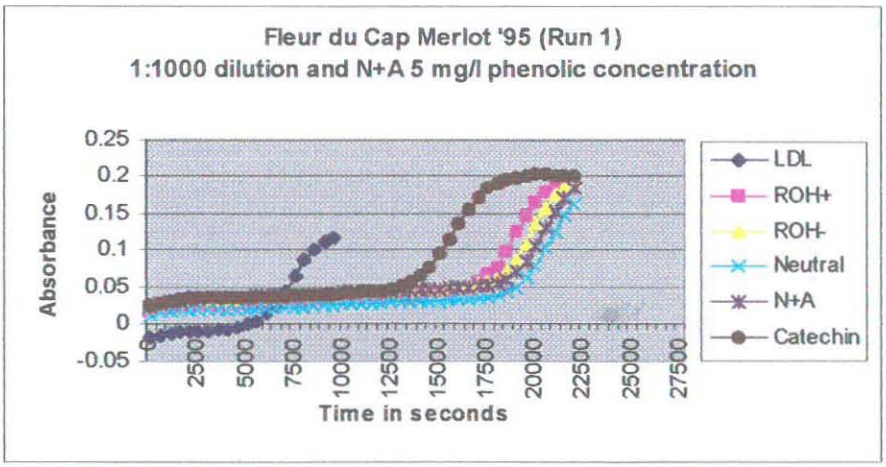


FIGURE APPENDIX F12. WINE 2. FLEUR DU CAP MERLOT '95

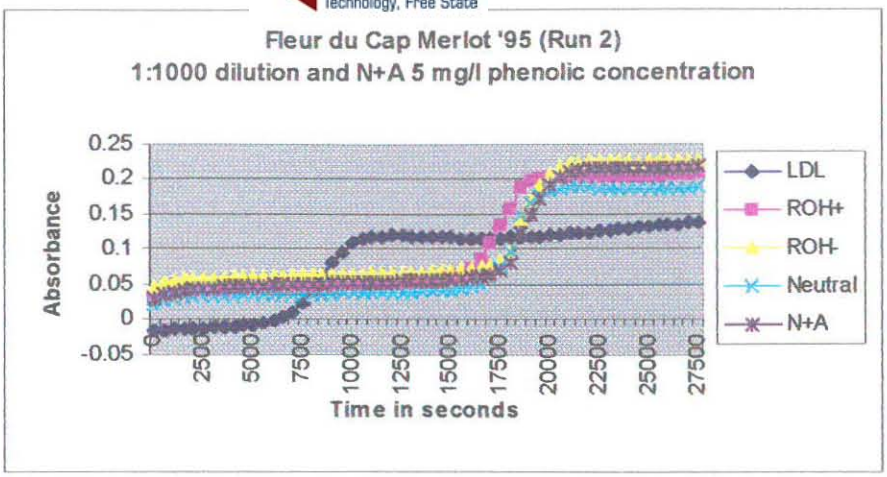


FIGURE APPENDIX F13. WINE 2. FLEUR DU CAP MERLOT '95

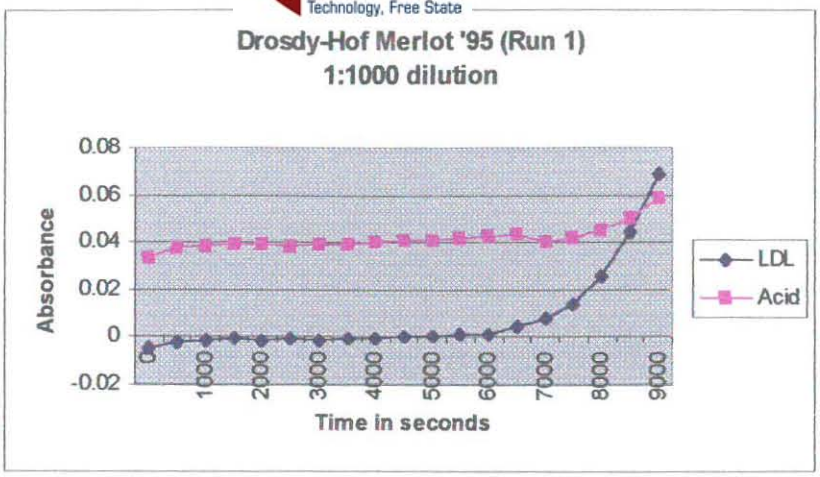


FIGURE APPENDIX F14. WINE 3. DROSDY-HOF MERLOT '95

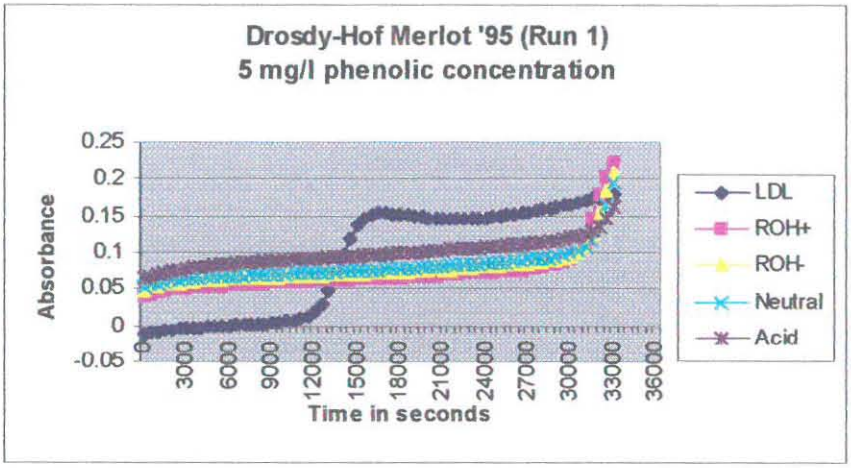


FIGURE APPENDIX F15. WINE 3. DROSDY-HOF MERLOT '95

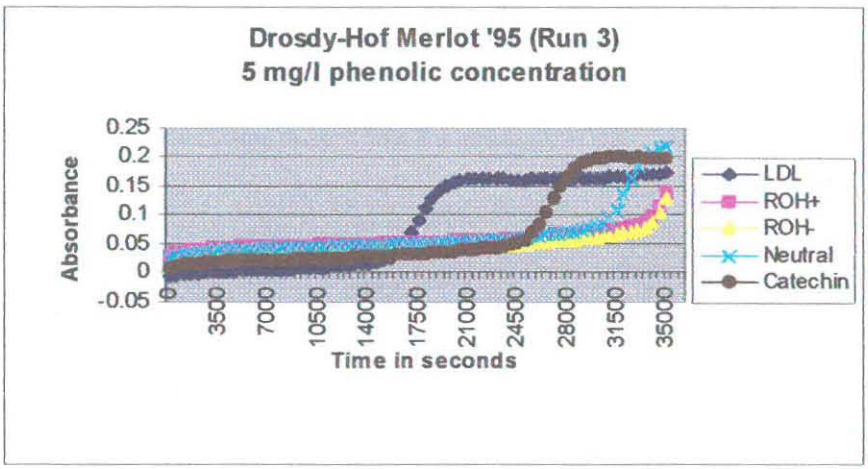


FIGURE APPENDIX F16. WINE 3. DROSDY-HOF MERLOT '95

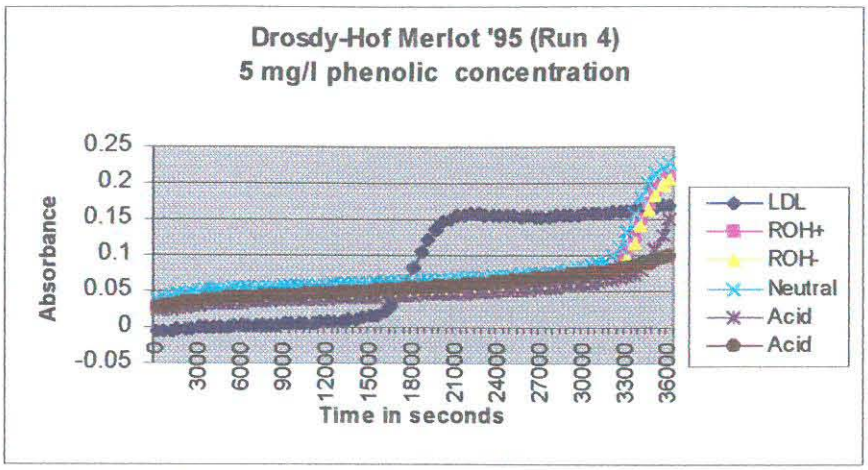


FIGURE APPENDIX F.17. WINE 3. DROSDY-HOF MERLOT '95

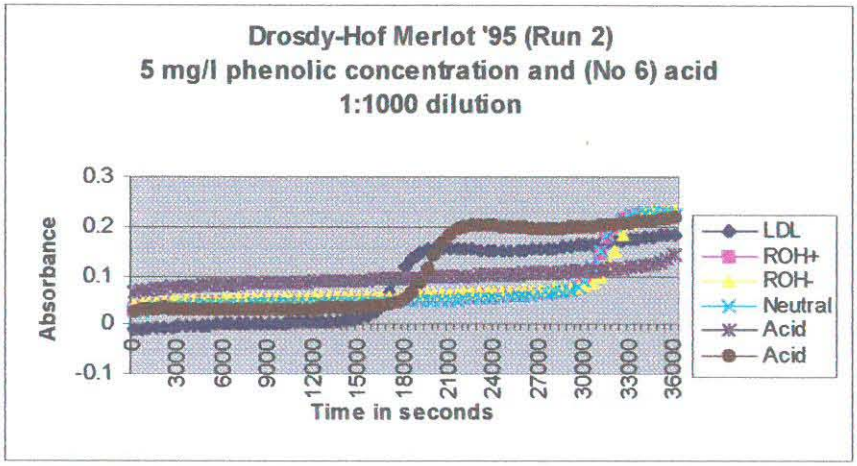


FIGURE APPENDIX F18. WINE 3. DROSDY-HOF MERLOT '95

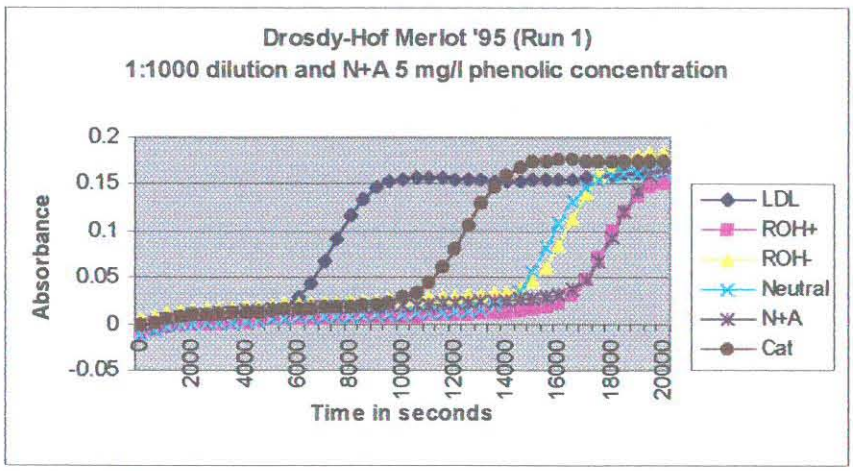


FIGURE APPENDIX F19. WINE 3. DROSDY-HOF MERLOT '95

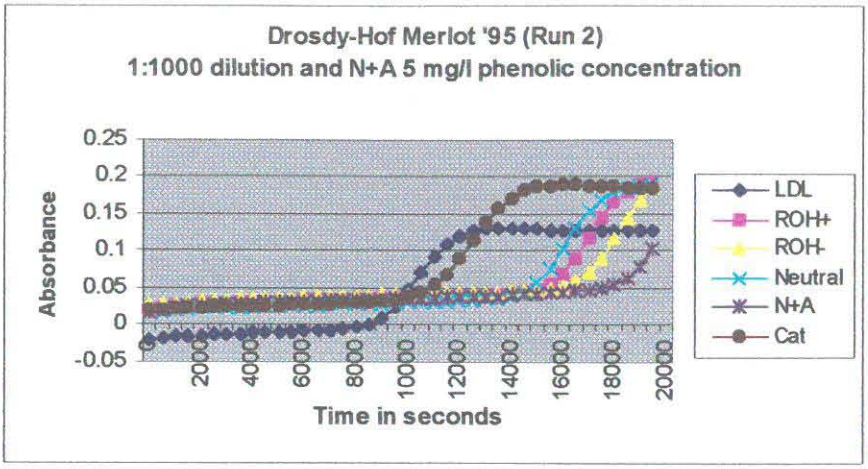


FIGURE APPENDIX F20. WINE 3. DROSDY-HOF MERLOT '95

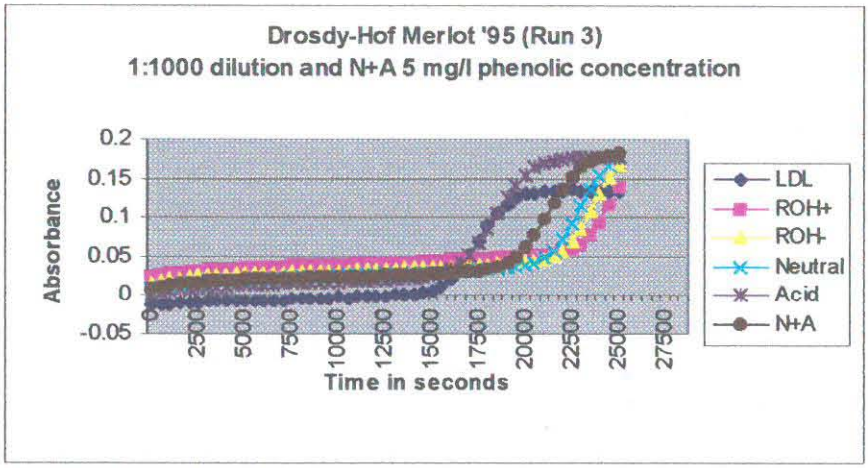


FIGURE APPENDIX F21. WINE 3. DROSDY-HOF MERLOT '95

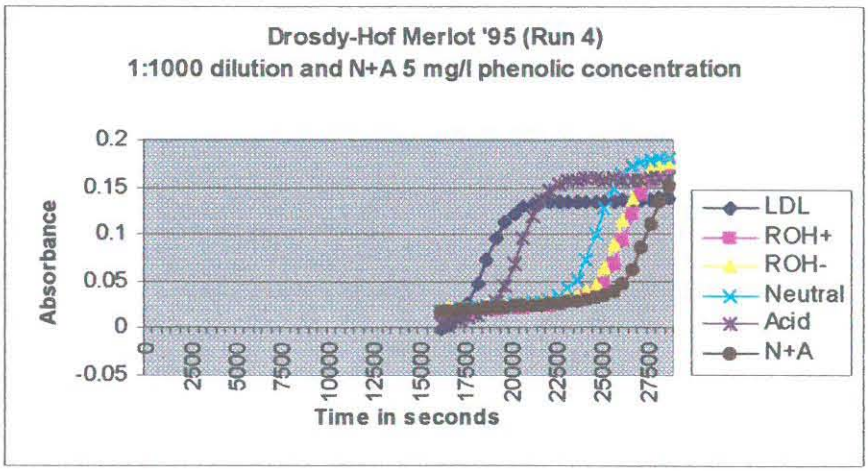


FIGURE APPENDIX F22. WINE 3. DROSDY-HOF MERLOT '95

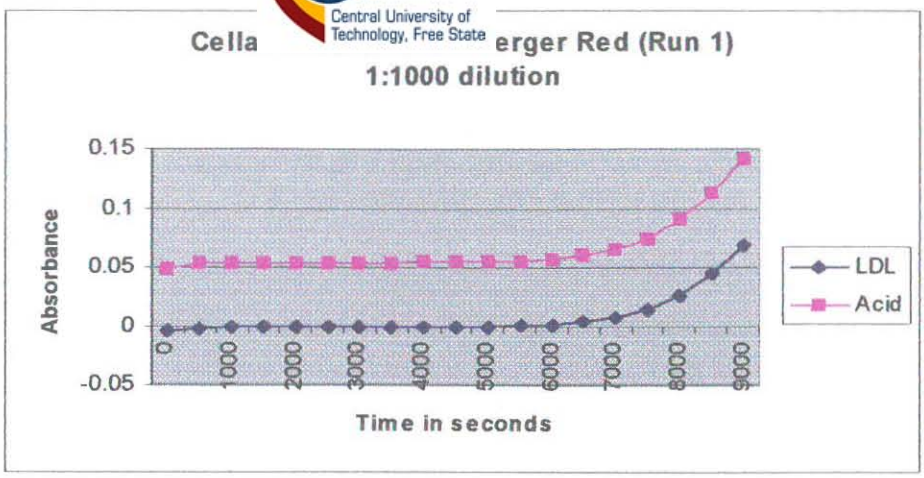


FIGURE APPENDIX F23. WINE 4. CELLAR CASK JOHANNISBERGER RED

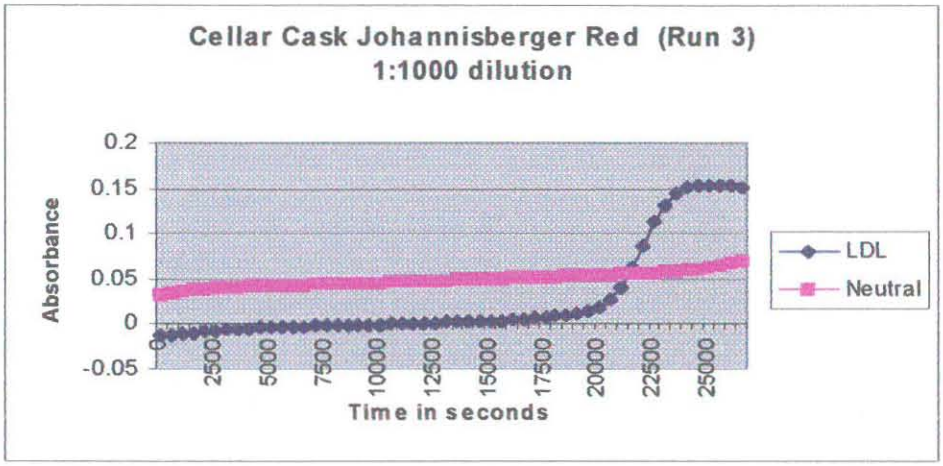


FIGURE APPENDIX F24. WINE 4. CELLAR CASK JOHANNISBERGER RED

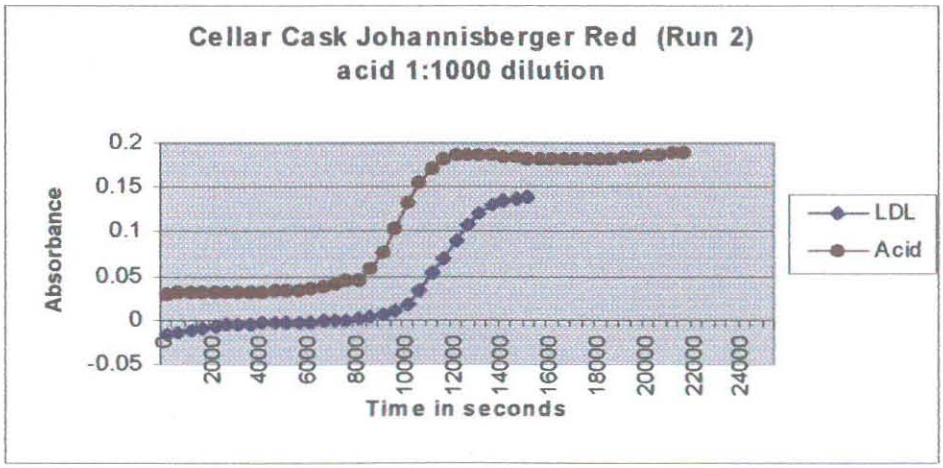


FIGURE APPENDIX F25. WINE 4. CELLAR CASK JOHANNISBERGER RED

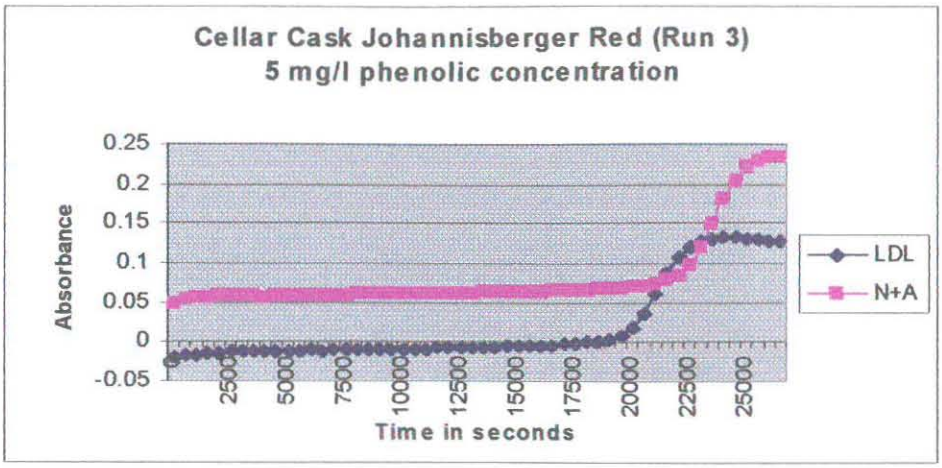


FIGURE APPENDIX F26. WINE 4. CELLAR CASK JOHANNISBERGER RED

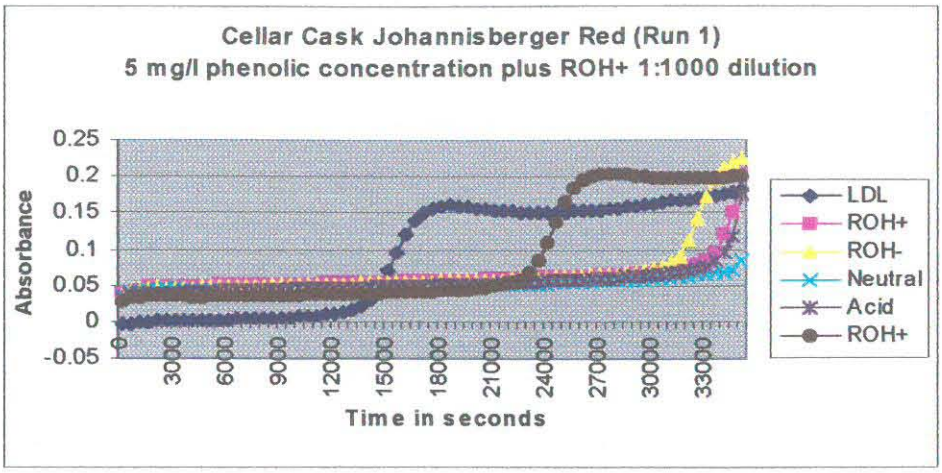


FIGURE APPENDIX F27. WINE 4. CELLAR CASK JOHANNISBERGER RED

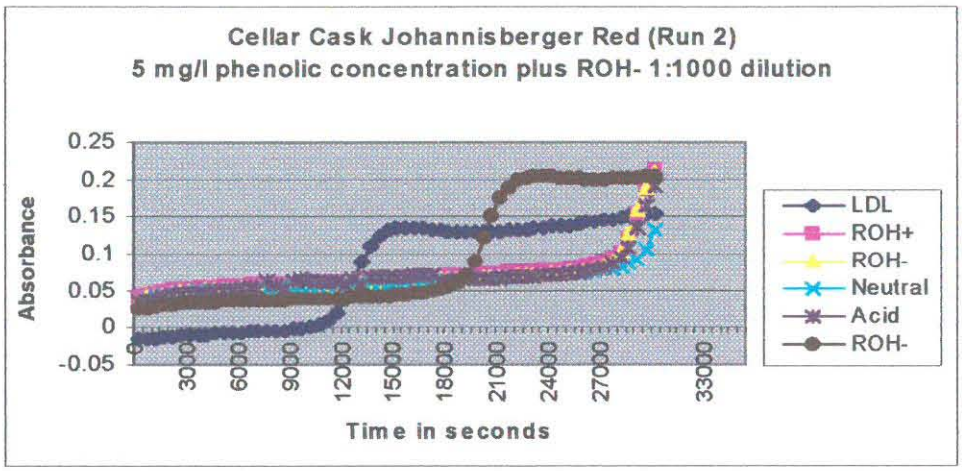


FIGURE APPENDIX F28. WINE 4. CELLAR CASK JOHANNISBERGER RED

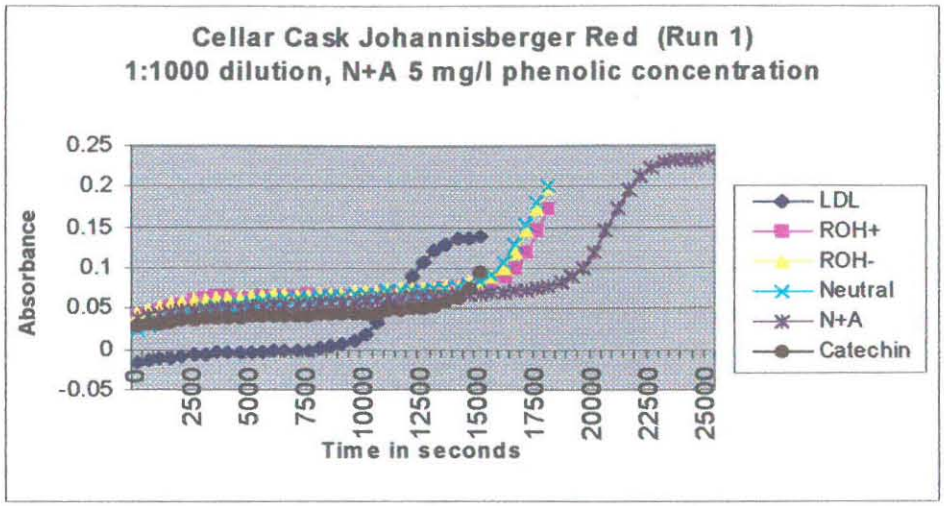


FIGURE APPENDIX F29. WINE 4. CELLAR CASK JOHANNISBERGER RED

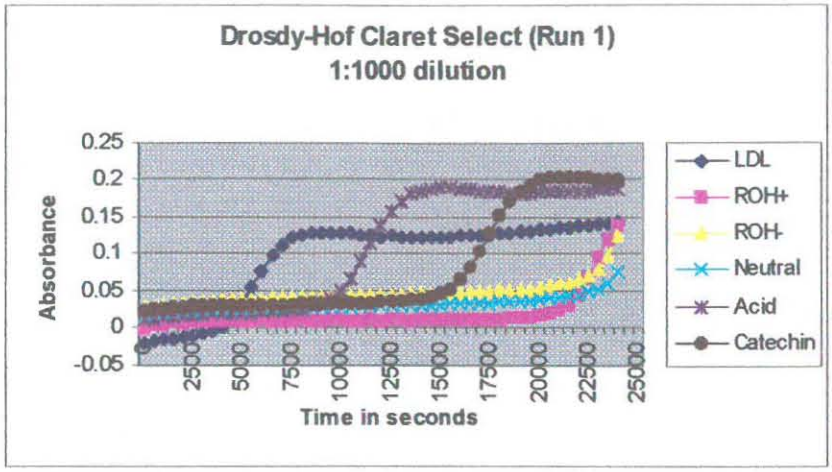


FIGURE APPENDIX F30.WINE 5. DROSDY-HOF CLARET SELECT

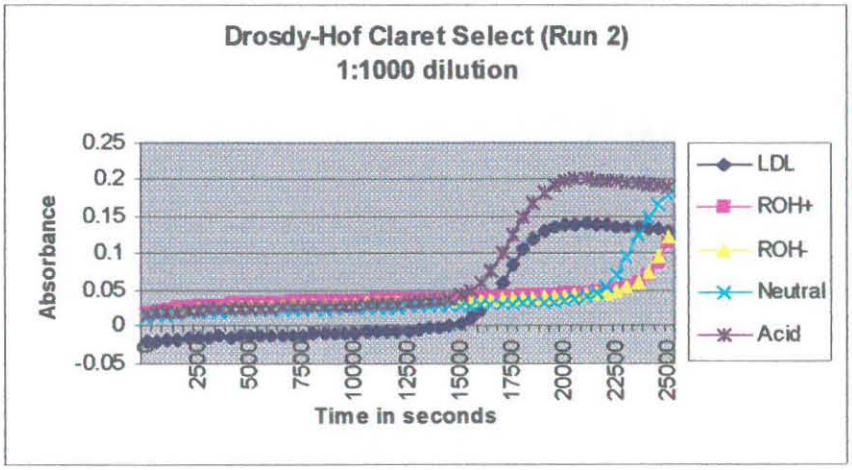


FIGURE APPENDIX F31.WINE 5. DROSDY-HOF CLARET SELECT

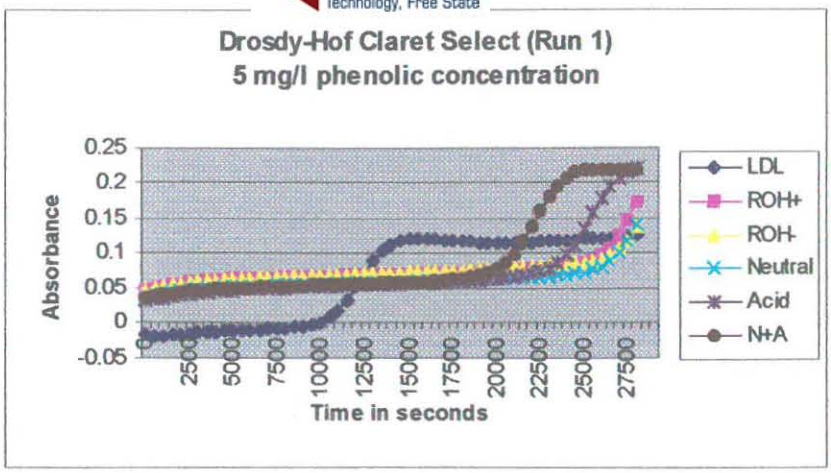


FIGURE APPENDIX F32.WINE 5. DROSDY-HOF CLARET SELECT

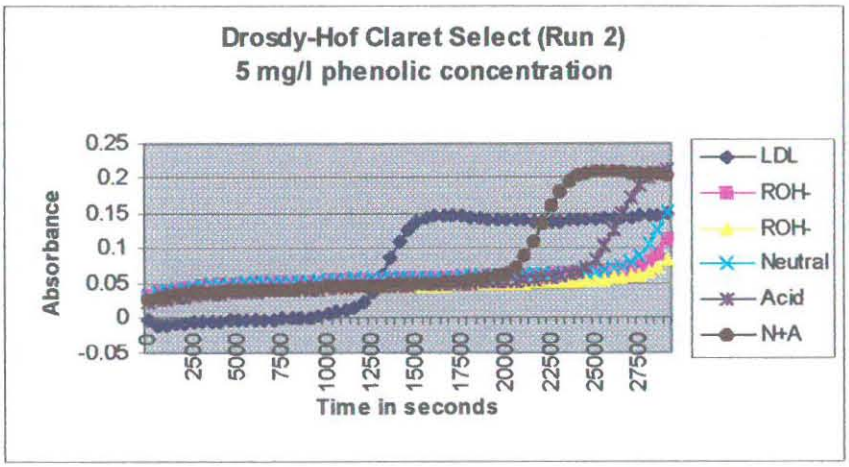


FIGURE APPENDIX F33.WINE 5. DROSDY-HOF CLARET SELECT

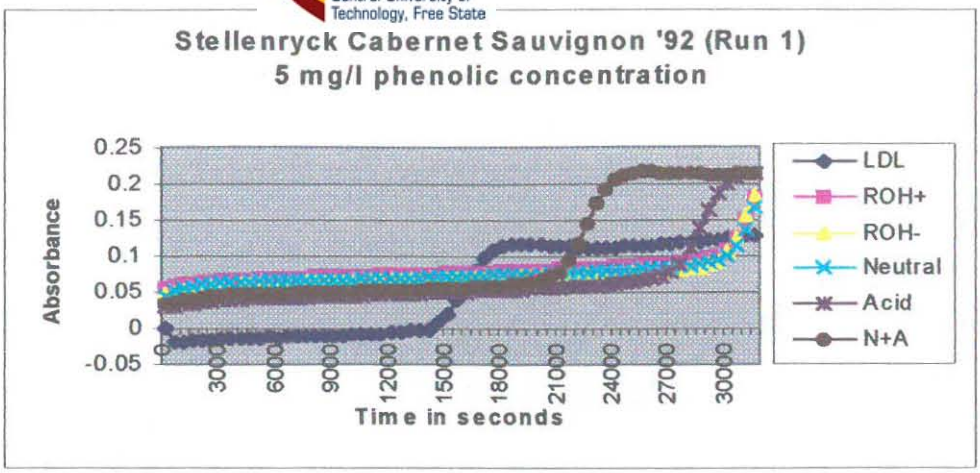


FIGURE APPENDIX F34.WINE 6. STELLENRYCK CABERNET SAUVIGNON '92

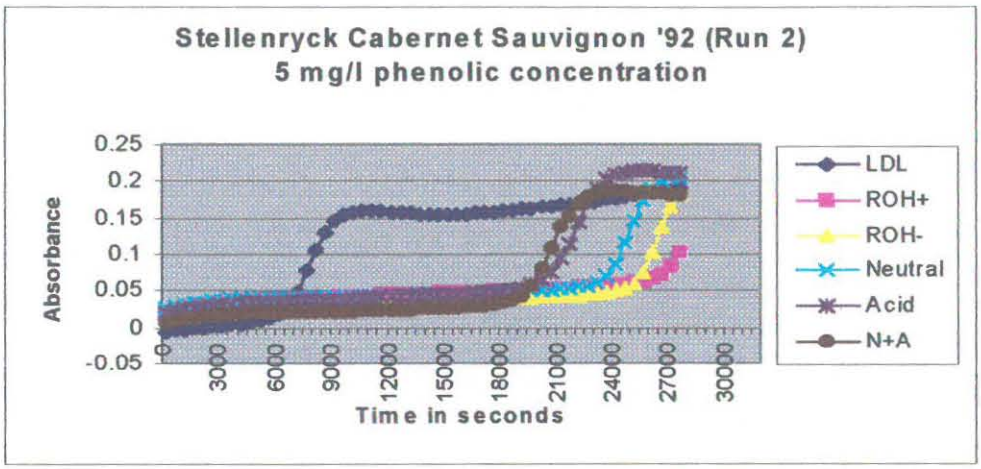


FIGURE APPENDIX F35.WINE 6. STELLENRYCK CABERNET SAUVIGNON '92

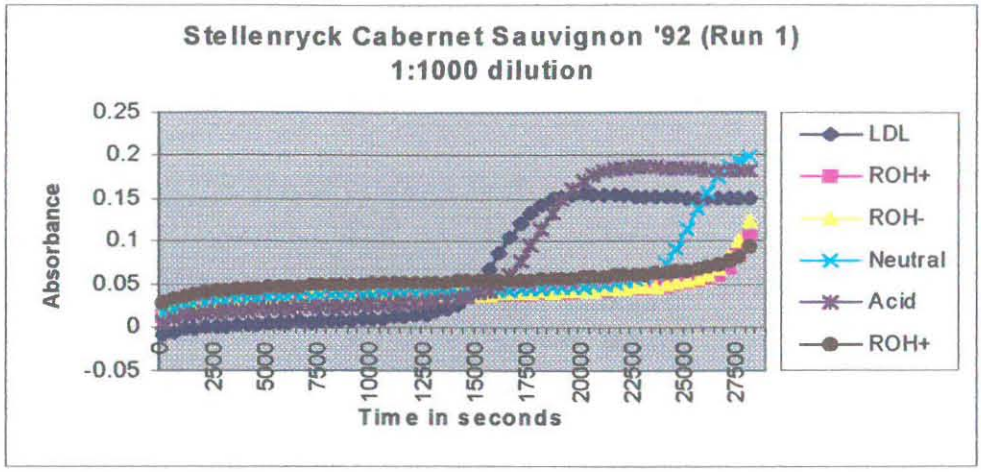


FIGURE APPENDIX F36.WINE 6. STELLENRYCK CABERNET SAUVIGNON '92

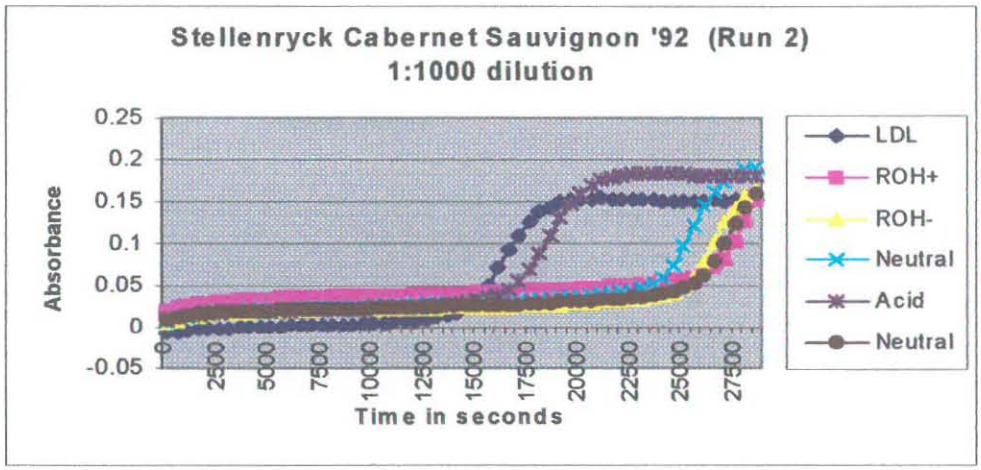


FIGURE APPENDIX F37.WINE 6. STELLENRYCK CABERNET SAUVIGNON '92

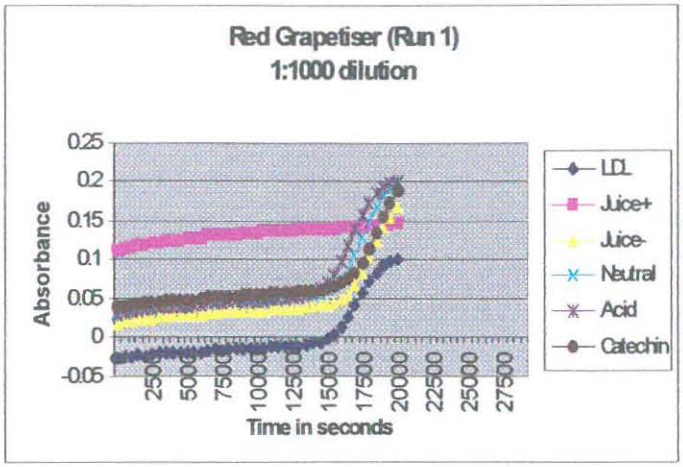


FIGURE APPENDIX F38.JUICE 7. RED GRAPETISER

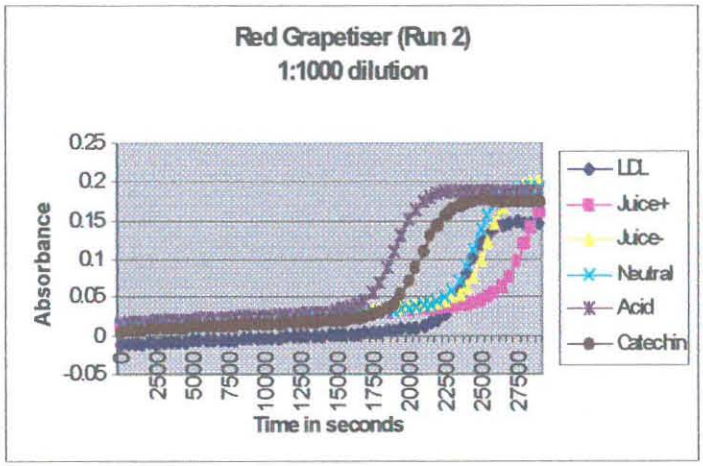


FIGURE APPENDIX F39.JUICE 7. RED GRAPETISER

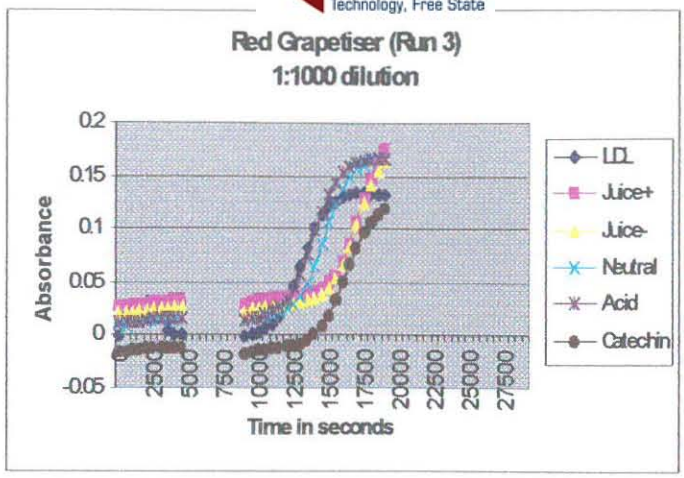


FIGURE APPENDIX F40.JUICE 7. RED GRAPETISER

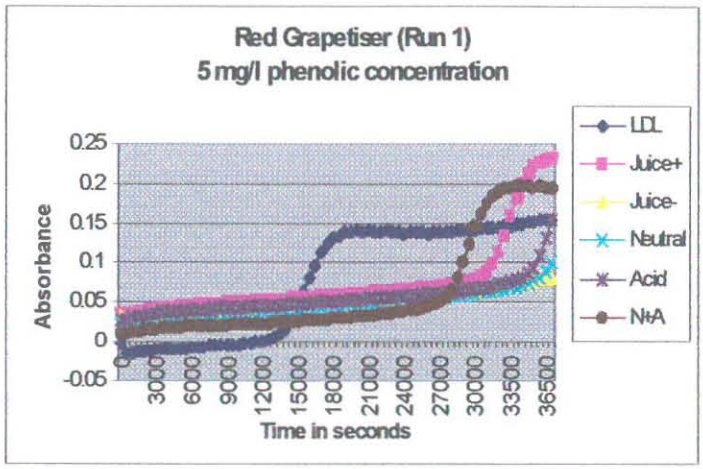


FIGURE APPENDIX F41.JUICE 7. RED GRAPETISER

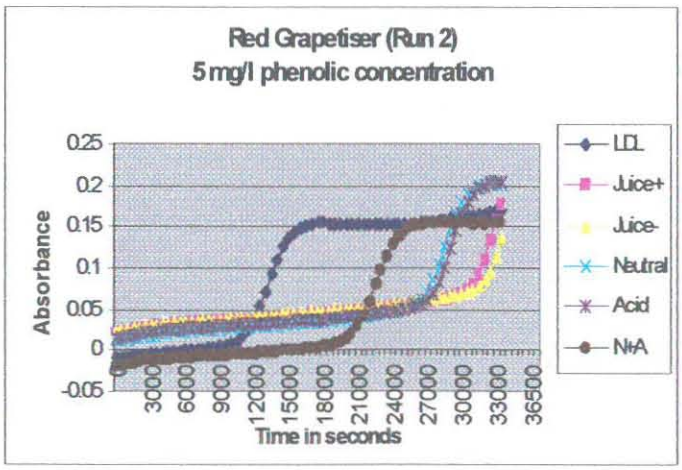


FIGURE APPENDIX F42.JUICE 7. RED GRAPETISER

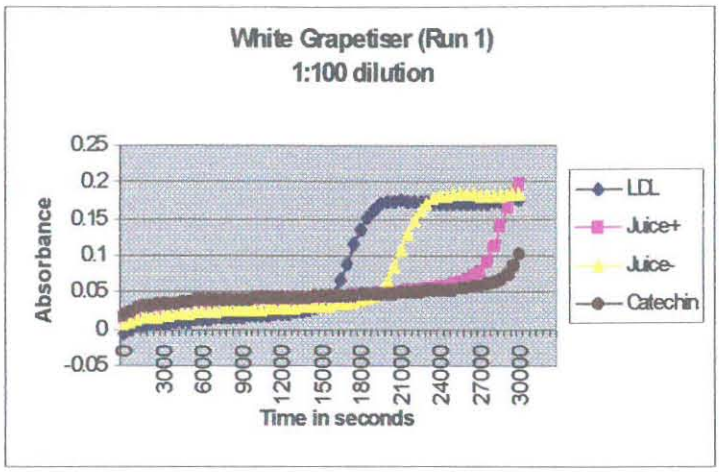


FIGURE APPENDIX F43.JUICE 8. WHITE GRAPETISER

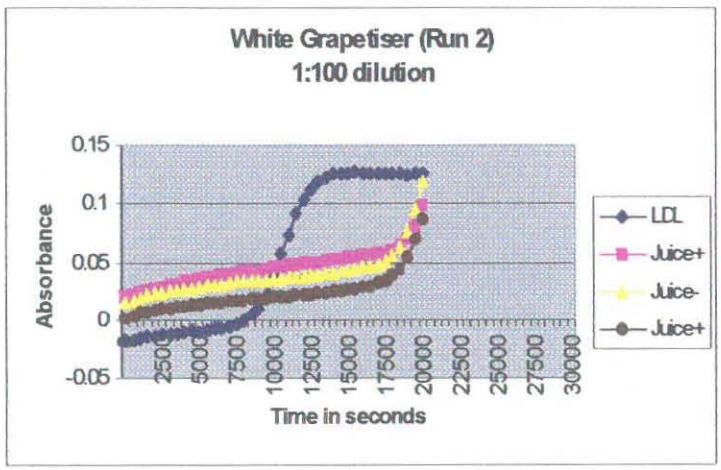


FIGURE APPENDIX F44.JUICE 8. WHITE GRAPETISER

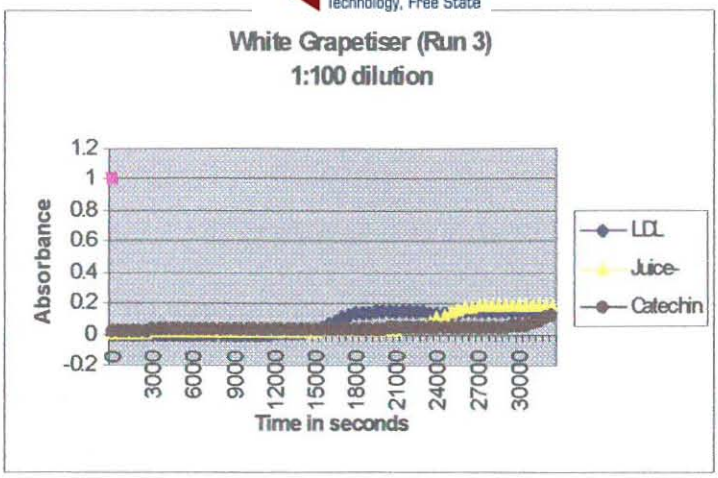


FIGURE APPENDIX F45.JUICE 8. WHITE GRAPETISER

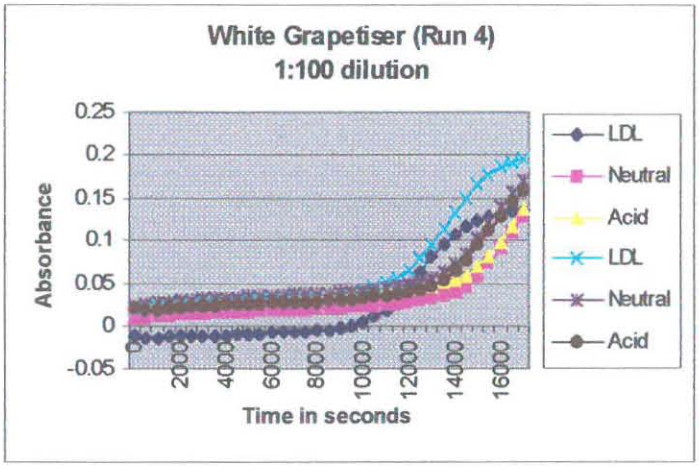


FIGURE APPENDIX F46.JUICE 8. WHITE GRAPETISER

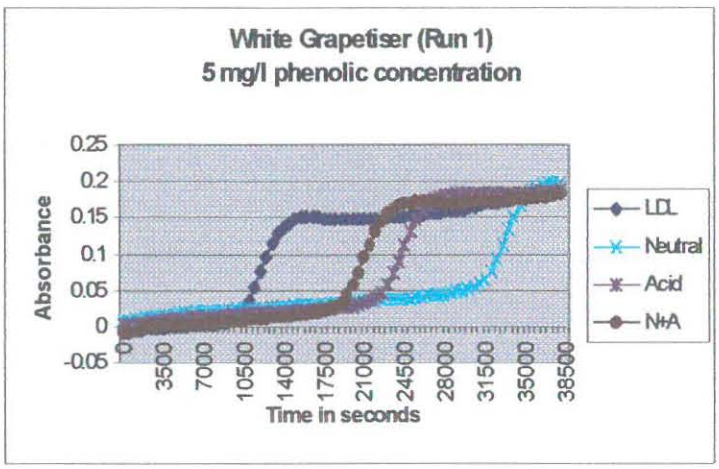


FIGURE APPENDIX F47.JUICE 8. WHITE GRAPETISER

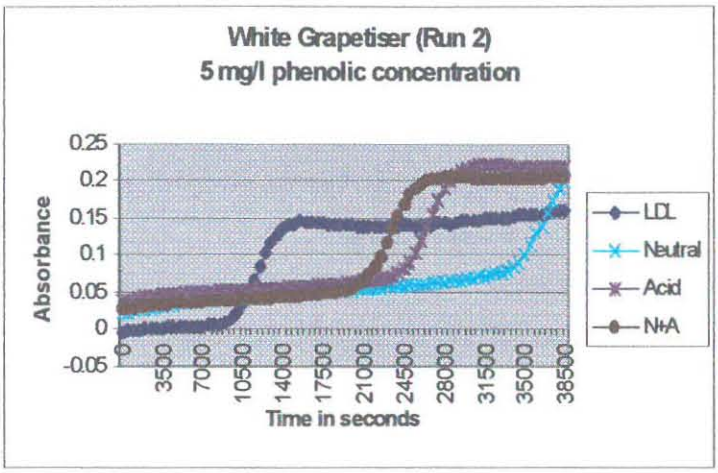


FIGURE APPENDIX F48.JUICE 8. WHITE GRAPETISER

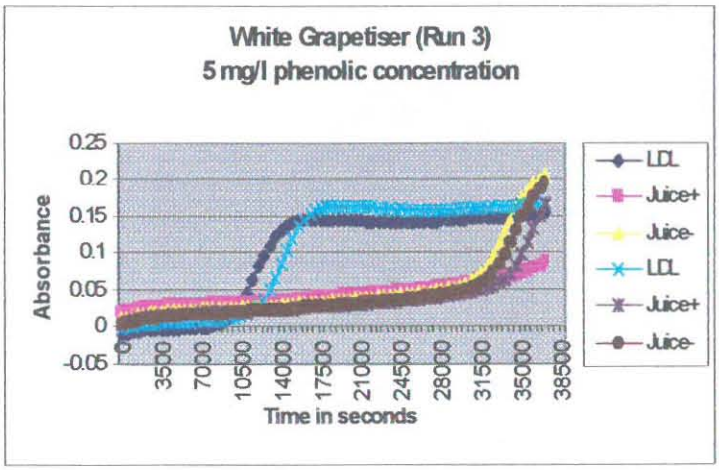


FIGURE APPENDIX F49.JUICE 8. WHITE GRAPETISER

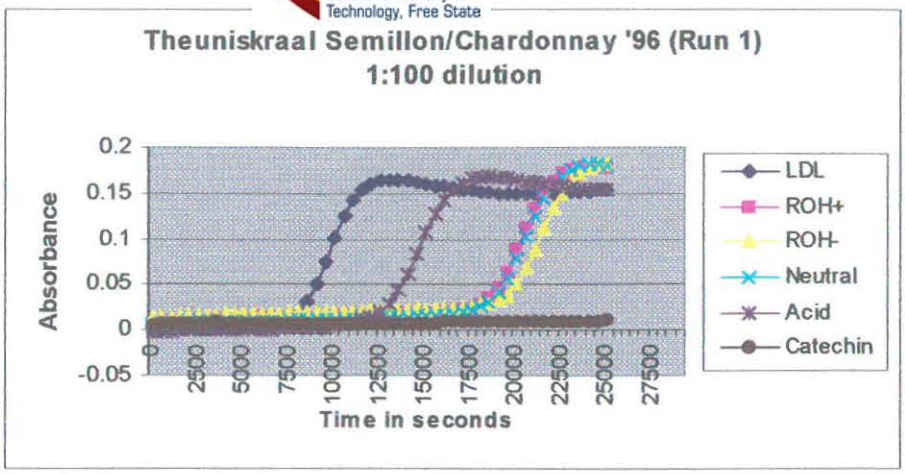


FIGURE APPENDIX F50. WINE 9. THEUNISKRAAL SEMILLON/CHARDONNAY '96

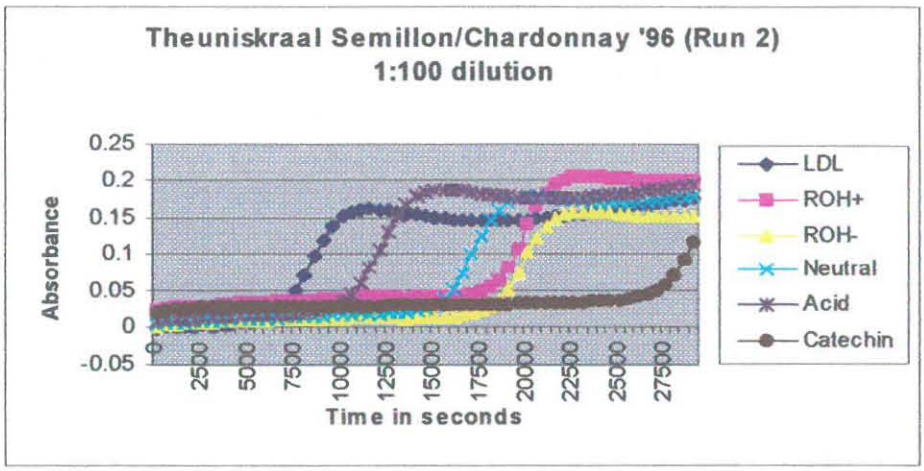


FIGURE APPENDIX F51. WINE 9. THEUNISKRAAL SEMILLON/CHARDONNAY '96

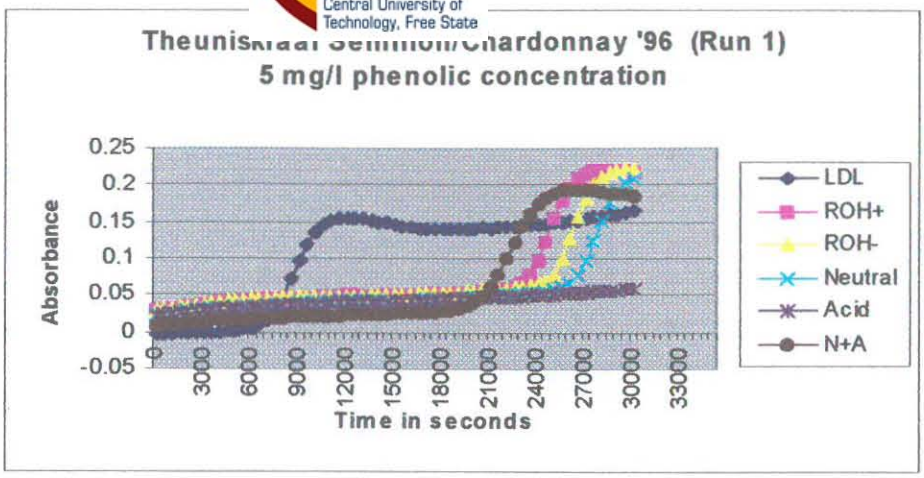


FIGURE APPENDIX F52. WINE 9. THEUNISKRAAL SEMILLON/CHARDONNAY '96

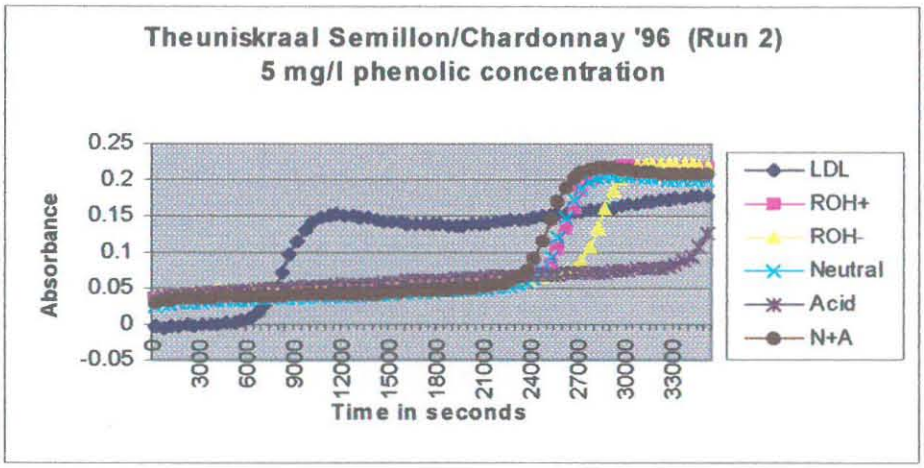


FIGURE APPENDIX F53. WINE 9. THEUNISKRAAL SEMILLON/CHARDONNAY '96

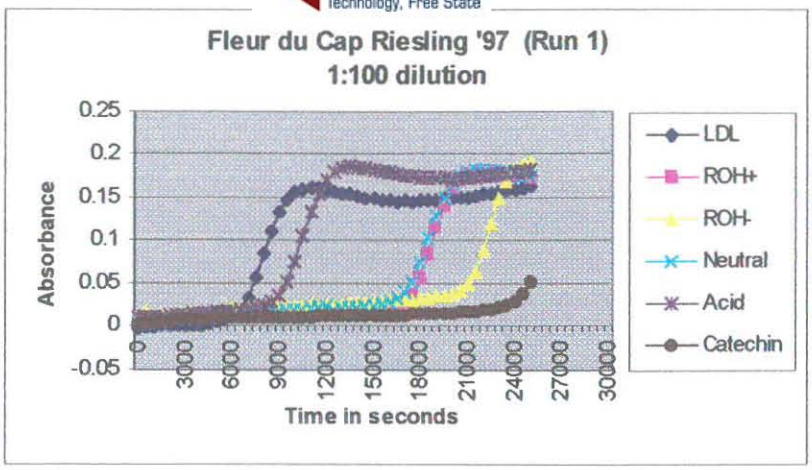


FIGURE APPENDIX F54. WINE 10. FLEUR DU CAP RIESLING '97

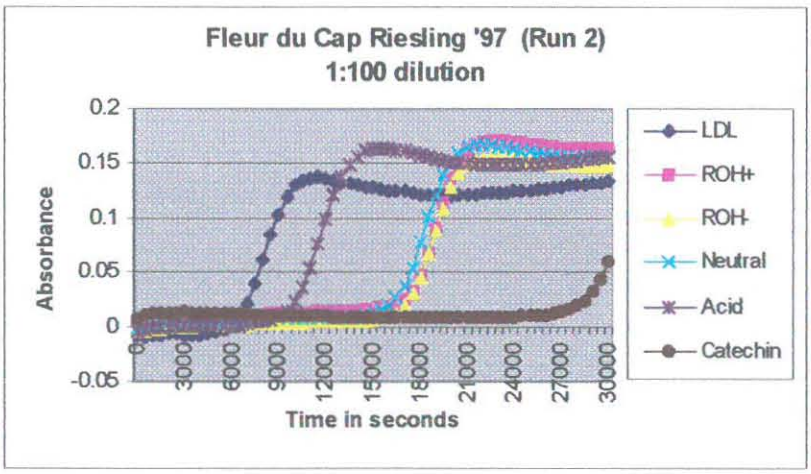


FIGURE APPENDIX F55. WINE 10. FLEUR DU CAP RIESLING '97

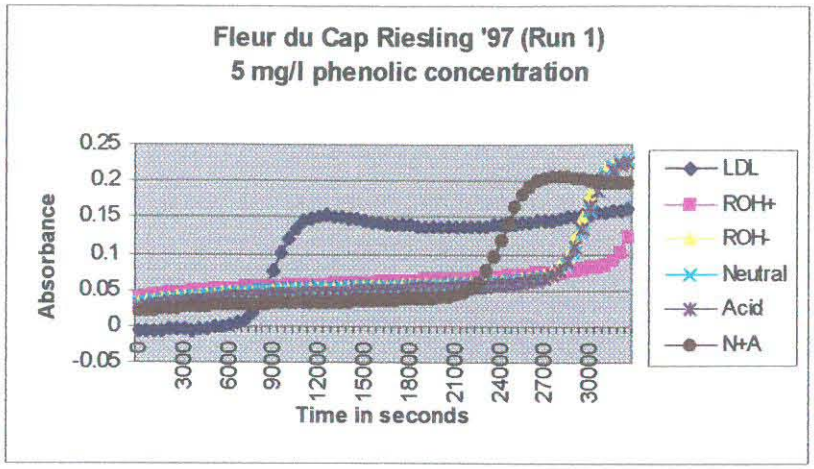


FIGURE APPENDIX F56. WINE 10. FLEUR DU CAP RIESLING '97

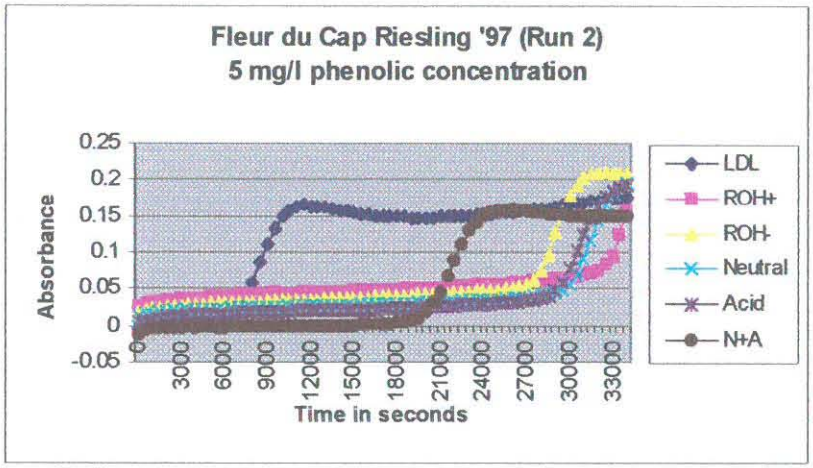


FIGURE APPENDIX F57. WINE 10. FLEUR DU CAP RIESLING '97

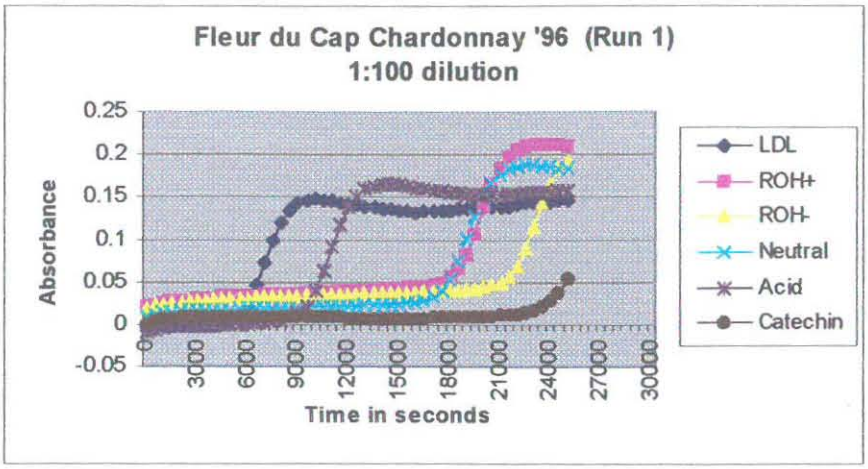


FIGURE APPENDIX F58. WINE 11. FLEUR DU CAP CHARDONNAY '96

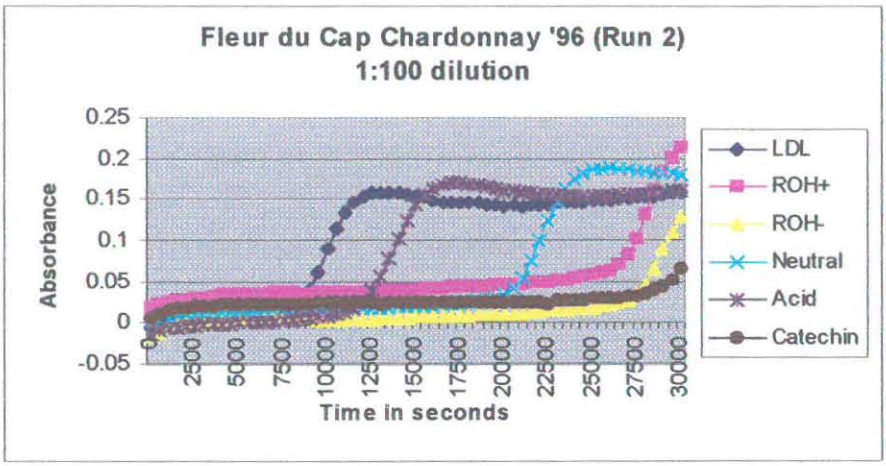


FIGURE APPENDIX F59. WINE 11. FLEUR DU CAP CHARDONNAY '96

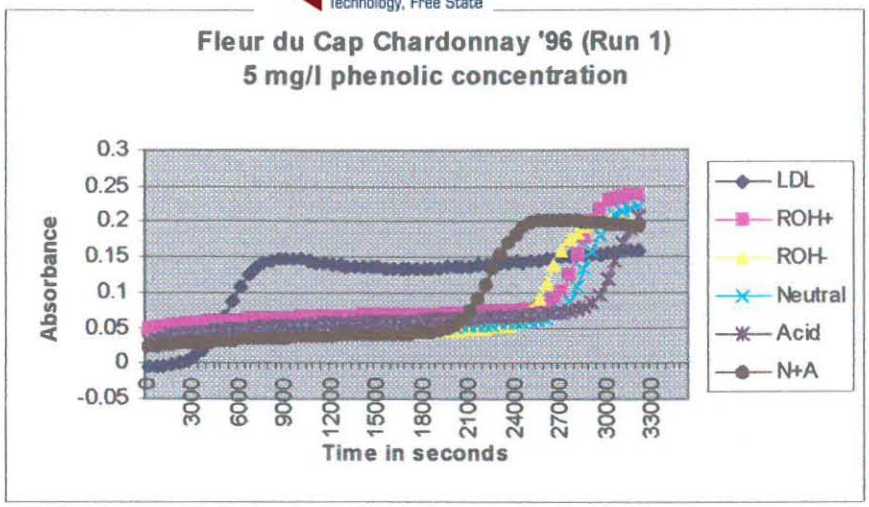


FIGURE APPENDIX F60. WINE 11. FLEUR DU CAP CHARDONNAY '96

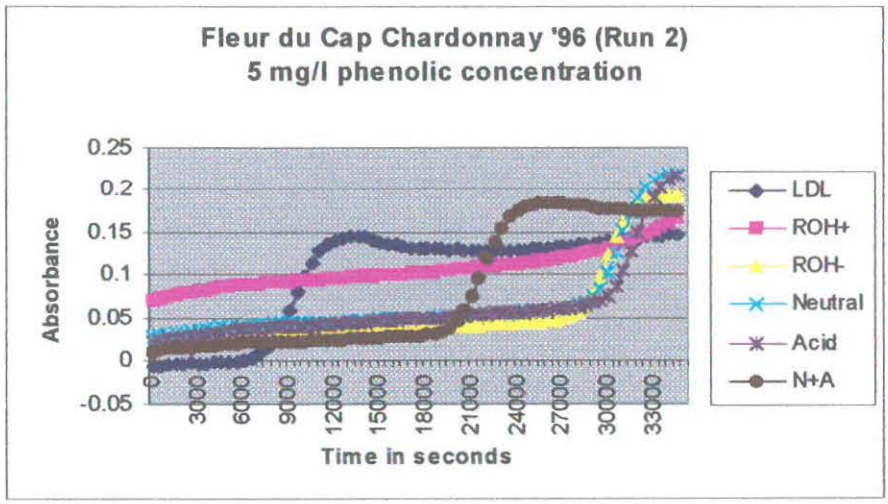


FIGURE APPENDIX F61. WINE 11. FLEUR DU CAP CHARDONNAY '96

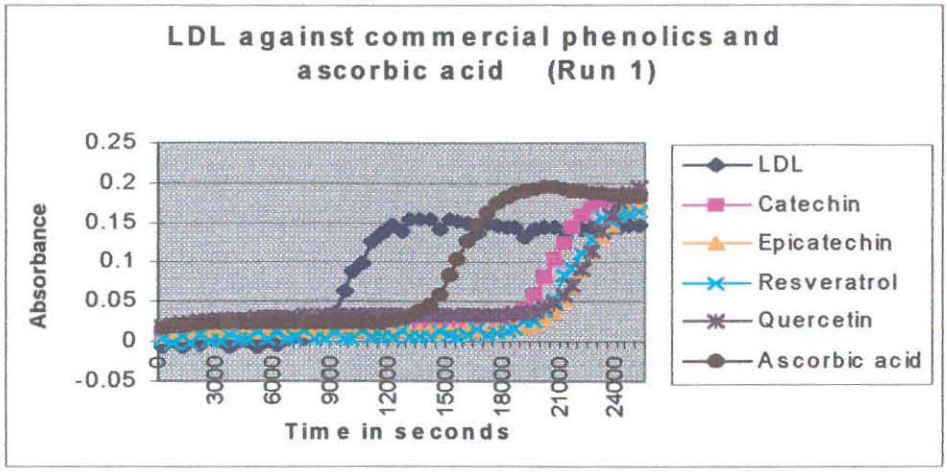


FIGURE APPENDIX F89. ASCORBIC ACID PLUS COMMERCIAL PHENOLICS

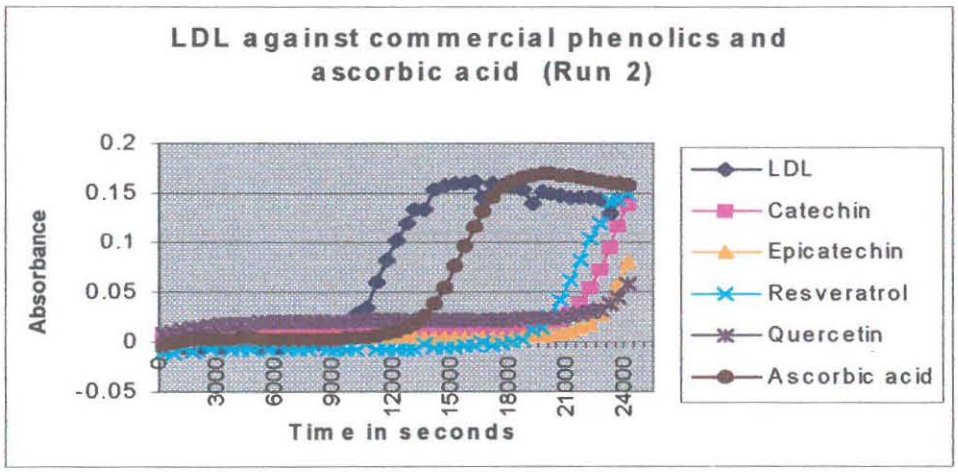


FIGURE APPENDIX F90. ASCORBIC ACID PLUS COMMERCIAL PHENOLICS