THE ANTIOXIDANT ACTIVITY OF SOUTH AFRICAN WINES IN DIFFERENT TEST SYSTEMS AS AFFECTED BY CULTIVAR AND AGEING

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DECLARATION

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

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Dalene de Beer Date

ABSTRACT

Phenolic compounds in wine, due to their antioxidant activity, are reportedly responsible for the health-promoting properties of wines. The effect of cultivar and in-bottle ageing on the antioxidant activity of South African wines in different types of antioxidant assays was, therefore, investigated.

The antioxidant activity of commercial South African red (Cabernet Sauvignon, Ruby Cabernet, Pinotage, Shiraz, Merlot) and white (Sauvignon blanc, Chenin blanc, Chardonnay, Colombard) cultivar wines was compared using the 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) radical cation ($ABTS^{\bullet+}$) scavenging, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging and microsomal lipid peroxidation (MLP) assays. The red wines was more effective than the white wines on an "as-is" and an equal total phenol content. The total antioxidant activity (TAA_{ABTS} and TAA_{DPPH}) of Ruby Cabernet was the lowest of the red wines, but the antioxidant potency (AP_{ABTS} and AP_{DPPH}) of red wine phenolic fractions did not differ ($P \geq 0.05$). Ruby Cabernet and Pinotage were the least effective inhibitors of MLP, while Merlot was the most effective of the red wines. Pinotage phenolic fractions had lower ($P < 0.05$) AP_{MLP} than that of other red wines. Of the white wines, Chardonnay and Chenin blanc had the highest and lowest effectivity respectively according to all antioxidant parameters. Ascorbic acid present in some wines increased and decreased their TAA and % MLP inhibition respectively. TAA and % MLP inhibition correlated well ($r \geq 0.7$, $P < 0.001$) with total phenol content of red and white wines, as well as with flavanol content of red wines and tartaric acid ester content of white wines. The % MLP inhibition also correlated well with flavanol content of white wines. No correlation ($P > 0.01$) was obtained between TAA or % MLP inhibition and monomeric anthocyanin content of red wines. In the deoxyribose assay, red wines were more pro-oxidant and exhibited lower hydroxyl radical scavenging and metal chelating abilities than white wines.

The effect of in-bottle ageing on antioxidant activity of wines was determined using the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays. The TAA and total phenol content of experimental red (Pinotage and Cabernet Sauvignon)

and white (Chardonnay and Chenin blanc) cultivar wines, decreased ($P < 0.05$) during 12 months of storage at 0, 15 and 30 ºC. The TAA_{ABTS} of Cabernet Sauvignon and Chardonnay, stored at 30 ºC were lower ($P < 0.05$) than at 0 ºC. The AP_{ABTS} and AP_{DPPH} of most wines also decreased during storage. The monomeric anthocyanin content of red wines decreased ($P < 0.05$) rapidly at 15 and 30 ºC. The flavanol content of wines (except Chenin blanc) increased during the first 9 months, decreasing again after 12 months, while minor changes in the flavonol and tartaric acid ester content of both red and white wines were observed. The TAA_{ABTS} exhibited a good correlation ($r \geq 0.7$, $P < 0.001$) with total phenol content of red and white wines, as well as with flavonol and tartaric acid ester content of red and white wines and flavanol content of white wines. The monomeric anthocyanin content of red wines correlated ($r = 0.50$, $P < 0.001$) weakly with TAA_{ABTS}. The decrease in the TAA_{ABTS} of wines could thus be mainly attributed to a decrease in their total phenol content.

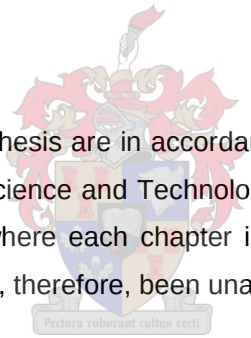
UITTREKSEL

Die antioksidant aktiwiteit van fenoliese komponente in wyn is waarskynlik verantwoordelik vir die gesondheidsvoordele daarvan. Die studie het dus gepoog om effek van kultivar en veroudering na bottelering op die antioksidant aktiwiteit van Suid-Afrikaanse wyne te ondersoek.

Die antioksidant aktiwiteit van kommersiële Suid-Afrikaanse rooi (Cabernet Sauvignon, Ruby Cabernet, Pinotage, Shiraz, Merlot) en wit (Sauvignon blanc, Chenin blanc, Chardonnay, Colombard) kultivarwyne is vergelyk deur middel van die 2,2'-azino-di-(3-etielbensotialosien-sulfoon suur)-radikaal katioon ($ABTS^{\bullet+}$) vernietigingstoets, 2,2-difeniel-1-pikriel-hidrasielradikaal ($DPPH^{\bullet}$) vernietigingstoets en mikrosomale lipied-peroksidasietoets (MLP). Die antioksidant aktiwiteit en die antioksidant kragtigheid (AK) van die rooiwyne was beter as dié van witwyne in al drie antioksidant toetse. Die totale antioksidant aktiwiteit (TAA_{ABTS} en TAA_{DPPH}) van Ruby Cabernet was die laagste van die rooiwyne, terwyl die AK_{ABTS} en AK_{DPPH} van rooiwyn fenoliese fraksies nie van mekaar verskil ($P \geq 0.05$) het nie. Van die rooiwyne, het Ruby Cabernet en Pinotage die laagste en Merlot die hoogste effektiwiteit in die MLP toets getoon. Die AK_{MLP} van Pinotage se fenoliese fraksies was die laagste van die rooiwyne. Die witwyne, Chardonnay en Chenin blanc, het onderskeidelik die beste en swakste antioksidant aktiwiteit en AK van die witwyne getoon in al drie antioksidant toetse. Askorbiensuur wat in sommige witwyne voorgekom het, het die TAA van hierdie wyne verhoog, maar hul % MLP inhibisie verlaag. Die TAA en % MLP inhibisie het goed gekorreleer ($r \geq 0.7$, $P < 0.001$) met die totale fenolinhoud van rooi- en witwyne, asook die flavanolinhoud van rooiwyne en die wynsteensuur-esterinhoud van witwyne. Die % MLP inhibisie het ook goed gekorreleer met die flavanolinhoud van witwyne. Geen korrelasie ($P > 0.1$) is waargeneem tussen antioksidant aktiwiteit van rooiwyne en hul monomeriese antosianien-inhoud. Rooiwyn was meer pro-oksidatief in die deoksieribose toets as witwyne, maar was die swakste hidroksieradikaal-vernietigers en metaalcheleerders.

Die effek van veroudering na bottelering op die antioksidant aktiwiteit van wyne soos bepaal met die $ABTS^{\bullet+}$ en $DPPH^{\bullet}$ vernietigingstoetse, is ondersoek. Die TAA en die totale fenolinhoud van eksperimentele rooi- (Pinotage en Cabernet Sauvignon) en witwyne (Chardonnay en Chenin blanc) het afgeneem ($P < 0.05$) tydens opberging na bottelering by 0, 15 en 30 ºC oor 12 maande. Opberging by 30 ºC het 'n groter vermindering ($P < 0.05$) in die TAA_{ABTS} waarde vir Cabernet Sauvignon en Chardonnay veroorsaak as by 0 ºC. Die meeste wyne se AP_{ABTS} en AP_{DPPH} waardes het ook verminder ($P < 0.05$) na 12 maande. Drastiese vermindering ($P < 0.05$) in die monomeriese antosianieninhoud van rooiwyne is opgemerk tydens opberging by 15 en 30 ºC. Tydens die eerste 9 maande se opberging het die flavanolinhoud van wyne toegeneem ($P < 0.05$) en daarna afgeneem ($P < 0.05$) tot by 12 maande, terwyl flavonol- en wynsteensuuresterinhoud van beide rooi- en witwyne min verandering ondergaan het. Die totale fenolinhoud van rooi- en witwyne, asook die flavonol en wynsteensuur-esterinhoud van rooi-en witwyne en die flavanolinhoud van witwyne, het goed gekorreleer ($r \geq 0.7$, $P < 0.001$) met die TAA_{ABTS}. In teenstelling met die resultate vir kommersiële kultivarwyne, was die TAA_{ABTS} van rooiwyne swak gekorreleer ($r = 0.5$, $P < 0.001$) met hul monomeriese antosianieninhoud. Die afname in TAA_{ABTS} van wyne tydens veroudering kon dus meestal toegeskryf word aan die afname in hul totale fenolinhoud.

CONTENTS



The language and style in this thesis are in accordance with the requirements of the International Journal of Food Science and Technology. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

List of abbreviations

$A^{\bullet}$	radical formed from antioxidant molecule
AH	antioxidant molecule
ABAP	azinobis-(2-amidinopropane)
$ABTS^{\bullet+}$	2,2'-azino-di-(3-ethylbenzthialozinesulphonic acid) radical cation
AP	antioxidant potency
BHT	butylated hydroxytoluene
CAE	caffeic acid equivalents
CE	catechin equivalents
DAC	4-dimethylaminocinnamaldehyde
$DMPD^{\bullet}$	*N,N*-dimethyl-*p*-phenylenediamine dihydrochloride radical
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
DR	deoxyribose
EC_{50}	concentration of antioxidant needed to scavenge 50% of DPPH radicals
EDTA	ethylenediaminetetraacetic acid
F-C	Folin-Ciocalteau
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalents
H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography
IC_{50}	concentration of antioxidant needed to inhibit lipid peroxidation by 50%
LDL	low-density lipoprotein
MDA	malonaldehyde
MLP	microsomal lipid peroxidation
Mv-3-glc	malvidin-3-glucoside
$^{\bullet}NO$	nitric oxide radical
$O_2^{\bullet-}$	superoxide anion radical
$^{\bullet}OH$	hydroxyl radical
$ONOO^-$	peroxynitrite
ORAC	oxygen-radical absorbance capacity

PUFA	polyunsaturated fatty acid
QE	quercetin equivalents
$R^{\bullet}$	alkyl radical
RH	polyunsaturated fatty acid
$RO^{\bullet}$	alkoxyl radical
ROH	alcohol
$ROO^{\bullet}$	peroxyl radical
ROOH	fatty acid hydroperoxide
ROS	reactive oxygen species
RSE	Radical Scavenging Efficiency
TAA	total antioxidant activity
TAS	total antioxidant status
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity

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CHAPTER 1

INTRODUCTION

The detrimental effects of free radicals in the form of reactive oxygen species on biologically important molecules are implicated in the pathogenesis of human diseases such as cancer, atherosclerosis, ischemic heart disease and neurodegenerative diseases (Halliwell & Gutteridge, 1990; Shahidi & Wanasundara, 1992; Leake, 1998). Polyunsaturated fatty acids in cellular structures such as liposomes, erythrocytes and cell membranes are subject to oxidation by reactive oxygen species in the body (O'Brien, 1987; Luc & Fruchart, 1991; Frei, 1995). Free radicals are generated in biological systems through endogenous metabolic processes, as well as by food components, drugs, UV radiation and pollution (Halliwell & Gutteridge, 1990). Protection against oxidative damage by reactive oxygen species is afforded by endogenous antioxidants and those obtained from fruits, vegetables, and beverages such as wine and tea (Kinsella *et al*., 1993).

The protective role of plant antioxidants such as phenolic compounds, carotenoids and ascorbic acid, *in vivo* has not been studied extensively, but some evidence exists that they are absorbed from sources such as fruit, vegetables and beverages such as tea (Hollman *et al*., 1995; Manach *et al*., 1998; Miyazawa *et al*., 1999; Richelle *et al*., 1999) and wine (Lapidot *et al*., 1998). Intake of phenolic substances from red wine and other sources has been shown to enhance the antioxidant status of human plasma serum after oral ingestion or intravenous application (Cao *et al*., 1998; Duthie *et al*., 1998; Prior & Cao, 1999).

In recent years, considerable scientific and commercial interest has developed concerning the antioxidant activity of wine and specifically its phenolic compounds (Frankel *et al.*, 1995; Campos & Lissi, 1996; Simonetti *et al.*, 1997; Ghiselli *et al.*, 1998; Fogliano *et al*., 1999; Saint-Cricq de Gaulejac *et al*., 1999). The relatively low level of coronary heart disease, despite the high intake of saturated fat observed in the French population (the "French Paradox"), is believed to be related to the consumption of red wine (Renaud &

De Lorgeril, 1992). The protective effect of wine towards coronary heart disease seems to be largely due to ethanol (Renaud & De Lorgeril, 1992; Gurr, 1996), but the role of its phenolic components is also of great importance (Gorinstein *et al*., 1998; Nigdikar *et al*., 1998). Important phenolic compounds in wine include anthocyanins, flavonols, flavanols, hydroxybenzoic acids, hydroxycinnamic acids and hydroxystilbenes such as resveratrol (Macheix *et al*., 1990). The phenolic compounds in wine exhibit a broad spectrum of beneficial pharmacological properties believed to be related to their antioxidative properties (Kinsella *et al*., 1993). Properties such as anti-atherogenic activity (Renaud & De Lorgeril, 1992; Kinsella *et al*., 1993), anti-tumour activity (Clifford *et al*., 1996), anti-ulcer activity (Saito *et al*., 1998), regulation of platelet aggregation (Ghiselli *et al*., 1998; Keevil *et al*., 2000) and anti-inflammatory activity (Tomera, 1999) have been demonstrated. Phenolic compounds in wine could also have a regenerating effect on endogenous antioxidants in biological systems by reducing the oxidised forms of α-tocopherol and ascorbate *in vivo* (Kinsella *et al*., 1993; Facino *et al*., 1998; Pryor, 2000).

The focus of the South African wine industry is mainly on white grape cultivars due to the large amounts of white wine produced for brandy production. However, the amount of red wine produced has increased from 12.6% of the total wine production in 1993 to 21.0% in 2000 (Anonymous, 2001). Consumption of wine in South Africa was 9.5 L per capita in 1998, which is approximately one sixth of the per capita consumption of wine in France (Anonymous, 2001). The market for South African wines abroad has increased dramatically since 1994, although South Africa's production is still only 3.2% of the total world production (Anonymous, 2001). In this regard, the cultivation of the unique South African cultivar, Pinotage, is continuously increasing (Anonymous, 2001).

Red wine is proposed to be considered in nutritional recommendations as it could contribute to increased antioxidant intake (Ursini *et al*., 1999). The implication of this for the wine industry is the development of niche markets where the antioxidant content or antioxidant potential of wines may be major factors in determining the acceptability and marketability of wines. South

African wines could be expected to have different antioxidative properties to those produced elsewhere as the antioxidant activity depends on the specific polyphenol content (Frankel *et al*., 1995; Meyer *et al*., 1997; Soleas *et al*., 1997). The latter is determined by factors such as the phenolic composition of the grapes used, vineyard practices, terroir, vinification techniques, wood ageing and in-bottle ageing (Macheix *et al*., 1990).

Most studies in France, the United States of America, Spain and Italy have only made a broad distinction between the antioxidant properties of red and white wine without considering cultivar (Frankel *et al*., 1995; Simonetti *et al.*, 1997; Fogliano *et al.*, 1999). In some cases only red wine have been considered (Saint-Cricq de Gaulejac *et al*., 1999; Pellegrini *et al*., 2000). Due to its much lower total phenol content, white wine has a lower antioxidant activity (Simonetti *et al*., 1997). Some researchers have reported that the phenolic compounds predominantly found in white wine, namely flavonols, are more effective against low-density lipoprotein (LDL) oxidation than those found in red wine (Vinson & Hontz, 1995; Hurtado *et al*., 1997). Furthermore, the correlation between antioxidant activity and the content of different classes of polyphenolic compounds has only recently been considered (Kroyer & Krauze, 1995; Gardner *et al*., 1999; Kondo *et al*., 1999; Saint-Cricq de Gaulejac *et al*., 1999; Burns *et al*., 2000).

The effect of ageing on antioxidant activity has only been addressed in a few studies (Larrauri *et al*., 1999; Manzocco *et al*., 1998). The in-bottle ageing of red wines in particular have a great impact on their sensory quality and acceptability. However, decreases in the content of some groups of phenolic compounds can occur during the ageing process, which impacts on the antioxidant properties of such wines (Nagel & Wulf, 1979).

The objective of this study was to investigate the antioxidant activity of South African red and white wines and determine the effect of cultivar, phenolic composition and in-bottle ageing. A selection of red (Pinotage, Cabernet Sauvignon, Merlot, Shiraz and Ruby Cabernet) and white (Chenin Blanc, Chardonnay, Sauvignon Blanc and Colombar) wines from the major South African wine grape cultivars was screened for antioxidant activity using various methods to allow comparison with international studies on wine. In order to evaluate the antioxidant properties of South African wines, a series of

assays was used to quantify their radical scavenging ability, metal chelating ability, pro-oxidant activity, as well as their ability to inhibit oxidation in a biological membrane system. Accelerated and normal storage conditions were used to evaluate the effect of ageing on the antioxidant properties of two red (Cabernet Sauvignon and Pinotage) and two white wines (Chenin Blanc and Chardonnay).

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CHAPTER 2

PHENOLIC COMPOUNDS: A REVIEW OF THEIR POSSIBLE ROLE AS *IN VIVO* ANTIOXIDANT COMPONENTS OF WINE

2.1 INTRODUCTION

Chronic diseases such as atherosclerosis and cancer, which are the leading causes of death in the Western world, are likely to be mediated by free radical and lipid peroxidation mechanisms (Halliwell & Gutteridge, 1990). Plant phenolic components, such as those occurring in wine, could protect susceptible populations from degenerative diseases involving oxidative damage due to their antioxidant action (Frei, 1995). A possible illustration of such a scenario is the relatively low incidence of coronary heart disease in the French population. This phenomenon, normally referred to as the French Paradox, is related to the consumption of red wine (Renaud & De Lorgeril, 1992).

This review will discuss the principles of oxidative stress and the resultant cellular damage caused by lipid peroxidation, an important factor in the genesis of chronic disease conditions. Different groups of phenolic compounds will be detailed with specific reference to their antioxidant activity in different model systems and their possible protective action against free radicals. The activity of different compounds in these model systems will also be related to their chemical structures. A summary of information available about the absorption and bioavailability of phenolic compounds from dietary sources will be discussed. The phenolic compounds present in wine and factors influencing their content such as the phenolic composition of the grapes, vinification processes and maturation, will finally be discussed.

2.2 REACTIVE OXYGEN SPECIES AND FREE RADICALS

Oxidative reactions within the cell are tightly controlled and protective mechanisms are in place to destroy oxidant by-products of normal cell metabolism. Oxidative stress occurs when oxidant by-products of metabolism and other free radicals overcome the antioxidant defence system (Davies, 1995). During certain pathophysiological states or when antioxidant deficiencies occur and normal control mechanisms are not sufficient, oxidant by-products of normal metabolism can cause damage to DNA, proteins and lipids. These adverse effects appear to play a major role during ageing and degenerative diseases such as cancer, cardiovascular disease, cataract formation, immune system defects and brain dysfunction (Cutler, 1991; Hertog *et al*., 1995; Keli *et al*., 1996).

Four endogenous sources accounts for the oxidant by-products in cells, namely mitochondrial energy production, activities of phagocytic cells, peroxisomal fatty acid metabolism and the activities of certain metabolic enzymes (Frei, 1994). Exogenous sources, such as excess dietary iron or copper and cigarette smoke also contributes to oxidative stress (Frei, 1994).

Significant amounts of superoxide anion radical ($O_2^{\bullet-}$) together with other reactive oxygen species (ROS) are produced in the body by phagocytes (Halliwell & Gutteridge, 1990). Under normal conditions these ROS are accurately directed against invading micro-organisms, but when chronic inflammation occurs the surrounding tissues are exposed to these harmful ROS (Davies, 1995). Mitochondria produce $O_2^{\bullet-}$ and other ROS by reducing molecular oxygen during the production of energy necessary for cellular processes (Halliwell & Gutteridge, 1990). A number of enzymes including xanthine oxidase, aldehyde oxidase, NADPH-oxidase and cytochrome P-450 enzymes also contribute to $O_2^{\bullet-}$ production (Kanner *et al*., 1987; Halliwell & Gutteridge, 1990). The oxidation of reduced metals such as Fe^{2+} by oxygen in Fenton-type reactions also contributes to $O_2^{\bullet-}$ production (Aust *et al*., 1990).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet} \qquad [1]$$

The reaction rate of $O_2^{\bullet-}$ in aqueous solution is relatively low (Halliwell & Gutteridge, 1990). Superoxide anion radicals can, however, be transformed to more reactive species by superoxide dismutase, as well as during reaction with iron. Superoxide dismutase is an endogenous enzyme catalysing the dismutation of superoxide to hydrogen peroxide (H_2O_2) (Halliwell & Gutteridge, 1990). The superoxide anion radical can also play a role in the production of $^{\bullet}OH$ during Haber-Weiss reactions by reducing transition metal ions as explained in the following reactions (Frei, 1994):

$$M^{(n+1)+} + O_2^{\bullet-} \rightarrow M^{n+} + O_2 \qquad [2]$$

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO^- + {}^{\bullet}OH \qquad [3]$$

The role of H_2O_2 and hydroxyl radicals in the initiation of lipid peroxidation is very important. Several organelles such as mitochondria, microsomes and peroxisomes, as well as cytosolic enzymes, are H_2O_2 generators (Boveris *et al*., 1972). The level of H_2O_2 in cells is controlled by superoxide dismutase and glutathione peroxidase. H_2O_2 is not a very strong oxidant, but can be converted to a potent oxidising compound, namely the hydroxyl radical, by a one-electron reduction.

Another important ROS is the nitric oxide radical ($^{\bullet}NO$). This radical species plays a central role in the regulation of vascular homeostasis (Rubbo *et al*., 1995). Synthesis of $^{\bullet}NO$ occurs during oxidation of L-arginine by nitric oxide synthases in the endothelial cells of the vascular wall. Reaction of $^{\bullet}NO$ with $O_2^{\bullet-}$ yields another ROS, namely peroxynitrite ($ONOO^-$). The most important role of $^{\bullet}NO$ *in vivo* is the promotion of vascular relaxation, inhibition of platelet aggregation, inhibition of platelet and leukocyte adhesion to the vessel wall, as well as the inhibition of smooth-muscle cell proliferation (Rubbo *et al*., 1995). Peroxynitrite and $^{\bullet}NO$ can, however, also have negative *in vivo* effects as these free radical species can participate in lipid peroxidation and damage to other biomolecules (Rubbo *et al*., 1995).

2.3 LIPID PEROXIDATION

Lipid peroxidation is an autoxidation process with detrimental effects occurring in foods and the cells of the body. In foods, it can lead to rancidity and loss of nutritional value. In the cell, however, it can affect membrane structure, function, and cause damage to biologically important molecules such as DNA and proteins resulting in chronic diseases such as atherosclerosis and cancer (Cutler, 1991; Hertog *et al*., 1995; Keli *et al*., 1996). Peroxidation of lipids in foods mostly occurs enzymatically, but peroxidation of lipids in the cell is generally initiated by ROS (Kanner *et al*., 1987). These ROS are produced as a result of oxidative reactions required to obtain energy for normal metabolic processes within the cell. Endogenous antioxidants are present to protect against excessive levels of free radicals produced by the metabolism of oxygen (Halliwell, 1995).

2.3.1 Free radical chain reaction mechanism

Autoxidation is the spontaneous reaction between atmospheric oxygen and organic compounds. This process generally follows an autocatalytic free radical chain reaction mechanism. Metal ions and light are generally pro-oxidative, while a variety of natural and synthetic compounds can act as antioxidants (Halliwell & Chirico, 1993).

The overall reaction is the addition of oxygen to an organic compound. Three distinct steps can be distinguished in the free radical chain reaction, namely initiation, propagation and termination (Chan, 1987; Shahidi & Wanasundara, 1992):

Initiation: $X^{\bullet} + RH \rightarrow R^{\bullet} + XH$ [4]

Propagation: $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$ [5]

$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$ [6]

Termination: $ROO^{\bullet} + ROO^{\bullet} \rightarrow \text{non-radical products}$ [7]

$ROO^{\bullet} + R^{\bullet} \rightarrow \text{non-radical products}$ [8]

$R^{\bullet} + R^{\bullet} \rightarrow \text{non-radical products}$ [9]

where $X^{\bullet}$ = initiating radical species

RH = polyunsaturated fatty acid (PUFA)

ROOH = fatty acid hydroperoxide

$R^{\bullet}$ = alkyl radical

$RO^{\bullet}$ = alkoxyl radical

$ROO^{\bullet}$ = peroxyl radical

Lipid peroxidation is initiated by many mechanisms. The initiating radical, $X^{\bullet}$, abstracting a hydrogen from an polyunsaturated fatty acid (PUFA) [4], can be a transition metal ion, such as Fe^{2+}, Fe^{3+} or Cu^{+}, a radical formed from an azo-initiator, a ROS or a radical generated by photolysis (Chan, 1987). Oxygenation of the alkyl radical formed during initiation, yielding a peroxyl radical, is the first step of the propagation phase [5]. The peroxyl radicals will abstract another hydrogen from a PUFA [6]. The propagation reactions can be repeated indefinitely until the reaction is terminated when radicals combine in addition reactions to form stable non-radical products [7,8,9] (Chan, 1987). Figure 1 illustrates the mechanism of initiation and propagation of the autoxidation of a PUFA, as well as the generation of secondary products of lipid peroxidation.

2.3.2 Reaction kinetics

The different stages of the autoxidation reaction of a PUFA are illustrated in Figure 2. The kinetic behaviour of lipid peroxidation is such that the reaction rate is slow at first and increases as the reaction continues due to the

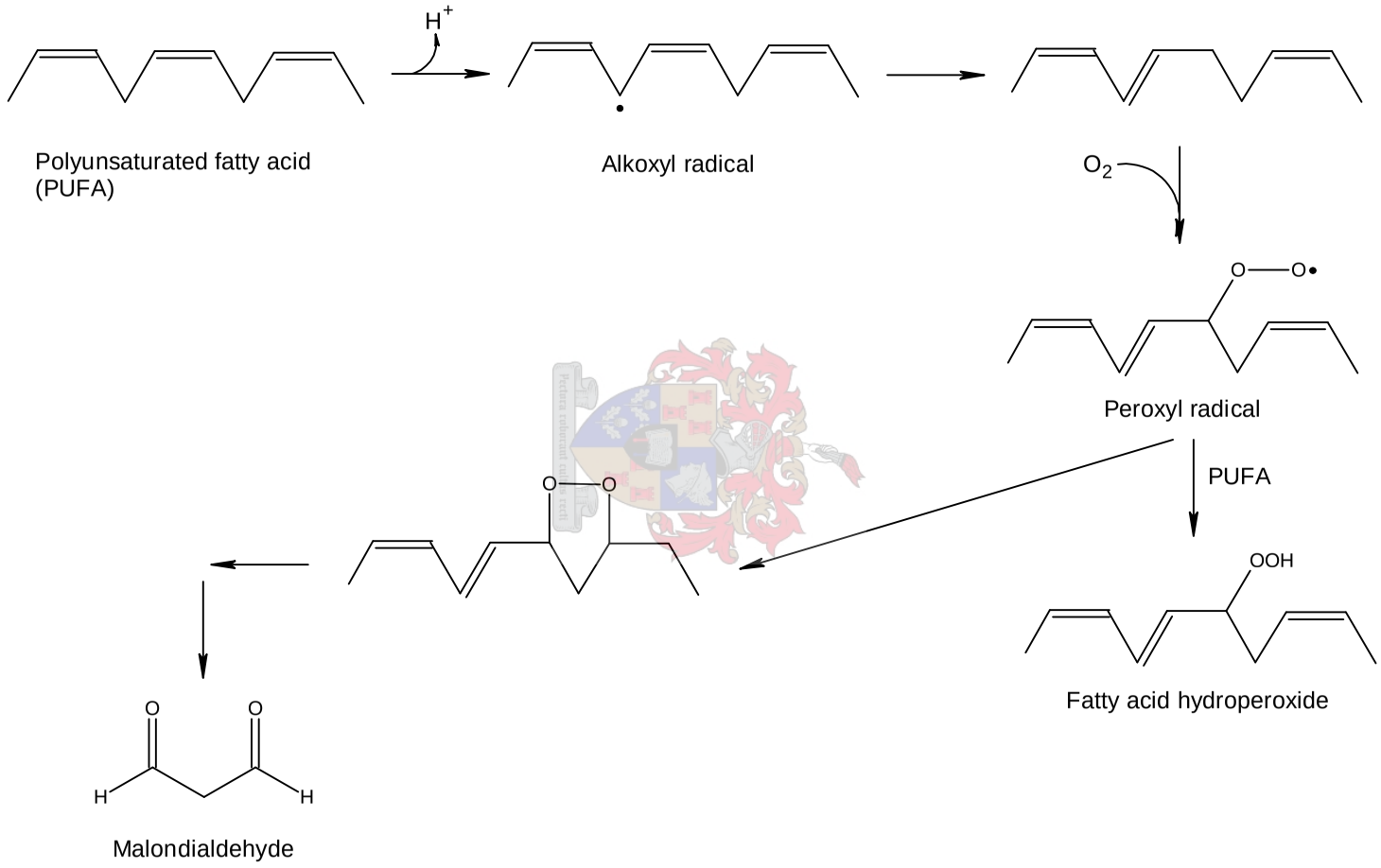


Figure 1 Autoxidation of a polyunsaturated fatty acid (Beuge & Aust, 1978).

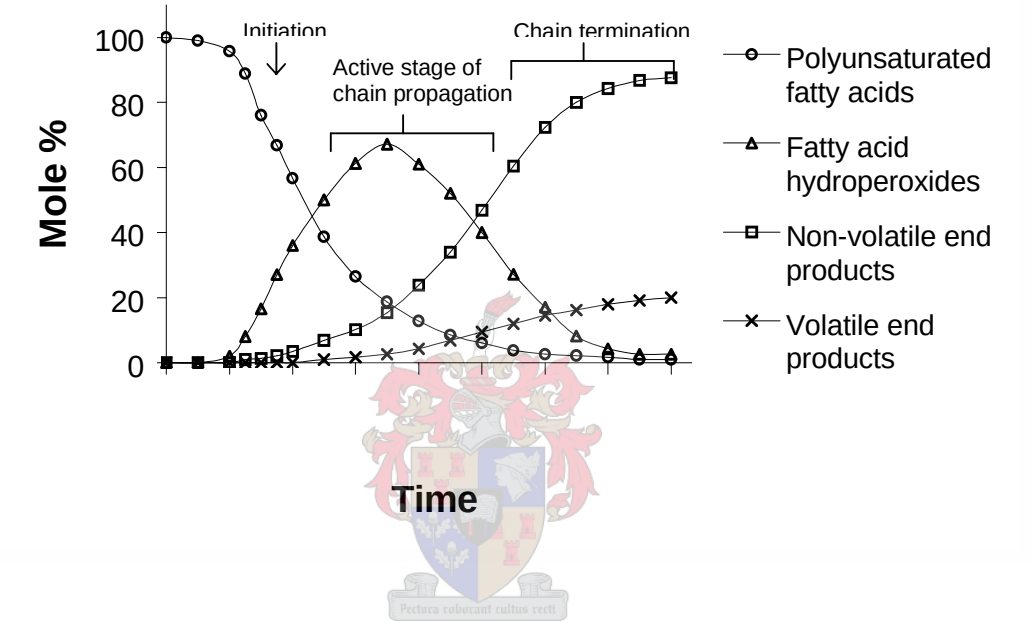


Figure 2 Stages of the autoxidation reaction of a polyunsaturated fatty acid (Gardner, 1987).

autocatalytic nature of the reaction mechanism. Due to the slow initial reaction rate an induction period is generally observed where no reaction products can be detected (Gardner, 1987). The concentration of initiating radicals is generally very small and an overall rate constant for initiation [6], k_i, can be defined:

$$R_i = 2k_i[X^\bullet] \qquad [10]$$

where R_i = overall rate of the initiation reaction [4]
k_i = rate constant for the initiation reaction [4]

Two rate constants can be defined for the reaction during the propagation phase. One for the oxygenation reaction [5], k_o, which is very fast and one for the abstraction of a hydrogen molecule [6], k_p, which is the rate-determining step in the propagation phase (Chan, 1987).

The termination reaction, involving $R^\bullet$, is generally very fast and only the reaction combining two peroxyl radicals is rate-determining with a rate constant, k_t. Applying steady-state kinetics to the reactions as defined above, i.e. assuming that the concentrations of $R^\bullet$ and $ROO^\bullet$ are constant during the reaction and that the partial pressure of O_2 is high enough to disregard termination reactions involving $R^\bullet$, allows the derivation of the following equation (Chan, 1987):

$$\frac{d[R^\bullet]}{dt} = R_i - k_o[R^\bullet][O_2] + k_p[ROO^\bullet][RH] = 0 \qquad [11]$$

For the calculation of the rate of autoxidation the following equations can also be derived from [11]:

$$R_i = k_o[R^\bullet][O_2] + k_p[ROO^\bullet][RH] \qquad [12]$$

$$\frac{d[ROO^\bullet]}{dt} = k_o[R^\bullet][O_2] - k_p[ROO^\bullet][RH] - 2k_t[ROO^\bullet]^2 = 0 \qquad [13]$$

Substituting [12] in [13] it follows that:

$$R_i - 2k_t[ROO^{\bullet}]^2 = 0 \qquad [ROO] = \left(\frac{R_i}{2k_t}\right)^{\frac{1}{2}} \qquad [14]$$

The rate of the autoxidation reaction is, therefore:

$$\frac{d[ROOH]}{dt} = k_p[ROO^{\bullet}][RH] = k_p[RH]\left(\frac{R_i}{2k_t}\right)^{\frac{1}{2}} \qquad [15]$$

This is a general kinetic equation for a free radical chain reaction containing only one bimolecular termination process. In practice the kinetics involved is usually much more complex. The system involved could consist of an aqueous phase and a lipid phase or a membrane system containing various different kinds of substrates. A complete understanding of the kinetics involved is, therefore, not possible as partition coefficients of antioxidants in the different phases would influence the effectivity of the inhibition of autoxidation by antioxidant molecules (Liao & Yin, 2000).

2.3.3 Initiation of lipid peroxidation

The direct oxygenation of fatty acids are not likely to contribute significantly to initiation of lipid peroxidation due to the endothermic nature of the reaction. Possible routes of initiation includes excitation of triplet oxygen to the singlet state, decomposition of lipid hydroperoxides, enzyme activity or reaction of lipids with free radicals.

The electronic structure of oxygen contains two unpaired electrons in the triplet state. The reaction of oxygen with molecules in the triplet state is, therefore, spin forbidden. Excitation of a triplet oxygen molecule results in the singlet state [17]. Excitation occurs after reaction of triplet oxygen with a molecule sensitised by light [16] (Halliwell & Gutteridge, 1990). In the singlet state oxygen can react with PUFA's via the ene reaction to form lipid hydroperoxides (Kanner *et al*., 1987).

$$^{0}S \xrightarrow{hv} {}^{1}S \rightarrow {}^{3}S \quad [16]$$

$$^{3}S + {}^{3}O_2 \rightarrow {}^{0}S + {}^{1}O_2 \quad [17]$$

where ^{0}S = unactivated sensitiser molecule
^{1}S = sensitiser molecule in singlet state
^{3}S = sensitiser molecule in triplet state
$^{3}O_2$ = oxygen in triplet state
$^{1}O_2$ = oxygen in singlet state

Decomposition of lipid hydroperoxides can occur by several reactions, but the reaction of lipid hydroperoxides with transition metal ions such as iron is the most common as the activation energies are lower and metal ions are present in low concentrations in most biological and other systems (Halliwell & Gutteridge, 1990; Marnett & Wilcox, 1995; Sevanian & Ursini, 2000). Peroxyl and alkoxyl radicals formed in this way can abstract hydrogen to initiate lipid peroxidation.

$$ROOH + Fe^{2+}-complex \rightarrow RO^{\bullet} + OH^{-} + Fe^{3+}-complex \quad [18]$$

$$ROOH + Fe^{3+}-complex \rightarrow ROO^{\bullet} + H^{+} + Fe^{2+}-complex \quad [19]$$

Iron-dependent initiation of lipid peroxidation occurs through complexes of iron with ATP, carbohydrates, DNA, membrane lipids or proteins such as ferritin, hemoglobin, methemoglobin and cytochrome P-450 (Halliwell & Gutteridge, 1990). Fe(II)-complexes stimulate membrane peroxidation more than Fe(III)-complexes. No enzyme activity is necessary for the stimulation of lipid peroxidation with iron.

Enzymes such as cyclooxygenase and lipoxygenase catalyse the controlled peroxidation of fatty acids to produce hydroperoxides and endoperoxides during endogenous metabolism (Halliwell & Gutteridge, 1990). These hydroperoxides can be decomposed, forming peroxyl radicals capable of initiating lipid peroxidation.

Initiation of lipid peroxidation can also occur by direct attack on a double bond of a PUFA by endogenous or exogenous free radicals (Kanner *et al*.,

1987). Endogenous free radicals include ROS such as $O_2^{\bullet-}$, H_2O_2 and hydroxyl radicals. Sources of exogenous free radicals are air pollution and smoking (Davies, 1995).

2.3.4 Products of lipid peroxidation

Lipid peroxidation is believed to play an important role in many conditions of cellular damage due to changes in membrane fluidity, increased permeability of membranes and cytotoxicity of lipid peroxidation products (Halliwell & Gutteridge, 1990). The most common products include hydroperoxides, aldehydes, hydroxy acids, hydroperoxy acids and epoxides (Gardner, 1987).

Hydroperoxides are intermediary products of lipid peroxidation that undergo decomposition by heat or in the presence of iron. A product of decomposition, namely alkoxyl radicals, can undergo β-scission to produce aldehydes (Chan, 1987). Intramolecular reaction of the alkoxyl radicals with double bonds lead to the formation of other secondary products of lipid peroxidation such as epoxides and polyhydroxylated derivatives of fatty acids. Malondialdehyde (MDA), formed as a secondary product of lipid peroxidation (Figure 1), is commonly used as a marker of peroxidation through its reaction with thiobarbituric acid (TBA) (Hoyland & Taylor, 1991; Guillén-Sans & Guzmán-Chozas, 1998). Other markers of lipid peroxidation includes conjugated dienes and lipid hydroperoxides (Figure 1) (Slater, 1984).

2.4 ANTIOXIDANTS

Many definitions exist to describe the term “antioxidant”. Halliwell (1995) defines an antioxidant as any substance that when present at low concentrations relative to those of an oxidisable substrate, significantly delays or prevents the oxidation of that substrate. This definition is especially relevant in biological systems.

Antioxidants can be classified into two groups, namely chain-breaking (primary) antioxidants and preventative (secondary) antioxidants. Chain-breaking antioxidants act by scavenging free radicals and donating hydrogen. Preventative antioxidants are generally metal chelators and

reductants capable of sparing other antioxidants *in vivo*. Other functions of antioxidants include peroxide decomposition, singlet oxygen quenching and enzyme inhibition (Namiki, 1990).

The most common water-soluble antioxidant compounds in plants and foods are the phenolic compounds. These secondary metabolites of plants are characterised by an aromatic ring possessing one or more hydroxyl substituents. The flavonoids, one of the classes of phenolic compounds, contain a C_6-C_3-C_6 flavone skeleton (Figure 3) in which the three-carbon bridge is cyclised with oxygen (Robards *et al*., 1999). These flavonoids can be hydroxylated and methoxylated in various positions. Glycosylation with glucose, galactose, rhamnose, xylose or arabinose on the 3-, 5- and 7-hydroxyl are common (Macheix *et al*., 1990). Different groups can be identified that contains the same basic structure, such as phenolic acids, flavanols, flavonols, anthocyanidins, procyanidins and others. The major phenolic groups will be discussed in the next section.

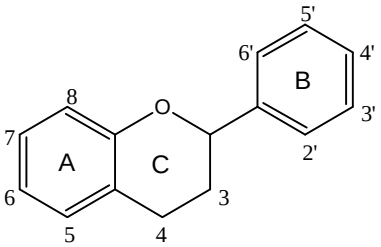
2.4.1 Phenolic acids

The simplest phenolic compounds commonly found in plants are the derivatives of benzoic and cinnamic acids (Figure 3). Hydroxybenzoic acids are gallic acid, ellagic acid, vanillic acid, protocatechuic acid, salicylic, 4-hydroxybenzoic and syringic acid (Macheix *et al*., 1990). Small amounts of quinic acid esters and glucosides of hydroxybenzoic acids have also been found in fruits. The hydroxycinnammic acids, namely *p*-coumaric, caffeic, ferulic and sinapic acids, rarely occur in the free form in fruits. The soluble derivatives have one of the alcoholic groups esterified with an organic acid, glucose or other phenolic compound (Macheix *et al*., 1990). Chlorogenic acid, the quinic acid ester of caffeic acid, is the main phenolic component of potatoes and coffee (Clifford, 2000a).

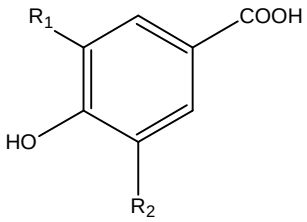
2.4.2 Flavonoids

2.4.2.1 Flavanols

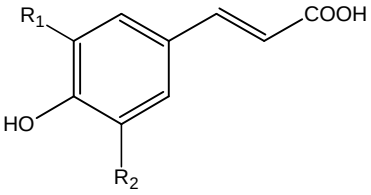
Flavanols (Figure 3) occurs in fruit, wine, tea and chocolate (Arts *et al*., 2000a; Arts *et al*., 2000b). This class of compounds differs from other flavonoids as



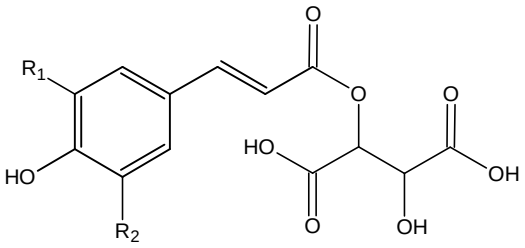
Flavone skeleton



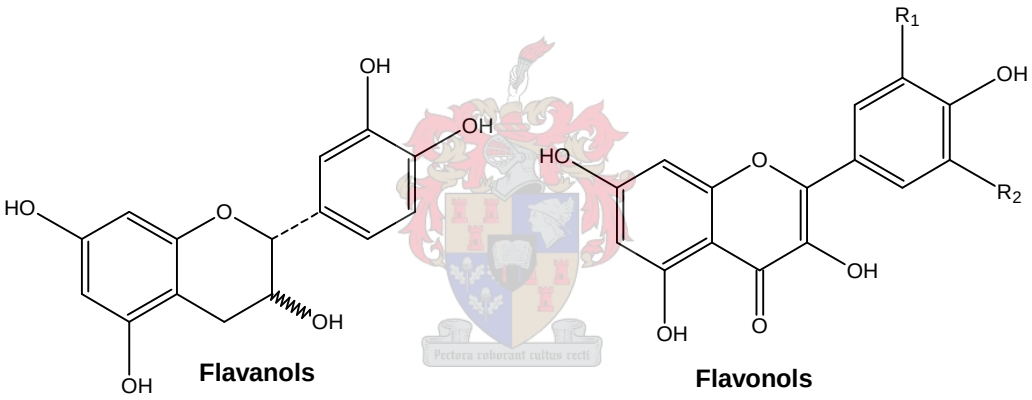
Hydroxybenzoic acids

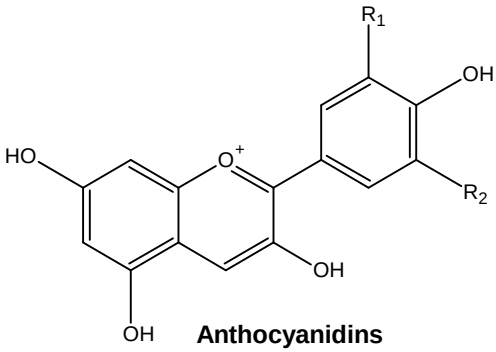


Hydroxycinnamic acids

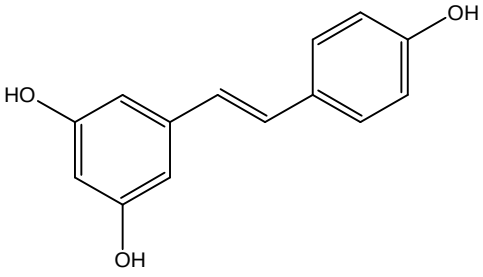


Tartaric acid esters





Anthocyanidins



Stilbenes

R_1 = R_2 = H, OH, O-Me or O-sugar

Figure 3 Structures of some groups of phenolic compounds.

they do not generally occur as glycosides (Macheix *et al*., 1990). Derivatives of the basic flavanols with gallic acid such as (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate and (+)-gallocatechin also occur commonly in tea prepared from *Camellia sinensis* (Balentine *et al*., 1997).

2.4.2.2 Flavonols

Flavonols (Figure 3) occur in fruit and vegetables, as well as in beverages such as wine and tea (Hollman & Arts, 2000). The flavonols generally occur as glycosides with the sugar attached preferably to the 3-position or more rarely to the 7-position. The sugar moiety is usually glucose, although glycosides of galactose, rhamnose, arabinose and xylose are also encountered. The most common flavonols in plants include quercetin, kaempferol, myricetin, isorhamnetin and rutin (the rutinose glycoside of quercetin) (Ribéreau-Gayon, 1972).

2.4.2.3 Anthocyanidins

Anthocyanidins (Figure 3) and anthocyanins (the glycoside derivatives of anthocyanidins) are common in red, blue and purple fruit and flowers (Mazza, 1995). The basic anthocyanidins include cyanidin, malvidin, delphinidin, peonidin and pelargonidin. Glycosylation of anthocyanins, as in the case of flavonols, usually occurs in the 3-position and/or 5-position (Macheix *et al*., 1990). Glycosides of anthocyanidins with glucose are the most common although those with galactose, rhamnose and arabinose have also been reported (Clifford, 2000b). Acylation of anthocyanidins or anthocyanins can occur with cinnamic acids such as caffeic, ferulic, *p*-coumaric and sinapic acids, as well as aliphatic acids such as acetic, malic, malonic, oxalic and succinic acids (Clifford, 2000b).

Variation in the colour of anthocyanin molecules as a function of pH is caused by their ionic nature. At low pH (pH < 1) all the anthocyanin molecules are in the coloured flavylium cation form (Ribéreau-Gayon, 1972), while at higher pH the molecules are mainly in the colourless carbinol pseudobase form or the blue quinoidal anhydrobase form (Figure 4).

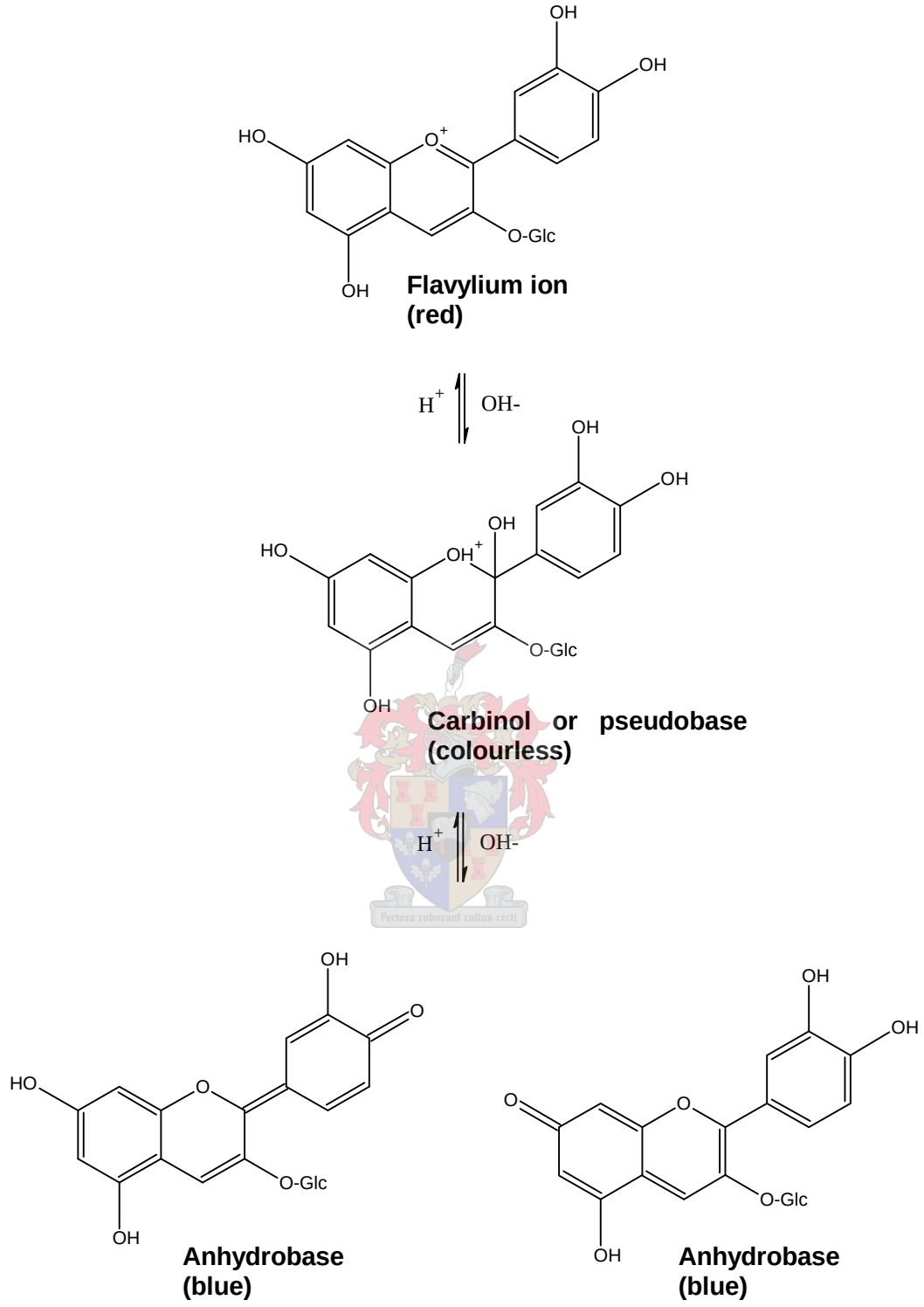


Figure 4 Structural modification of anthocyanins as a function of pH (Ribereau-Gayon, 1972).

2.4.2.4 Proanthocyanidins

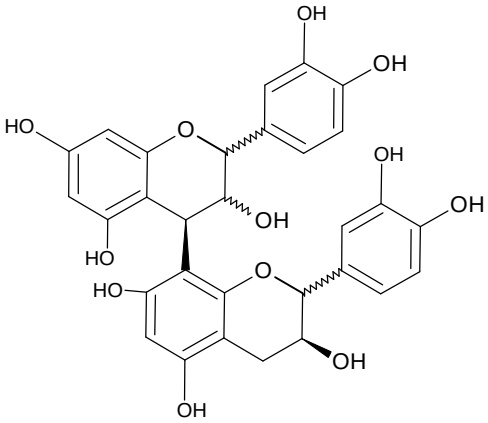
The name of this group of compounds is derived from the fact that these compounds yield anthocyanidins when treated with heat in the presence of a mineral acid (Porter *et al*., 1986). Proanthocyanidins are complex flavonoids naturally present in cereals, legumes, some fruits and beverages such as wine, tea and cocoa (Santos-Buelga & Scalbert, 2000). The structure of this group of compounds is based on flavanol sub-units [(+)-catechin and (-)-epicatechin] linked through the 4- and 8-positions or through the 4- and 6-positions (Haslam, 1996). Procyanidin dimers such as B1, B2, B3, B4 (Figure 5), B5, B6, B7, B8 and A2 occur along with small amounts of trimers such as C1 and C2 and tetramers (Macheix *et al*., 1990).

2.5 MEASUREMENT OF *IN VITRO* ANTIOXIDANT ACTIVITY

The antioxidant activity of a compound depends on the oxidisable substrate available and other factors in the environment such as metal ions, enzymes, temperature and pH (Halliwell, 1995). Different test systems are available to evaluate different antioxidant properties such as free radical scavenging, inhibition of lipid peroxidation and metal chelation. The following section evaluates test systems which will be used for the measurement of antioxidant activity of wine in this study, but the low-density lipoprotein peroxidation assay were also included in the discussion due to the important link between wine consumption and a reduced risk of coronary heart disease.

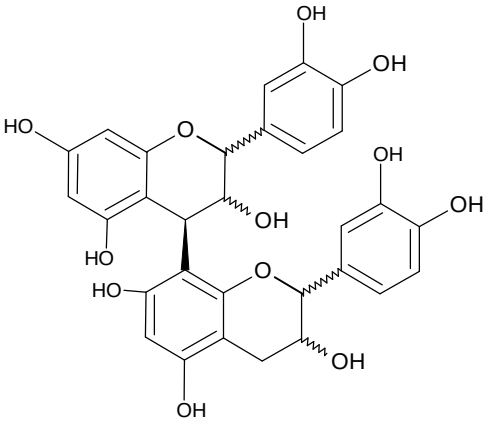
2.5.1 Radical scavenging test systems

Assays measuring free radical scavenging activity can be divided into those involving radicals that are pro-oxidants, i.e. oxidants with biological relevance, such as hydroxyl radicals, peroxyl radicals and $O_2^{\bullet-}$ (Figure 6), and those involving radicals that are not pro-oxidants (mostly synthetic radicals) (Figure 7) (Prior & Cao, 1999). The use of peroxyl radicals, hydroxyl radicals or $O_2^{\bullet-}$ in the oxygen-radical absorbance capacity (ORAC) (Cao *et al*., 1993; Cao & Prior, 1999), total radical-trapping antioxidant parameter (TRAP) (Wayner *et al*., 1985), superoxide scavenging (Robak & Gryglewski, 1988) and deoxyribose (Halliwell *et al*., 1987) assays makes these assays uniquely



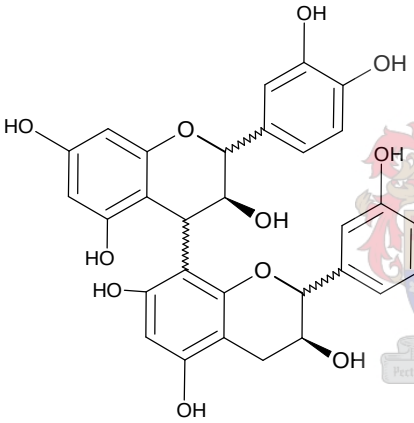
Procyanidin B1

(epicatechin-(4β-8)-catechin)



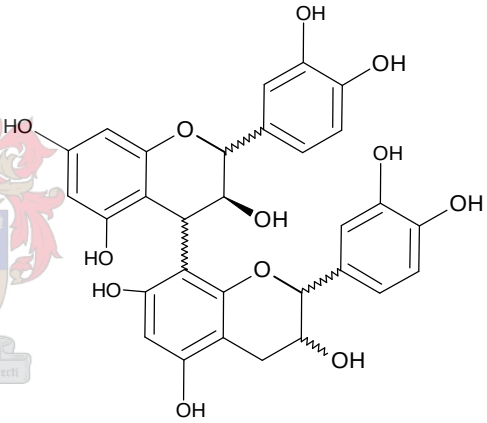
Procyanidin B2

(epicatechin-(4β-8)-epicatechin)



Procyanidin B3

(catechin-(4β-8)-catechin)



Procyanidin B4

(catechin-(4β-8)-epicatechin)

Figure 5 Structures of some procyanidin B-dimers.

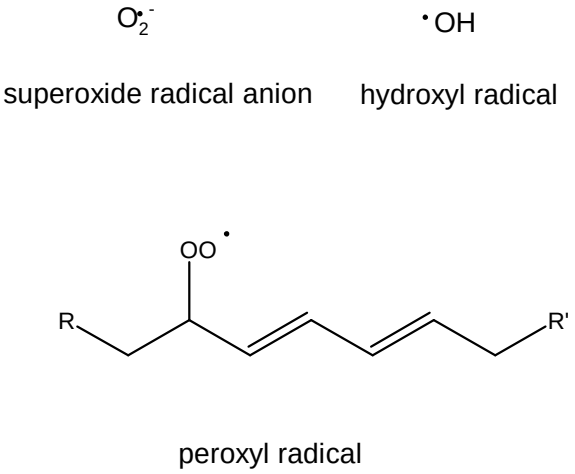


Figure 6 Radicals acting as pro-oxidants in cells.

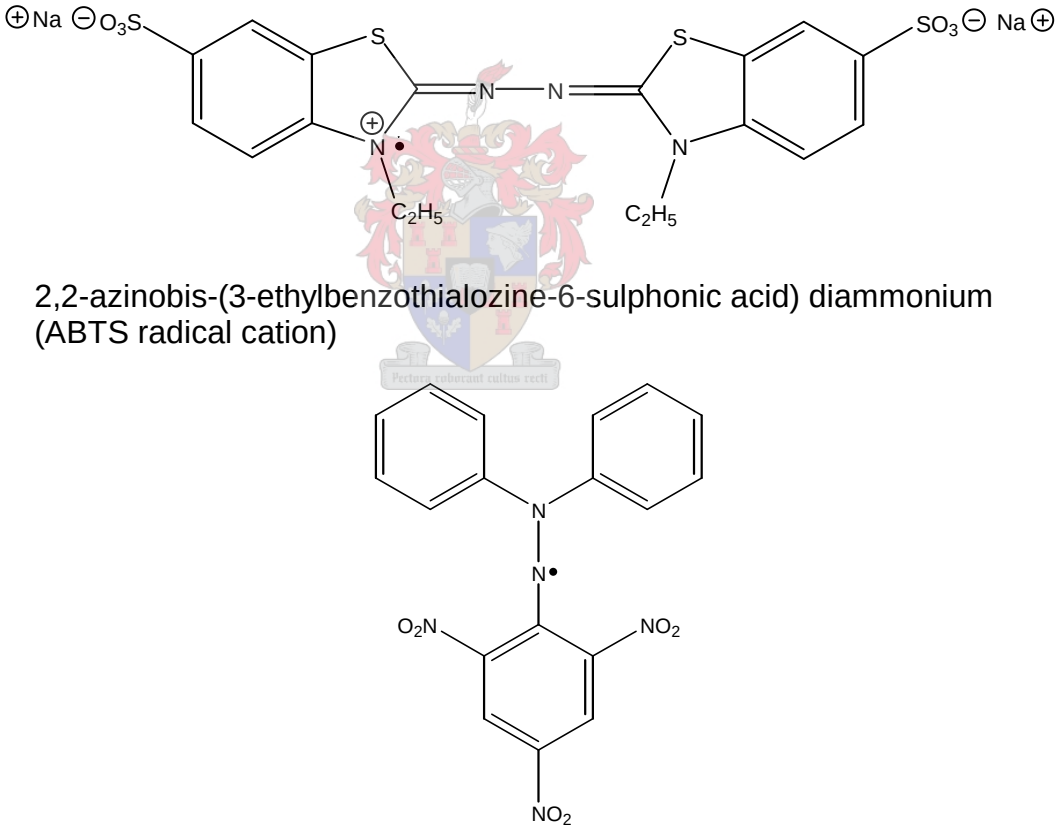


Figure 7 Synthetic radicals used in antioxidant assays.

suited to evaluate the activity of antioxidant mixtures against biologically relevant pro-oxidants (Cao *et al*., 1993). Methods utilising synthetic radicals include the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging (Brand-Williams *et al*., 1995), 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) radical cation ($ABTS^{\bullet+}$) scavenging (Miller *et al*., 1993) and *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride radical ($DMPD^{\bullet}$) scavenging (Fogliano *et al*., 1999) assays. Another assay, the ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996), determine antioxidant activity as a measure of the ability to reduce Fe^{3+}.

Several methods have been developed to measure the total antioxidant activity (TAA) of biological and food samples. These assays include the ABTS radical cation scavenging, FRAP, ORAC and TRAP assays. The synergistic action of antioxidant mixtures such as blood plasma or food makes this approach more significant than measuring the individual antioxidant molecules separately (Prior & Cao, 1999).

2.5.1.1 DPPH radical scavenging assay

The stable radical, $DPPH^{\bullet}$, is used to determine antioxidant activity in a relatively short time (Brand-Williams *et al*., 1995). The $DPPH^{\bullet}$ scavenging activity of an antioxidant gives an indication of hydrogen donating ability of the antioxidant as the major product of the reaction of $DPPH^{\bullet}$ with phenols is DPPH-H (2,2-diphenyl-1-picrylhydrazine) (Hogg *et al*., 1961). The hydrogen donating ability plays a role in the inhibition of lipid peroxidation as lipid radicals can be inactivated in this manner (Chan, 1987).

The DPPH radical has a characteristic absorption at 515 nm, which disappears when a hydrogen atom is donated by an antioxidant molecule. Absorbance can be read at a specific time (Yoshida *et al*., 1989; Chen & Ho, 1997) or at steady state (Brand-Williams *et al*., 1995; Sánchez-Moreno *et al*., 1998). The percentage scavenging of $DPPH^{\bullet}$ relative to a control is determined for a range of concentrations of a specific antioxidant by monitoring the absorption at the steady state.

$$\% \text{ Scavenging} = \frac{Abs_{C(t)} - Abs_{S(t)}}{Abs_{C(t)}} \times 100 \qquad [20]$$

where $Abs_{C(t)}$ = absorbance of control at time t

$Abs_{S(t)}$ = absorbance of sample with antioxidant at time t

An EC_{50} (concentration of antioxidant required to scavenge 50% of the $DPPH^{\bullet}$) can be determined from a plot of percentage scavenging against concentration of antioxidant (Brand-Williams *et al*., 1995). A low EC_{50} indicates more effective hydrogen donating ability than a higher EC_{50}. Kinetics of the reaction of $DPPH^{\bullet}$ with an antioxidant can also be studied. Kinetic parameters of interest are the stoichiometric factor and rate constant of the reaction (Bondet *et al*., 1997). As some antioxidants reacts fast and others slow, the time required for the reaction to reach a steady-state (T_{EC50}) can be used with the EC_{50} as a measure of antiradical efficiency (Sánchez-Moreno *et al*., 1998):

$$\text{Antiradical efficiency} = \frac{1}{EC_{50} \times T_{EC50}} \qquad [21]$$

where EC_{50} = concentration of sample needed to scavenge 50% of initial radicals

T_{EC50} = time required to reach steady state for sample at EC_{50} concentration

The $DPPH^{\bullet}$ scavenging assay has been used to screen the free radical scavenging ability of wines. The EC_{50} is expressed as the concentration of wine phenolic compounds needed to achieve 50% scavenging (Manzocco *et al*., 1998; Larrauri *et al*., 1999; Saint-Cricq de Gaulejac *et al*., 1999; Sánchez-Moreno *et al*., 1999). Red wines have EC_{50} values lower than white wines indicating the greater effectivity of red wine phenolic compounds to scavenge the $DPPH^{\bullet}$ (Sánchez-Moreno *et al*., 1999). Larrauri *et al*. (1999) found that the antiradical efficiency (this parameter incorporates the EC_{50} and

the time needed to scavenge all radicals) increases during in-bottle ageing of red wine.

2.5.1.2 ABTS radical cation scavenging assay

The $ABTS^{\bullet+}$ scavenging assay was first developed by Miller *et al*. (1993) for the determination of the total antioxidant status (TAS) of body fluids. In the original $ABTS^{\bullet+}$ scavenging method metmyoglobin activated to its ferryl state by hydrogen peroxide [22], is incubated with ABTS in an inactive form to generate a stable radical cation, $ABTS^{\bullet+}$ [23].

$$HX - Fe^{3+} + {}^{\bullet}X - [Fe^{4+} = O] \quad [22]$$

$$^{\bullet}X - [Fe^{4+} = O] + ABTS \rightarrow ABTS^{\bullet+} + HX - Fe^{3+} \quad [23]$$

where $HX\text{-}Fe^{3+}$ = metmyoglobin

$^{\bullet}X\text{-}[Fe^{4+}= O]$ = ferrylmyoglobin

The radical cation can be monitored by measurement of one of its characteristic absorption maxima at 640, 734 and 820 nm (Miller *et al*., 1993). Antioxidants added to this system can either scavenge the $ABTS^{\bullet+}$ formed, or interfere with the radical generating process (Miller & Rice-Evans, 1997b; Strube *et al*., 1997). A modification to this procedure was proposed by Re *et al*. (1999). The $ABTS^{\bullet+}$ is generated prior to addition of samples to the reaction mixture using a non-enzymatic process. This procedure enables the direct measurement of only the free radical scavenging ability of the antioxidant in question. Chemical oxidants used to generate $ABTS^{\bullet+}$ include manganese dioxide (Miller *et al*., 1996), potassium persulphate (Pellegrini *et al*., 1999; Re *et al*., 1999) and a thermolabile azo-compound, 2,2'-azobis-(2-amidinopropane) HCl (Van den Berg *et al*., 1999).

Measurement of the absorbance at a specific time after addition of the antioxidant enables the calculation of the percentage scavenging:

$$\% \text{ Scavenging} = \frac{Abs_{C(t)} - Abs_{S(t)}}{Abs_{C(t)}} \times 100$$

[24]

where $Abs_{C(t)}$ = absorbance of control at time t

$Abs_{S(t)}$ = absorbance of sample with antioxidant at time t

A standard plot of the percentage scavenging of a range of concentrations of Trolox, a water-soluble vitamin E analogue, is used to calculate a TEAC value (Trolox equivalent antioxidant capacity) for a pure compound or a TAA value (total antioxidant activity) for complex mixtures of antioxidants such as plant extracts. The TEAC of a compound indicates the concentration (in mM) of Trolox which has equivalent antioxidant activity to a 1 mM solution of the specific compound, while the TAA of a complex mixture indicates the concentration of Trolox which has equivalent antioxidant activity to the mixture on a mass basis. This enables the comparison of different antioxidants and mixtures on the same basis (Van den Berg *et al*., 1999).

2.5.2 Biologically relevant test systems

Test systems evaluating the biological relevance of an antioxidant need to closely mimic conditions found in the cell. When evaluating the antioxidant potency of a potential *in vivo* antioxidant it is important to utilise biologically relevant reactive oxygen species and sources generating such species (Halliwell, 1995).

Important questions when selecting a test system are (Halliwell, 1995):

1. Which biomolecule should be protected?
2. What is the mechanism of antioxidant activity of the proposed antioxidant?
3. How stable is the resultant radical from the radical scavenging reaction?
4. Would the antioxidant be toxic to the human body at the levels utilised in the assay?

Tests systems that were developed for these purposes include the superoxide scavenging (Robak & Gryglewski, 1988), the deoxyribose (Aruoma *et al*., 1987; Halliwell *et al*., 1987), peroxyl radical scavenging (Wayner *et al*., 1985; Cao & Prior, 1999; Kondo *et al*., 1999), the microsomal lipid peroxidation (Beuge & Aust, 1978) and the low-density lipoprotein peroxidation (Frankel *et al*., 1995; Vinson & Hontz, 1995; Teissedre *et al*., 1996)assays. These assays measure the products of lipid peroxidation such as thiobarbituric acid reactive substances (TBARS), conjugated dienes or lipid hydroperoxides.

2.5.2.1 Thiobarbituric acid reaction

The TBA reaction has been used as an indicator for lipid peroxidation since 1944 (Hoyland & Taylor, 1991; Guillén-Sans & Guzmán-Chozas, 1998). TBA reacts with two molecules of MDA (Figure 1), a secondary product of lipid peroxidation, yielding a complex with an absorption maxima at 532 nm when heated in an acidic medium (Sinnhuber *et al*., 1958). The extent of lipid peroxidation is generally expressed as the amount of MDA formed:

$$\text{Amount of MDA formed (moles)} = \frac{V \times Abs_{532}}{\varepsilon} \qquad [25]$$

where V = final volume of test solution in ml

Abs_{532} = absorbance at 532 nm

ε = molar extinction coefficient (Beuge & Aust, 1978)

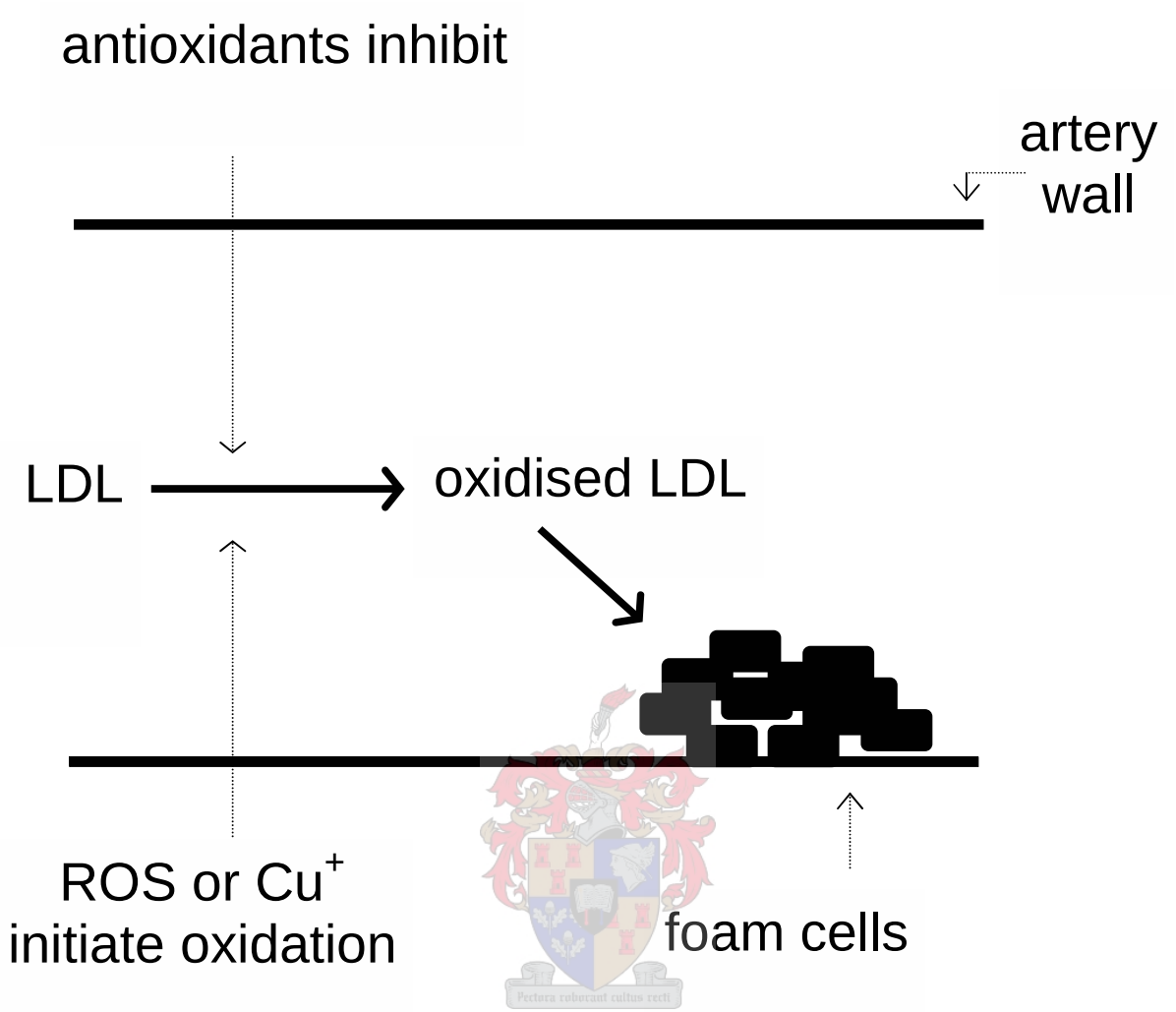
The reaction is subject to a number of interferences such as other aldehydes and some sugars that also react with TBA to produce the chromogen (Baumgartner *et al*., 1975; Knight *et al*., 1988). Other interferences include the formation of MDA from the decomposition of oxidised lipids during the acid heating step needed to release the bound forms of MDA from the sample (Draper & Hadley, 1990). This inclusion of MDA formed by decomposition of oxidised lipids during the TBA procedure, as well as the bound forms of MDA, may provide a better indication of the extent of lipid peroxidation than determination of free MDA alone (Draper & Hadley,

1990). Modifications have been made to minimise interferences in the TBA reaction. The most important one is the addition of metal chelators and antioxidants before reaction with TBA to prevent further peroxidation during the acid-heating step (Draper & Hadley, 1990; Esterbauer & Cheeseman, 1991). Precipitation of protein in biological samples with trichloroacetic acid is advocated to remove potential MDA precursors such as protein-MDA complexes or oxidised lipids (Esterbauer & Cheeseman, 1991). Despite all the difficulties and controversies surrounding the TBA reaction it is still the fastest and most effective way to measure the extent of lipid peroxidation when the necessary precautions are taken. Other indices of lipid peroxidation include conjugated dienes, lipid hydroperoxides, loss of lipid substrate, oxygen uptake and chemiluminescence (Slater, 1984).

2.5.2.2 Low-density lipoprotein peroxidation

Increasing evidence links the oxidative modification of low-density lipoprotein (LDL) with the early stages of atherosclerosis (Gey, 1990; Esterbauer *et al*., 1991; Luc & Fruchart, 1991). Oxidised LDL is associated with endothelial dysfunction resulting in an increased adherence of macrophages. The macrophages become large foam cells due to lipid accumulation, which together with T cells and smooth muscle cells, form a fatty streak (Figure 8) (Luc & Fruchart, 1991; Ross, 1993). The fatty streak precedes the development of intermediate lesions, which are composed of layers of macrophages and smooth muscle cells, which in turn develop into more advanced lesions called fibrous plaques. This “oxidation hypothesis” in the genesis of atherosclerosis has led to the development of test assays for ascertaining whether antioxidants can inhibit the peroxidation of LDL (Luc & Fruchart, 1991).

The assay protocol entails the incubation of isolated human LDL at physiological temperature and pH in the presence of a lipid peroxidation initiator (Teissedre *et al*., 1996). Products of lipid peroxidation are measured and the percentage inhibition determined:



LDL = low-density lipoprotein
ROS = reactive oxygen species

Figure 8 Diagrammatic representation of the mechanism of atherosclerosis (Gurr, 1996).

$$\%\,\text{Inhibition} = \frac{LPP_C - LPP_S}{LPP_S} \times 100 \qquad [26]$$

where LPP_C = amount of lipid peroxidation products formed in the control

LPP_S = amount of lipid peroxidation products formed in the sample with antioxidants

Measurement of the percentage inhibition obtained at different concentrations enables calculation of the IC_{50} value, i.e. the concentration of antioxidant required for 50% inhibition. Indexes of LDL peroxidation generally used include the peroxide value, electrophoretic mobility of oxidised LDL and the amount of either lipid hydroperoxides, TBARS, conjugated dienes or hexanal formed. Although consensus has not been reached on the specific oxidants that initiate LDL peroxidation *in vivo*, it is believed that transition metal ions are involved (Frei, 1995). Initiators used for LDL peroxidation *in vitro* include Cu^{2+} (Frankel *et al*., 1992; Teissedre *et al*., 1996), macrophages (Nardini *et al*., 1995), metmyoglobin (Castelluccio *et al*., 1995; Salah *et al*., 1995) and lipid-soluble azo-initiators (Noguchi *et al*., 1998).

Ex vivo assay protocols are used to monitor the effect of antioxidants or beverages such as wine on the susceptibility of LDL to undergo peroxidation. Results from these studies are contradictory as beneficial effects have been reported in some studies (Fuhrman *et al*., 1995; Nigdikar *et al*., 1998), while no effects were reported in others (De Rijke *et al*., 1996; Caccetta *et al*., 2000). Nigdikar *et al*. (1998) investigated the effect of ethylenediamine-tetraacetic acid (EDTA) used during the isolation of LDL. Different lag times for LDL peroxidation were reported when EDTA was omitted from the dialysis step in contrast to when EDTA was used continuously during dialysis. Differences in protocols used could, therefore, explain the contradictory results obtained. Another problem encountered is that LDL isolated from different volunteers will have different susceptibilities to peroxidation and should, therefore, be pooled to ensure homogeneity. Isolated LDL should also be used within two weeks to prevent changes due to autoxidation. These problems need to be considered when using the method to screen the

antioxidative properties of many samples (W.C.A. Gelderblom, PROMEC, MRC, South Africa, personal communication).

Wine, dealcoholised wine and wine fractions have been shown to inhibit LDL peroxidation (Frankel *et al*., 1995; Kerry & Abbey, 1997; Van Golde *et al*., 1999). This assay is used often to measure the antioxidant activity of wine due to the link between wine consumption and decreased risk of coronary heart disease (Renaud & De Lorgeril, 1992).

2.5.2.3 Microsomal lipid peroxidation

The microsomal lipid peroxidation assay is used to evaluate the effectiveness of antioxidants to scavenge radicals generated in a membranal lipid environment. Other assays to monitor lipid peroxidation in membranes include erythrocytes (Tsuda *et al*., 1994), hepatocytes (Morel *et al*., 1994), liposomes (Aruoma *et al*., 1997b; Yi *et al*., 1997) and mitochondria (Yen & Hsieh, 1998).

The microsomal lipid peroxidation assay utilises a rat liver microsomal fraction incubated in the presence of a lipid peroxidation initiator at physiological conditions (pH and temperature) (Beuge & Aust, 1978). The effect of antioxidants on the membranal lipid peroxidation is measured by monitoring the products of lipid peroxidation expressed as a percentage inhibition relative to a control:

$$\% \text{ Inhibition} = \frac{LPP_C - LPP_S}{LPP_S} \times 100 \qquad [27]$$

where LPP_C = amount of lipid peroxidation products in the control

LPP_S = amount of lipid peroxidation products in the presence of antioxidants

Products of lipid peroxidation can be determined as in the case of LDL peroxidation by measuring lipid hydroperoxides, TBARS or conjugated dienes. Measuring TBARS is the most commonly used method (Afanas'ev *et al*., 1989; Tsuda *et al*., 1994; Yen & Hsieh, 1998; Lapidot *et al*., 1999). The IC_{50} value, which is the concentration of antioxidant required to obtain 50%

inhibition, can be calculated from the percentage inhibition using a range of concentrations.

Lipid peroxidation can be enzymatically initiated by NADPH, ADP and Fe(III) ions through the production of H_2O_2 by NADPH oxidase (Boveris *et al*., 1972; Beuge & Aust, 1978). However, when using enzymatic initiation, the possible inhibition of the enzyme reaction by the compound tested should be considered (Halliwell, 1995). Non-enzymatic initiation of microsomal lipid peroxidation is iron-dependent and involves complexation of Fe(II) with the cytochrome P-450 enzyme (Plumb *et al*., 1997). The Fe-cytochrome P-450 complex promotes the breakdown of lipid hydroperoxides in the microsomal fraction to peroxyl radicals, which participates in propagating lipid peroxidation (Beuge & Aust, 1978). Fe(III) can also be used in conjunction with a redox-cycling agent such as ascorbate or cysteine. Using ascorbate as redox-cycling agent can, however, lead to overestimation of antioxidant activity if the phenoxyl radicals (formed when the phenolic compound scavenge a peroxyl radical) can be reduced back to the antioxidant molecule (Halliwell, 1995). Inhibition of lipid peroxidation in this model system occurs by scavenging of peroxyl radicals participating in chain propagation, as well as by the chelation of iron. Due to the complex mechanisms involved, the use of different initiation methods is advised when inhibition of microsomal lipid peroxidation is investigated. As *in vivo* lipid peroxidation is probably metal ion-dependent, the most biologically relevant evaluation of the ability to inhibit lipid peroxidation are obtained using metal ions as initiators (Halliwell, 1995).

The mechanism of lipid peroxidation in membranes is similar to that of lipid peroxidation in LDL. One difference is that the lag phase observed in membranes is negligible compared to that observed in LDL (Sevanian & Ursini, 2000). This difference in lag phase duration can be ascribed to a fast propagation rate in membranes, compared to a limited propagation rate in LDL. This phenomenon is due to the influence of cholesterol on the lipid structure and microviscosity of LDL (Sevanian & Ursini, 2000).

2.5.2.4 Deoxyribose assay

The deoxyribose assay involves the generation of hydroxyl radicals by the Fenton reaction (Halliwell *et al*., 1987). Depending on the assay protocol, the ability of antioxidants to scavenge hydroxyl radicals or promote generation of hydroxyl radicals in the presence of iron can be measured (Figure 9). The Fenton chemistry is relevant in cells, as some antioxidants can have pro-oxidant activity when iron is available. This is one of the potentially toxic effects cautioning against excessive flavonoid intake (Skibola & Smith, 2000). *In vivo*, iron is usually bound to transport proteins such as transferrin and ferritin, but during disease conditions or tissue damage iron can be mobilised to be available for Fenton chemistry (Halliwell & Gutteridge, 1990).

Hydroxyl radicals formed in the presence of H_2O_2 and Fe^{3+} reacts with deoxyribose [28,29,30]. The resultant breakdown products are measured utilising the TBA reaction [32,33], are generally used to quantify degradation of deoxyribose (Halliwell *et al*., 1987). When EDTA is added to the reaction mixture the hydroxyl radicals are formed in free solution where they can be readily scavenged by antioxidant molecules [31]. Antioxidants capable of recycling Fe^{3+} to Fe^{2+} will, however, promote hydroxyl radical production, thereby presenting pro-oxidant activity.

$$Fe^{2+}-EDTA + O_2 \rightarrow Fe^{3+}-EDTA + O_2^{\bullet -} \quad [28]$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad [29]$$

$$Fe^{2+}-EDTA + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH + Fe^{3+}-EDTA \quad [30]$$

$${}^{\bullet}OH + AH \rightarrow H_2O + A^{\bullet} \quad [31]$$

$${}^{\bullet}OH + DR \rightarrow \text{fragments} \xrightarrow{\text{heat+acid}} MDA \quad [32]$$

$$2TBA + MDA \rightarrow \text{chromogen} \quad [33]$$

where EDTA = ethylenediaminetetraacetic acid
AH = antioxidant molecule
$A^{\bullet}$ = radical formed from antioxidant molecule
DR = deoxyribose
TBA = thiobarbituric acid
MDA = malondialdehyde

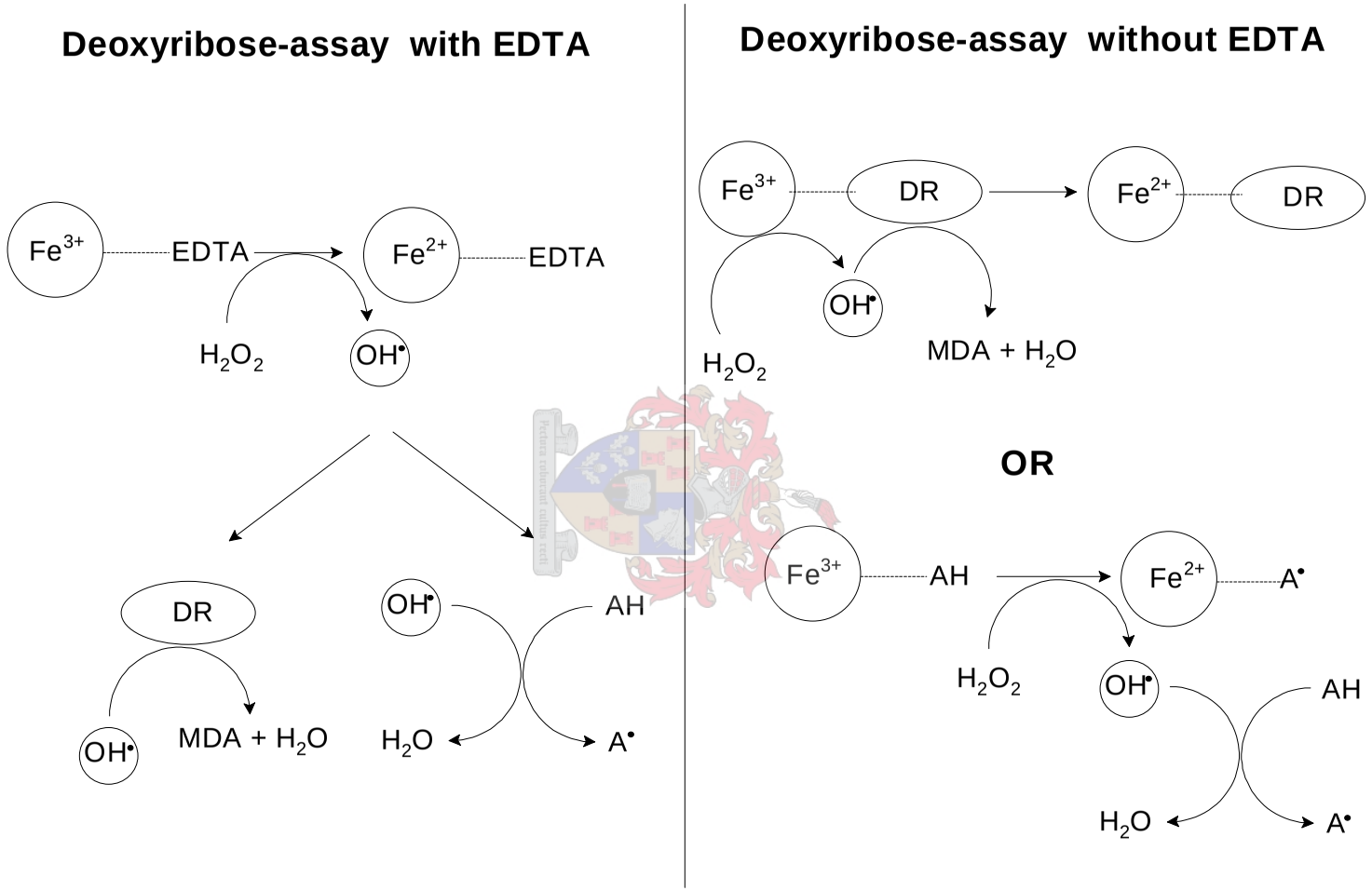


Figure 9 Diagram representing reactions involved in deoxyribose assay (see section 5.2.4 for abbreviations).

When EDTA is omitted from the reaction the Fe^{3+} ions weakly associates with the carbohydrate (Aruoma *et al*., 1987). Hydroxyl radicals produced then immediately reacts with the deoxyribose causing 'site-specific' damage. Antioxidants with a greater metal chelating ability than deoxyribose will be able to inhibit such 'site-specific' damage (Figure 9) (Aruoma *et al*., 1987).

When ascorbic acid is included in the reaction mixture, it acts as a pro-oxidant by recycling Fe^{2+} to Fe^{3+} to increase hydroxyl radical production (Aruoma *et al*., 1997a). Redox-cycling of iron by ascorbic acid precludes the redox-cycling of iron by antioxidant molecules in the reaction medium. In this case, only the hydroxyl radical scavenging ability of antioxidants added to the reaction mixture is measured (Aruoma *et al*., 1997a).

2.6 STRUCTURE-ACTIVITY RELATIONSHIPS

2.6.1 General considerations

The chemical structures of phenolic compounds are predictive of their antioxidant potential in terms of radical scavenging, hydrogen or electron donation and metal chelation. The antioxidant activity of a compounds is also affected by the stability of the resulting phenoxyl radical (Rice-Evans *et al*., 1997). The unique structure of phenolic compounds facilitates their role as free radical scavengers due to resonance stabilisation of the captured electron (Figure 10) (Shahidi & Wanasundara, 1992). Free radical scavenging occur by hydrogen donation to lipid radicals (reactions [34] and [35]) competing with chain propagation (reaction [36]) (Shahidi & Wanasundara, 1992):

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad [34]$$

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet} \quad [35]$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad [36]$$

$$A^{\bullet} + RH \rightarrow AH + R^{\bullet} \quad [37]$$

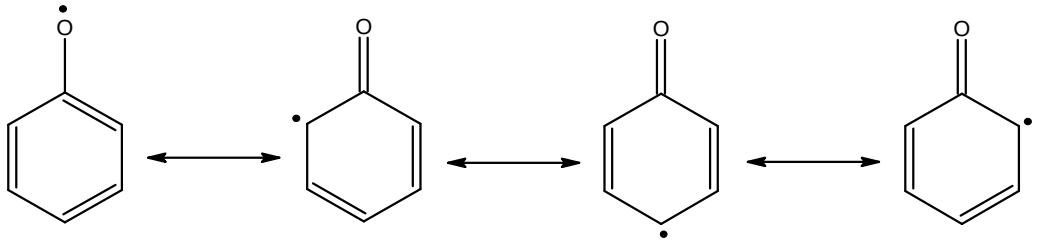


Figure 10 Stabilisation of the phenoxyl radical due to resonance delocalisation.

where $ROO^{\bullet}$ = peroxyl radical
AH = phenolic antioxidant
ROOH = fatty acid hydroperoxide
$A^{\bullet}$ = phenoxyl radical
$RO^{\bullet}$ = alkoxyl radical
ROH = alcohol
RH = PUFA
$R^{\bullet}$ = alkyl radical

Due to the exothermic nature of the reaction of an antioxidant molecule with a free radical species such as a peroxyl radical [34], the activation energy increases with increasing A-H bond dissociation energy. Therefore the hydrogen donating ability of an antioxidant increases as bond dissociation energy decreases. Substituents or groups such as methoxyl groups that increase the electron density of the phenolic ring will enhance antioxidant activity (Robards *et al*., 1999). However, due to steric hindrances, bulky substituents also reduce the rate of the propagation reaction involving the phenoxyl radical itself [37] (Shahidi & Wanasundara, 1992).

Many researchers have studied the structure-activity relationships of antioxidants in various test systems (Bors *et al*., 1990; Foti *et al*., 1996; Van Acker *et al*., 1996; Rice-Evans *et al*., 1997; Rice-Evans & Packer, 1998; Liao & Yin, 2000). All the structurally related effects could not be explained due to differences in mechanisms, end-points used, substrates and concentrations of antioxidants in the methods used. Most lipid oxidation test systems use metal ions as initiators, therefore, the structure-activity relationships derived from such test systems could include both free radical scavenging and metal chelation (Van Acker *et al*., 1996), although Van Acker *et al*. (1998) reported that iron chelation plays no role in the antioxidant activity of flavonoids using a microsomal lipid peroxidation system. In systems containing both lipid and aqueous phases, partitioning of compounds between these phases also play a contributing role in the antioxidant activity (Foti *et al*., 1996; Liao & Yin, 2000). More effective comparisons of structure related to activity can be made if only one aspect such as free radical scavenging activity or metal

chelating ability, is investigated at a time. The influence of structure on the antioxidant activity of phenolic compounds can be illustrated by considering the TEAC values of compounds differing in only one structural aspect.

2.6.2 Phenolic acids

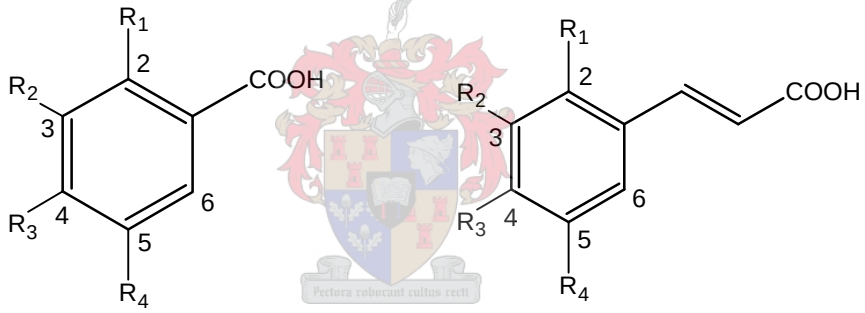
The antioxidant activity of phenolic acids is related to the acid moiety and the number and relative positions of hydroxyl groups on the aromatic ring structure (Rice-Evans *et al*., 1996; Hall III & Cuppett, 1997). Hydroxycinnamic acids are more effective antioxidants than hydroxybenzoic acids due to increased possibilities for delocalisation of the phenoxyl radical (Chen & Ho, 1997; Moon & Terao, 1998; Silva *et al*., 2000). Hydroxycinnamic acid esters such as caffeoyltartaric acid, *p*-coumaroyltartaric acid and chlorogenic acid were found to possess greater antioxidant activity than that of the parent hydroxycinnamic acids, possibly due to increased possibilities for electron delocalisation (Meyer *et al*., 1998; Silva *et al*., 2000).

Substituents increasing the electron density on the hydroxyl groups cause a decrease in the dissociation energy of the O-H bond. Therefore electron-donating substituents will increase the antioxidant activity as in the case of vanillic acid (TEAC = 1.43) relative to *p*-hydroxybenzoic acid (TEAC = 0.08) (Miller & Rice-Evans, 1997a). Substitution patterns of some hydroxybenzoic and hydroxycinnamic acids are shown in Table 1.

Interestingly the parent acids of hydroxybenzoic and hydroxycinnamic acids, namely benzoic and cinnamic acid, have no free radical scavenging activity (Miller & Rice-Evans, 1997a). Di- and trihydroxylation increases the activity over a single hydroxyl group with the position of the hydroxyl groups being the most important factor. Hydroxylation in the 2- and 4-positions or in the 3-, 4- and 5-positions confers the greatest antioxidant activity. Adjacent hydroxyl groups are less favourable for antioxidant activity than those *meta*-orientated with respect to each other (Miller & Rice-Evans, 1997a). A single methoxyl group adjacent to a hydroxyl group, however, has an activating effect on the antioxidant activity of a hydroxybenzoic acid due to steric hindrance (Hall III & Cuppett, 1997). The same tendency was not evident for the hydroxycinnamic acids where the methoxylation of *p*-coumaric acid (TEAC = 2.22), to produce ferulic acid (TEAC = 1.90), did not have the

Table 1 Substitution patterns for phenolic acids

Compounds	2	3	4	5	6
Hydroxybenzoic acids[1]					
Salicylic acid	OH	H	H	H	H
m-Hydroxybenzoic acid	H	OH	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H	H
Protocatechuic acid	H	OH	OH	H	H
Gallic acid	H	OH	OH	OH	H
Vanillic acid	H	O-Me	OH	H	H
Syringic acid	H	O-Me	OH	O-Me	H
Hydroxycinnamic acids[2]					
p-Coumaric acid	H	H	OH	H	H
Caffeic acid	H	OH	OH	H	H
Ferulic acid	H	O-Me	OH	H	H
Sinapic acid	H	O-Me	OH	O-Me	H



[1]Hydroxybenzoic acids

$R_1 = R_2 = R_3 = R_4$ = H, OH, O-Me

[2]Hydroxycinnamic acids

$R_1 = R_2 = R_3 = R_4$ = H, OH, O-Me

same incremental effect (Miller & Rice-Evans, 1997a).

2.6.3 Flavonoids

Earlier studies concerning the structure-activity relationships of flavonoids focussed on the relative positions of hydroxyl groups. The hydroxyl functions are reported to dissociate in the following sequence: 7-hydroxyl > 4'-hydroxyl > 5-hydroxyl (Agrawal & Schneider, 1983). The structural characteristics imparting the most antioxidant activity in flavonoids have been found to be (Bors *et al*., 1990):

1) the *ortho* 3',4'-dihydroxy moiety in the B-ring for electron delocalisation and stability of the phenoxyl radical (Figure 11);
2) the 2,3-double bond in combination with the 4-keto group for electron delocalisation from the C-ring (Figure 11); and
3) the 3- and 5-hydroxyl groups in the C- and A-ring respectively in combination with the 4-keto group in the C-ring for maximum scavenging potential (Figure 11).

Quercetin (TEAC = 4.72), one of the most effective flavonoid antioxidants, satisfies all the abovementioned criteria. Catechin (TEAC = 2.40), which lacks the 2,3-double bond and the 4-keto group is, therefore, a less effective free radical scavenger than quercetin (Rice-Evans & Packer, 1998). The substitution patterns of the B-ring of flavonoids have a large influence on the resonance delocalisation. Substitution patterns of some flavanols, flavonols and anthocyanins are shown in Table 2. The *o*-dihydroxy moiety in the B-ring of most flavonoids is an important structural criterion for effective free radical scavenging activity (Hall III & Cuppett, 1997). This function provides increased stability due to participation in electron delocalisation of the phenoxyl radical. Kaempferol (TEAC = 1.34), lacking the 3'-hydroxyl group has a much lower antioxidant activity than myricetin (TEAC = 3.10) (Rice-Evans *et al*., 1996). The effect of the *o*-dihydroxy structure on the antioxidant activity is enhanced in the presence of the 3-hydroxyl group of the C-ring (Rice-Evans & Packer, 1998). Kinetic studies providing rates of

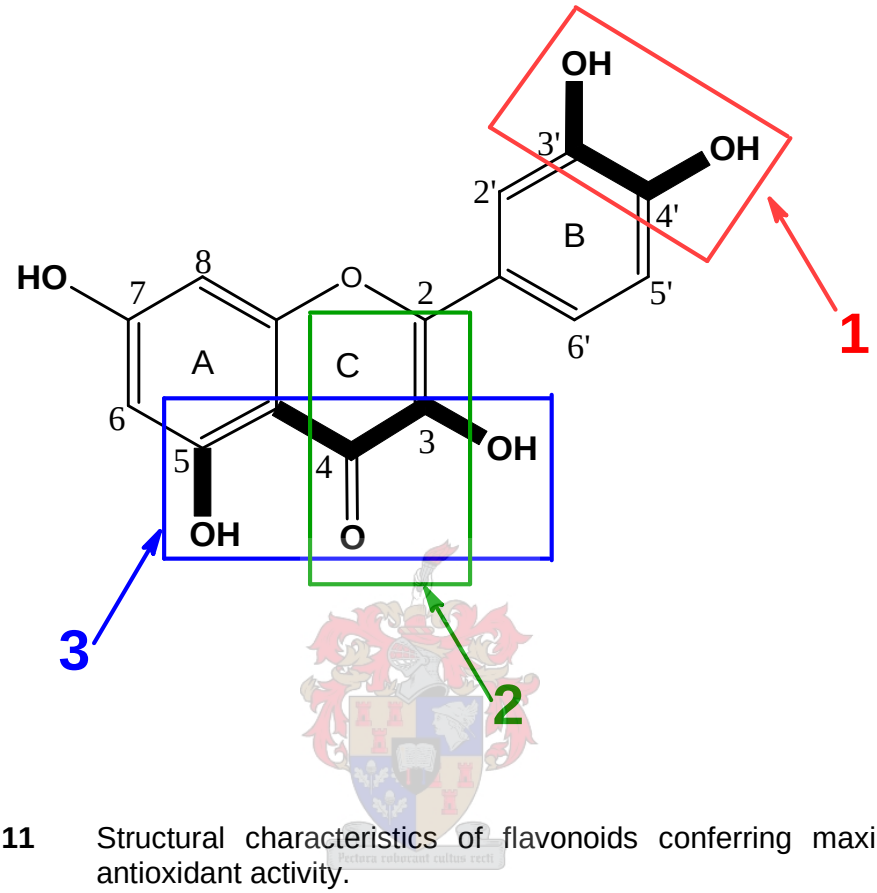


Figure 11 Structural characteristics of flavonoids conferring maximum antioxidant activity.

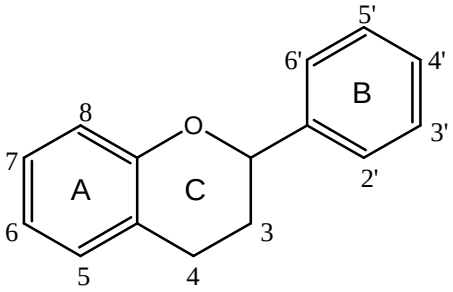
1 the ortho 3', 4'-dihydroxy moiety in the B-ring
2 the 2,3-double bond in combination with the 4-keto group
3 the 3- and 5-hydroxyl groups in the C- and A-ring respectively in combination with the 4-keto group in the C-ring

Table 2 Substitution patterns of flavonoids

Compounds	5	7	3	4	2'	3'	4'	5'
Flavanols								
(+)-Catechin	OH	OH	H,OH	H,H	H	OH	OH	H
(-)-Epicatechin	OH	OH	H,OH	H,H	H	OH	OH	H
Flavonols								
Quercetin	OH	OH	OH	O	H	OH	OH	H
Dihydroquercetin	OH	OH	H,OH	O	H	OH	OH	H
Myricetin	OH	OH	OH	O	H	OH	OH	H
Kaempferol	OH	OH	OH	O	H	H	OH	H
Rutin	OH	OH	O-Rut[a]	O	H	OH	OH	H
Anthocyanins								
Malvidin	OH	OH	OH	H	H	O-Me	OH	O-Me
Malvidin-3-Glc	OH	OH	O-Glc[b]	H	H	O-Me	OH	O-Me
Cyanidin	OH	OH	OH	H	H	OH	OH	H
Cyanidin-3-Glc	OH	OH	O-Glc	H	H	OH	OH	H
Peonidin	OH	OH	OH	H	H	O-Me	OH	H
Peonidin-3-Glc	OH	OH	O-Glc	H	H	O-Me	OH	H
Delphinidin	OH	OH	OH	H	H	OH	OH	OH
Delphinidin-3-Glc	OH	OH	O-Glc	H	H	OH	OH	OH
Petunidin	OH	OH	OH	H	H	O-Me	OH	OH
Petunidin-3-Glc	OH	OH	O-Glc	H	H	O-Me	OH	OH

[a] O-Rutinose

[b] O-Glucose



Flavone skeleton

decay of flavonoid phenoxyl radicals shows that those with the slowest decay rates in the order of 10^5 to10^6 $M^{-1}.s^{-1}$ all contain the 3',4'-dihydroxy structure (Bors *et al*., 1990).

The significant reduction in antioxidant activity due to glycosylation at the 3-position of the C-ring confirms the importance of this structural feature as found in quercetin (TEAC = 4.72) and rutin (TEAC = 2.40) (Rice-Evans *et al*., 1996). Removal of the 3-hydroxyl group, as in the case of flavanones, also significantly reduces the antioxidant activity. Retaining the *o*-dihydroxy structure of the C-ring, but removing the 2,3-double bond eliminates the means to delocalise the electrons of the phenoxyl radical from the B-ring to the A-ring. An example of this effect can be observed when comparing the antioxidant activity of quercetin and catechin (Rice-Evans *et al*., 1996). In the absence of the 4-keto function reduction of the 2,3-double bond has little effect on the antioxidant activity (Rice-Evans & Packer, 1998). Therefore the 3-hydroxyl group in conjunction with the 2,3-double bond and the 4-keto function is critical for maximum antioxidant activity. Pulse radiolysis studies have shown that the 2,3-double bond and the 4-keto group in the B-ring are the main determinants of the oxidation potential of flavonoids (Bors *et al*., 1995). All substances containing these characteristics were found to have a higher redox potential than ascorbic acid. These antioxidants could have the added activity of reducing ascorbyl radicals to ascorbic acid, thus preserving ascorbic acid in biological systems (Salah *et al*., 1995; Viana *et al*., 1996).

The importance of the 5,7-dihydroxy moiety in the A-ring can be seen by the effect of glycosylation in the 7-position on the antioxidant activity. A significant decrease of activity is observed if the 7-hydroxyl group of hesperitin (TEAC = 1.37) is glycosylated as in hesperidin (TEAC = 1.08) (Rice-Evans *et al*., 1996). Van Acker *et al*. (1996), however, found that substitution at the 7-position does not influence the half-peak potential.

Flavonoids and phenolic acids mainly act as free radical scavengers. Flavonols, however, have a bimodal function by acting as free radical scavengers and metal chelators. Chelation occurs at the 3-hydroxy-4-keto group or the 5-hydroxy-4-keto group of the flavonol (Hudson & Lewis, 1983; Rice-Evans *et al*., 1997). The 3',4'-dihydroxy structure present in many flavonoids can also contribute to metal chelation. Glycosylation at any of

these functional hydroxyl positions decreases the metal chelating activities (Rice-Evans & Packer, 1998).

The elucidation of quantitative structure-activity relationships of antioxidants using physicochemical parameters such as heat of formation, the number of hydroxyl groups and redox potentials have been attempted (Cao *et al*., 1997; Lien *et al*., 1999). These attempts have only been partially successful due to difficulties in calculating or determining these physicochemical properties for complex molecules and the use of a limited number of antioxidants in regression analysis. These quantitative structure-activity relationships can be used to calculate the expected activity of antioxidants in the $ABTS^{\bullet+}$ scavenging assay (Lien *et al*., 1999).

2.6.4 Comparative activities in selected antioxidant assays

Antioxidant and pro-oxidant activities of selected phenolic compounds in the $ABTS^{\bullet+}$ scavenging, LDL peroxidation and deoxyribose assays are reported in Table 3.

In general the flavonoids are more effective free radical scavengers than the phenolic acids, although the activity of gallic acid is approximately the same as that of (+)-catechin (Rice-Evans *et al*., 1996; Miller & Rice-Evans, 1997a). Two of the anthocyanidins, namely delphinidin and cyanidin, as well as the flavanol gallate esters, are the most effective flavonoids in the $ABTS^{\bullet+}$ scavenging assay (Rice-Evans *et al*., 1996; Re *et al*., 1999).

Most flavonoids and some phenolic acids are more effective inhibitors of LDL peroxidation than ascorbic acid and α-tocopherol, important *in vivo* antioxidant components of LDL (Vinson *et al*., 1995). The flavanols are the most effective group of flavonoids in this assay (Teissedre *et al*., 1996).

Pro-oxidant activity has been exhibited by some flavonoids in the presence of EDTA in the deoxyribose assay. Phenolic acids exhibit no pro-oxidant activity except for gallic and caffeic acids (Moran *et al*., 1997). (+)-Catechin, a flavanol, and some flavonols, namely quercetin, myricetin and kaempferol, also exhibited pro-oxidant activity (Puppo *et al*., 1992).

Table 3 Relative antioxidant activity of selected phenolic compounds

Compounds	TEAC[a]	EC_{50} (DPPH)[b]	IC_{50} (LDL)[c]	Prooxidant activity[d]
Phenolic acids				
Gallic acid	3.01 [e]		1.25 [i]	+ [j]
p-Coumaric acid	2.22 [f]	ineffective [h]	>16 [i]	- [j]
Ferulic acid	1.90 [f]	407 [h]		- [j]
Vanillic acid	1.43 [e]			- [j]
Syringic acid	1.36 [e]	218 [h]		
Caffeic acid	1.26 [f]	110 [h]	0.24 [i]	+ [j]
Protocatechuic acid	1.19 [e]	172 [h]		
p-Hydroxybenzoic acid	0.08 [e]	ineffective [h]		- [j]
Flavanols				
Epicatechin gallate	4.90 [g]		0.14 [i]	
Epigallocatechin gallate	4.80 [g]		0.08 [i]	
Epigallocatechin	3.80 [g]		0.10 [i]	
(-)-Epicatechin	2.50 [g]	135 [h]		
(+)-Catechin	2.40 [g]	149 [h]	0.19 [i]	+ [k]
Flavonols				
Quercetin	4.72 [f]	91 [h]	0.23 [i]	+ [k]
Myricetin	3.10 [g]		0.48 [i]	+ [k]
Rutin	2.40 [g]	136 [h]	0.51 [i]	
Kaempferol	1.34 [f]		1.82 [i]	+ [k]
Anthocyanidins				
Delphinidin	4.44 [f]			
Cyanidin	4.40 [f]		0.21 [i]	
Peonidin	2.22 [g]			
Malvidin	2.06 [f]			
Malvidin-3-glucoside	1.78 [g]			
Pelargonidin	1.30 [g]			
Plasma antioxidants				
Ascorbic acid	0.99 [f]		1.45 [i]	+ [j]
α-Tocopherol	0.97 [f]	304 [h]	2.40 [i]	
Synthetic antioxidants				
Trolox	1.00 [f]	284 [h]	1.26 [i]	- [l]

[a] Trolox equivalent antioxidant activity in mM as measured by the 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) (ABTS) radical scavenging assay.
[b] Concentration (μM) needed to scavenge 50 % of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.
[c] Concentration needed for 50% inhibition of low-density lipoprotein oxidation.
[d] Ability to promote hydroxyl radical generation in the deoxyribose assay.
[e] Miller & Rice-Evans, 1997a.
[f] Re *et al*., 1999.
[g] Rice-Evans *et al*., 1996.
[h] Winterton, 1999.
[i] Vinson *et al*., 1995.
[j] Moran *et al*., 1997.
[k] Puppo *et al*., 1992.
[l] Aruoma *et al*., 1990.

Synergistic effects are possible when mixtures of phenolic compounds are present. Catechin and sulphur dioxide, for example, shows greater activity in the $ABTS^{\bullet+}$ scavenging assay than the sum of their respective activities (Saucier & Waterhouse, 1999). Certain phenolic compounds can regenerate ascorbic acid or α-tocopherol from reduced forms (Liao & Yin, 2000; Van Acker *et al*., 2000). This recycling activity keeps the levels of these endogenous antioxidants high enough to protect membranes of low-density lipoprotein where they are naturally present (Esterbauer *et al*., 1991; Van Acker *et al*., 2000).

2.7 ABSORPTION AND BIOAVAILABILITY

The absorption, disposition and excretion of most phenolic compounds have not yet been extensively studied in humans. Information about the metabolic fate and pharmacokinetics of these potential *in vivo* antioxidants is still limited. A number of studies concerning the detection of plasma and urinary metabolites of certain phenolic compounds, such as quercetin, quercetin glycosides, kaempferol, catechin, epicatechin, ferulic acid, caffeic acid and cyanidin glucosides, have been reported (Table 4). Phenolic compounds in wine, present as soluble forms, should be more bioavailable than from fruits and vegetables where they are present as polymeric, insoluble or tightly bound and compartmentalised forms (Soleas *et al.*, 1997a). Some phenolic compounds in wine are also present in polymeric forms such as procyanidins or copigments of anthocyanins and flavanols (Rios *et al*., 2000).

Intakes of phenolic acids and flavonoids in humans have been estimated to range from as much as 170 mg/day (Kühnau, 1976) to 23 mg/day (Hertog *et al*., 1993). Values obtained by Kühnau (1976) were probably an overestimation as it were based on techniques now considered inappropriate. Estimations by Hertog *et al*. (1993), on the other hand, were restricted to a few selected flavonols and flavones after hydrolysis and, therefore, possibly represent an underestimation of total intake. Average intakes are also linked to dietary habits of the populations studied. In countries where red wine or coffee is consumed regularly intakes may be much higher. Recently,

Table 4 Bioavailability of phenolic compounds in human and rat *in vivo* studies

Source	**Amount administered**	**Human / Rat**	**Absorption**	**Excretion**	**Metabolites**	**Maximum plasma concentrations**	**Kinetics**	**References**
Phenols from red wine, whisky or new make spirit	100 ml	Human	Absorption detected by increase in plasma total phenol content and antioxidant capacity	Urine conc. = 32 μg GAE / l (red wine), 22 μg GAE / l (whisky) and 14 μg GAE / l (new make spirit)	-	-	-	Duthie *et al*., 1998
Red wine (catechin)	120 ml red wine (RW) or dealcoholised red wine (DRW)	Human	Absorption detected by presence of metabolites in plasma	-	Catechin glucuronides and sulfates and 3-MC glucuronides (20% of total catechin at 1h) in plasma	91 nM total catechin after 1h (red wine); 81 nM total catechin after 1h (dealcoholised red wine)	Max. absorption after 1h; elimination half-life ($t_{1/2}$) less for RW than for DRW	Donovan *et al*., 1999
Red wine (catechin)	120 ml dealcoholised red wine reconstituted in water (DRW) or alcohol and water (ARW)	Human	Absorption detected by presence of metabolites in plasma	-	Conjugate forms predominant in plasma	40 - 130 nM total catechin (DRW); 30 - 110 nM total catechin (ARW)	Max. absorption after 1h; elimination half-life ($t_{1/2}$) less for ARW than for DRW	Bell *et al*., 2000

Table 4 (continued …) Bioavailability of phenolic compounds in human and rat *in vivo* studies

Source	Amount administered	Human / Rat	Absorption	Excretion	Metabolites	Maximum plasma concentrations	Kinetics	References
Quercetin and catechin	0.25% of diet directly into stomach	Rat	Absorption detected by presence of metabolites in plasma	-	Catechin glucuronides and Q sulfo- and glucurono-sulfo derivatives; methylation rate higher for Q	50 mM Q metabolites (12h); 38 mM catechin metabolites (12h)	Absorption of catechin faster than Q; plasma conc. of Q stable between 8 and 24h	Manach *et al*., 1999
Quercetin, rutin or onion	100 mg Q, rutin (100 mg Q equiv.), onions (89 mg Q equiv.)	Human (ileo-stomy volun-teers)	24 ca. 9% from Q, 17 ca. 15% from rutin and 52 ca. 15% from onion	Illeostomy effluent and urine - 0.12% for Q, 0.07% for rutin and 0.31% for onion (13h)	-	-	-	Hollman *et al*., 1995
Quercetin or rutin	16.4 or 8.2 or 4.1 mmol / kg diet	Rat	Absorption detected by presence of metabolites in plasma, ileal contents and cecal contents	Ileal contents, cecal contents (similar amounts of quercetin recovered for Q and rutin diets), faeces	Glucuronide of Q and methylated Q in plasma	~ 115 mM flavonols with 16.4 mmol / kg Q diet	-	Manach *et al*., 1995

Table 4 (continued …) Bioavailability of phenolic compounds in human and rat *in vivo* studies

Source	**Amount administered**	**Human / Rat**	**Absorption**	**Excretion**	**Metabolites**	**Maximum plasma concentrations**	**Kinetics**	**References**
Quercetin or rutin	0.2% Q or or 0.4% rutin	Rat	Absorption detected by presence of metabolites in plasma	Cecal contents	Conjugated derivatives of Q and its methylated forms, isorhamnetin and tamarixetin, found in plasma	-	Q more rapidly absorbed than rutin	Manach *et a*l., 1997
Quercetin from onions	Fried onions containing 64 mg Q equiv.	Human	Absorption detected by presence of metabolites in plasma	-	-	196 ng / ml	Max. plasma conc. after 2.9h; $t_{1/2}$ (elimination) = 16.8h	Hollman *et al*., 1996
Onions (contains Q glucosides), apples (contains glucose and non-glucose Q glycosides) or rutin (Q-rutinoside)	225 µmol Q equiv. from onion, 325 µmol Q equiv. from apple or 331 µmol rutin	Human	Absorption detected by presence of metabolites in plasma and urine	Urine (1.39% for onion, 0.44% for apple and 0.35% for rutin)	-	224 ng / ml for onions, 92 ng / ml for apples and 90 ng / ml for rutin; $t_{1/2}$ (elimination) = 28h for onions and 23h for apples	Max. plasma concentration after 0.7h for onions, 2.5h for apples and 9h for rutin	Hollman *et al*., 1997b

Table 4 (continued …) Bioavailability of phenolic compounds in human and rat *in vivo* studies

Source	Amount administered	Human / Rat	Absorption	Excretion	Metabolites	Maximum plasma concentrations	Kinetics	References
Quercetin from onions	225 g fried onions	Human	Absorption detected by increased plasma antioxidant status	-	-	248 ng Q / ml plasma after 2h	Max. plasma concentration after 2h	McAnlis *et al*., 1999
Quercetin and kaempferol from tea and onions	1600 ml concentrated black tea (49 mg Q and 27 mg kaempferol) or 129 g fried onions (13mg Q) per day	Human	Absorption detected by presence of metabolites in plasma and urine	Excretion of Q in urine is 8x baseline after tea and 4x baseline after onions. Excretion of kaempferol is 25x baseline after tea	-	Plasma concentrations of Q after tea is 4x baseline and after onions is 3x baseline and of kaempferol after tea is 6x baseline	-	De Vries *et al*., 1998
Flavonols from onions and tea	77.3 or 110.4 mg / day	Human	Absorption detected by presence of metabolites in plasma and urine	0.29 - 0.47% excreted in urine	-	91.9 ng total flavonols / ml plasma at high dose; 171 ng total flavonols / ml 24h urine at high dose	-	Noroozi *et al*., 2000

Table 4 (continued …) Bioavailability of phenolic compounds in human and rat *in vivo* studies

Source	Amount administered	Human / Rat	A. Absorption	Excretion	Metabolites	Maximum plasma concentrations	Kinetics	References
Quercetin-4'-Glc and quercetin-3-Glc	331 μmol Q-4'-Glc or 325 μmol Q-3-Glc	Human	Absorption detected by presence of metabolites in plasma	-	-	Peak plasma concentration = 4.5 μM after Q-4'-Glc and 5.0 μM after Q-3-Glc	Peak concentration reached after 27 min. for Q-4'-Glc and after 37 min. for Q-3-Glc; $t_{1/2}$ (elimination) = 17.7h for Q-4'-Glc and 18.5h for Q-3-Glc	Olthof *et al*., 2000
Quercetin from a high flavonol meal	87 mg quercetin equiv.	Human	Absorption detected by presence of metabolites in plasma	-	-	28 – 142 nM (basal); 344 nM (after 3h)	Max. plasma concentration after 2-3h with return to baseline after 20h	Manach *et al*., 1998

Table 4 (continued …) Bioavailability of phenolic compounds in human and rat *in vivo* studies

Source	Amount administered	Human / Rat	Absorption	Excretion	Metabolites	Maximum plasma concentrations	Kinetics	References
Fruit juice	0.75, 1 and 1.5 l / day apple or blackcurrant juice for 7 days; 4.8, 6.4 and 9.6 mg Q / day	Human	Absorption detected by urinary excretion of Q and reduced plasma lipid oxidation	0.47% of Q excreted in urine (from 3 - 7 days)	-	-	$t_{1/2}$ (elimination) estimated at ~ 24 h	Young *et al.*, 1999
Epicatechin from black chocolate	40 and 80 g of black chocolate	Human	Absorption detected by presence of metabolites in plasma	-	-	203 ng / ml at 80 g chocolate	Max. plasma concentration after 2.57 h; $t_{1/2}$ (elimination) for 80 g = 2.3 h	Richelle *et al.*, 1999
Epicatechin	172 μmol / kg body weight	Rat	Absorption detected by presence of metabolites in plasma	-	EC, methylated EC and glucuronide and sulfate conjugates of EC and MEC found in plasma	Free EC = 1.2 mM; MEC sulfate / glucuronide = 11.5 mM; EC glucuronide = 10.7 mM	Max. plasma concentration of all metabolites reached within 2 h	Piskula & Terao, 1998

Table 4 (continued …) Bioavailability of phenolic compounds in human and rat *in vivo* studies

Source	Amount administered	Human / Rat	Absorption	Excretion	Metabolites	Maximum plasma concentrations	Kinetics	References
Cyanidin-3-Glc and cyanidin-3,5-diGlc or tea catechin (EGCg)	2.7 mg / kg body weight Cy-3-Glc + 0.25 mg / kg body weight Cy-3,5-diGlc; 2.6 mg / kg body weight EGCg	Human	Absorption detected by presence of metabolites in plasma	-	No glucuronides or sulfates or aglycone of anthocyanins, but peonidin-3-Glc (methylated Cy-3-Glc) found; glucuronides and sulfates of EGCg	29 nM Cy-3-Glc after 60 min; 725 nM EGCg after 60 min.	Absorption of anthocyanins faster than tea catechin (EGCg)	Miyazawa *et al*., 1999
Cyanidin-3-Glc and cyanidin-3,5-diGlc or tea catechin (EGCg)	320 mg / kg body weight Cy-3-Glc + 40 mg / kg body weight Cy-3,5-diGlc; 320 mg / kg body weight EGCg	Rat	Absorption detected by presence of metabolites in plasma	-	No glucuronides or sulfates or aglycone of anthocyanins, but peonidin-3-Glc found (methylated Cy-3-Glc); glucuronides and sulfates of EGCg	1560 mg / l of Cy-3-Glc and 195 µg / of Cy-3,5-diGlc; 3620 mg / l of EGCg	Absorption of anthocyanins faster than tea catechin (EGCg)	Miyazawa *et al*., 1999

Table 4 (continued …) Bioavailability of phenolic compounds in human and rat *in vivo* studies

Source	Amount administered	Human / Rat	Absorption	Excretion	Metabolites	Maximum plasma concentrations	Kinetics	References
Anthocyanins from red wine	300 ml water, white wine or red wine (218 mg Mv-3-Glc equiv.)	Human	Absorption detected from red wine as urinary metabolites	1.5 - 5.1% exctreted in urine after 12 h	Detection of anthocyanin dimers and unidentified derivatives of Mv-3-Glc in urine	-	Max. anthocyanin level in urine after 6 h	Lapidot *et al*., 1998
Ferulic acid from tomato	8 g / kg body weight fresh tomato (ca. 21 -44 mg ferulic acid)	Human	Absorption detected by urinary metabolites	11 - 25% excreted in urine	Ferulic acid and feruloyl-glucuronide	-	Maximal urinary excretion after 7 h	Bourne & Rice-Evans, 1998
Gallic acid from black tea	5 cups per day of black tea	Human	Absorption detected by urinary metabolites	urine	4-O-methyl gallic acid, 3-O-methyl gallic acid, 3,4-O-dimethyl gallic acid	-	-	Hodgson *et al*., 2000

Abbreviations: GAE = gallic acid equivalents; max. = maximum; conc. = concentration; equiv. = equivalents; Q = quercetin; Q-4'-Glc = quercetin-4'-glucoside; Q-3-Glc = quercetin-3-glucoside; EC = epicatechin; MEC = methyl epicatechin; Mv-3-Glc = malvidin-3-glucoside; Cy-3-Glc = cyanidin-3-Glc; Cy-3,5-diGlc = cyanidin-3,5-diglucoside; peonidin-3-Glc = peonidin-3-glucoside; EGCg = epigallocatechin gallate

Teissedre & Landrault (2000) estimated the average intake of phenolic compounds by the French population per day from red wine. They determined the content of gallic acid, caffeic acid, *p*-coumaric acid, caffeoyltartaric acid, protocatechuic acid, catechin, epicatechin, procyanidin dimers B1, B2, B3 and B4, malvidin-3-glucoside and total phenol content in 60 commercially available French varietal wines (50 red wines and 10 white wines). The estimated daily consumption of 180 ml red wine (1995 consumption figures) equates to between 400 mg phenols (as gallic acid equivalents) per person per day, while 180 ml white wine equates to only 44 mg phenols per person per day.

The bioavailability of a compound is not only affected by the extent of absorption, but factors such as distribution, metabolism (bioconversion in the gut and the liver) and elimination also play important roles in determining the extent of possible *in vivo* activity (Wiseman, 1999). Degradation of phenolic compounds in gastric and intestinal fluids could decrease the amount available for absorption. A study by Martínez-Ortega *et al*. (2001) showed that phenolic compounds in wine are more stable with respect to gastric and intestinal fluids than in hydroalcoholic phenolic solutions. Absorption of phenolic compounds occurs mostly in the small intestines before microbial degradation, but some absorption could also occur after microbial degradation in the colon (Rice-Evans & Packer, 1998). After compounds and degradation products are absorbed into the bloodstream from the intestines, biotransformations by enzymes in the liver and the kidneys can occur. Conjugates formed are usually methylated derivatives, glucuronides, sulphates and products with both glucuronide and sulphate moieties (Hollman *et al*., 1997a). The speed of elimination of phenolic compounds and their derivatives plays a role in their activity *in vivo* (Wiseman, 1999).

Bacteria in the colon are able to hydrolyse β-glycosidic bonds to release aglycones from glycosides (Bokkenheuser *et al*., 1987). Glucuronidases and sulphatases are also released by colonic bacteria to remove glucuronic acid and sulphates from conjugates. Ring fission of flavonoids in various positions by microbial enzymes produces a variety of phenolic acids (Hollman *et al*., 1997a). These are absorbed and can contribute to the biological activity, as

they also possess antioxidant activity (Manach *et al*., 1998). The specific hydroxylation pattern of the flavonoids determines susceptibility to ring fission and products of ring fission (Rice-Evans & Packer, 1998). After ring fission, flavanols yields valerolactones, phenylpropionic acids and benzoic acids (Figure 12), whereas flavonols such as quercetin gives rise to hydroxycinnamic acids, phenylpropionic acids, phenylacetic acids and benzoic acids (Figure 13). Other tissues containing enzymes capable of biotransformation of flavonoids are the kidney and small intestine. Examples of conjugates identified in plasma after ingestion of phenolic compounds are given in Table 4.

A few studies have correlated the presence of flavonoid metabolites with intake of specific compounds (De Vries *et al*., 1998; Hodgson *et al*., 2000; Noroozi *et al*., 2000). Gallic acid metabolites which can be used as markers of black tea intake has been identified (Hodgson *et al*., 2000). The measurement of metabolites of quercetin and kaempferol in urine and plasma can be used to distinguish between high and low flavonol consumption in epidemiological studies (De Vries *et al*., 1998). Short-term intake of these flavonols can also be inferred from the plasma concentrations of metabolites as elimination has been shown to be less than 24 h (Hollman *et al*., 1996; Young *et al*., 1999; Olthof *et al*., 2000). Plasma flavonols concentration and 24 h urine excretion were significantly correlated to dietary intake of flavonols (Noroozi *et al*., 2000). This will enable the determination of dietary intakes of flavonols for use in epidemiological studies, eliminating the need to rely on food intake questionnaires (Noroozi *et al*., 2000).

Many studies show increases in plasma antioxidant activity measured by a variety of methods after the ingestion of phenolic compounds (Cao *et al*., 1998a; Cao *et al*., 1998b; Duthie *et al*., 1998; Serafini *et al*., 1998; McAnlis *et al*., 1999; Sung *et al*., 2000). This suggests that phenolic compounds are absorbed and circulates in plasma in bioactive forms. A significant correlation between daily intake of total antioxidant capacity and fasting plasma antioxidant capacity were found in subjects on controlled diets high in fruits and vegetables (Cao *et al*., 1998a). Consumption of red wine (Cao *et al*., 1998b; Duthie *et al*., 1998), alcohol-free red wine (Serafini *et al*., 1998), onions (McAnlis *et al*., 1999), spinach and strawberries (Cao *et al*., 1998b)

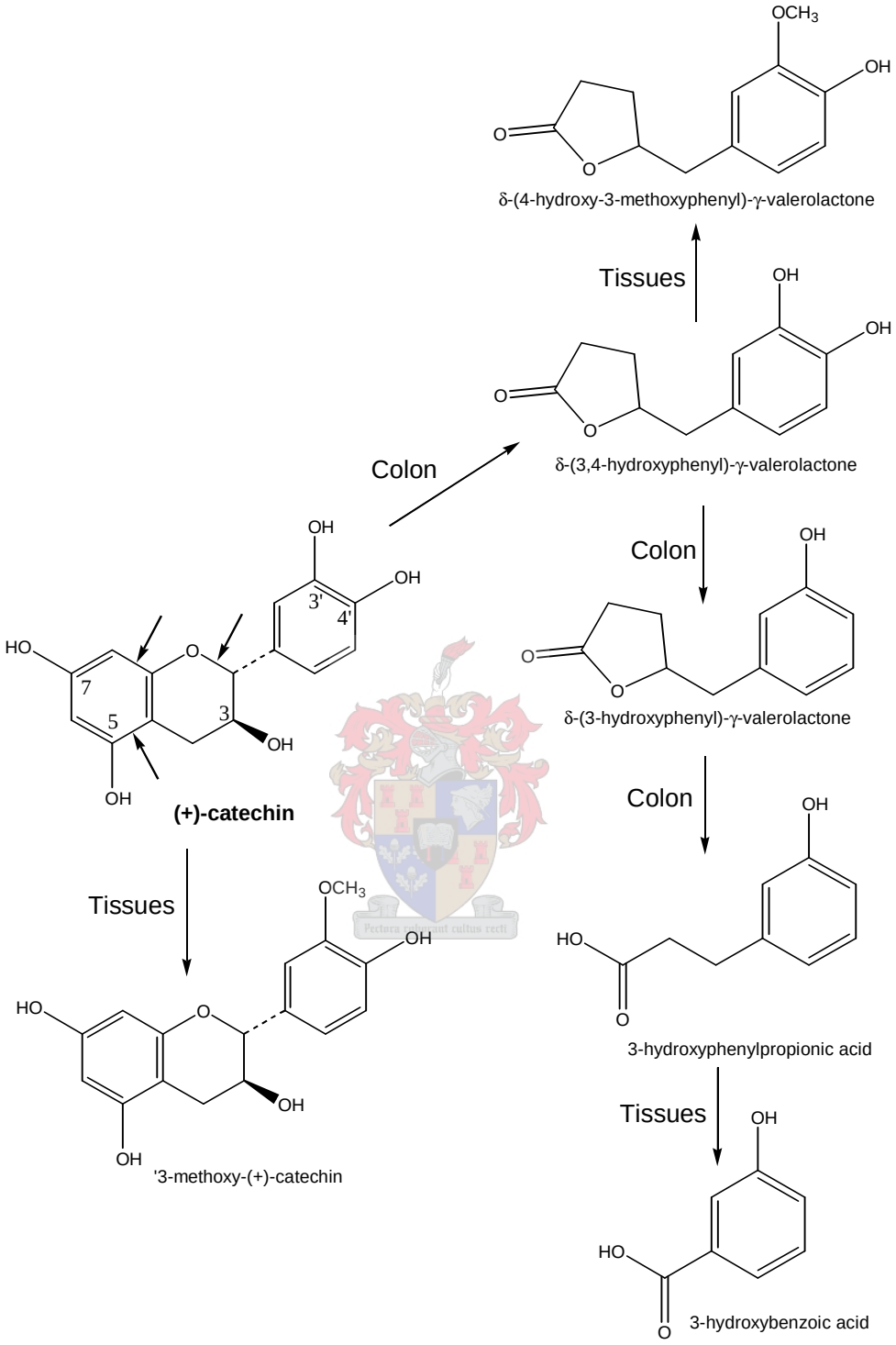


Figure 12 Metabolic conversions of catechin by body tissues and colon (Rice-Evans & Packer, 1998).

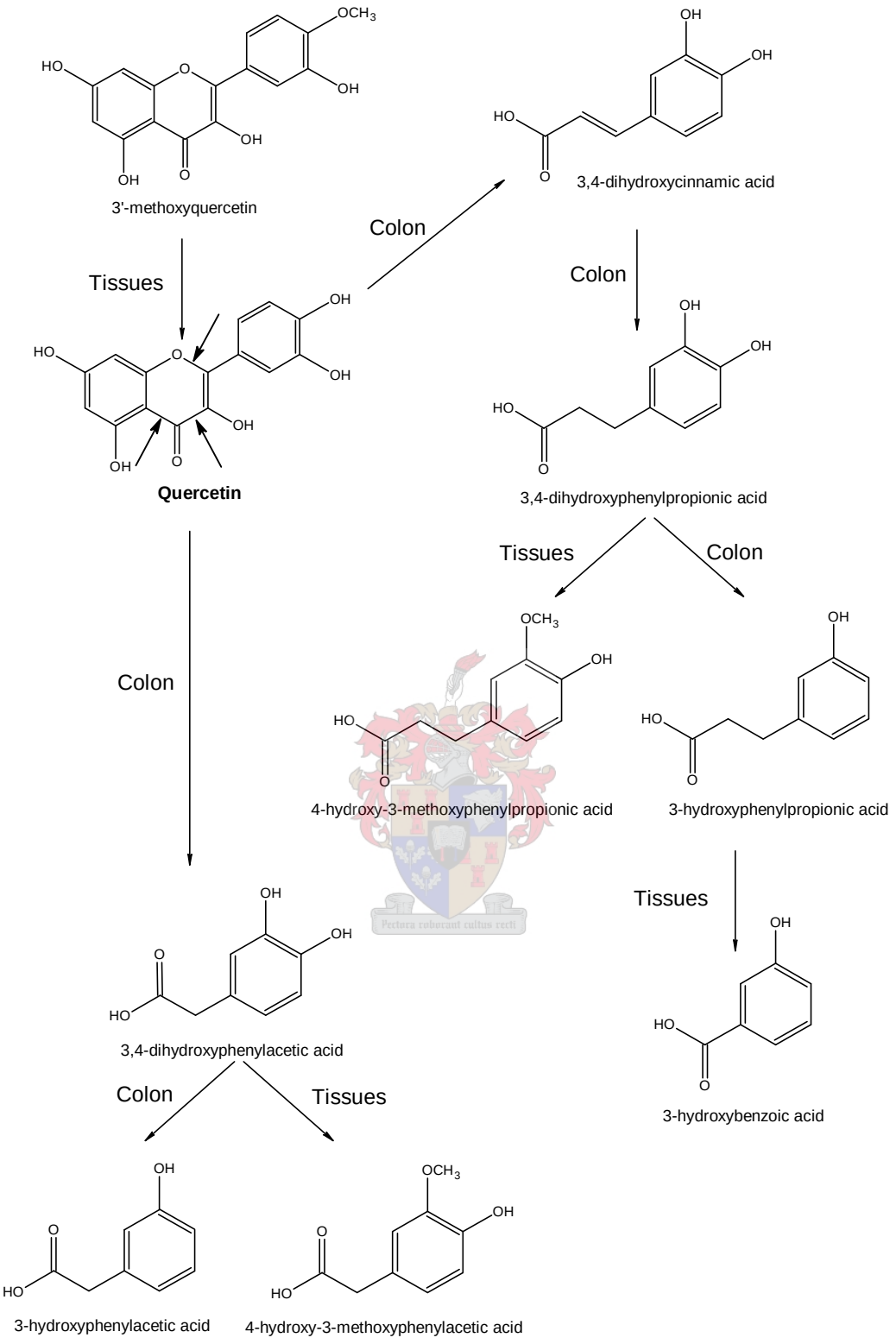


Figure 13 Metabolic conversions of quercetin in body tissues and colon (Rice-Evans & Packer, 1998).

and green tea (Sung *et al*., 2000) have been shown to increase the antioxidant status of plasma.

As long as information on the absorption, distribution, metabolism and excretion of phenolic compounds is limited to a few specific compounds it will remain unclear whether phenolic compounds are retained in the body in bioactive forms for sufficient time to provide *in vivo* protection.

2.8 METHODS FOR ESTIMATION OF PHENOLIC COMPOSITION

Different high performance liquid chromatography (HPLC) (Ricardo da Silva *et al*., 1990; Roggero *et al*., 1990; Kantz & Singleton, 1991; Jagota & Cheatham, 1992; Lamuela-Raventós & Waterhouse, 1994; Goldberg & Soleas, 1999) and gas chromatography-mass spectroscopic (Soleas & Goldberg, 1999) methods exist to determine the content of specific compounds. Indirect methods employed for the characterisation of wines estimate the content of specific groups of phenolic compounds based on their general chemical structure (Singleton & Rossi, 1965; McMurrough & McDowell, 1978; Mazza *et al*., 1999; Burns *et al*., 2000). In this section the indirect spectrophotometric methods that will be used for the characterisation of wines in the present study are discussed.

2.8.1 Total phenols

Methods used for the determination of total phenols include reaction with the Folin-Ciocalteau reagent (F-C reagent), permanganate titration, colorimetry with iron salts and ultraviolet absorbance at 280 nm (Singleton *et al*., 1999). Every method has its advantages and disadvantages, but the Folin-Ciocalteau method has been the most widely used (Frankel *et al*., 1995; Meyer *et al*., 1998; Simonetti *et al*., 1997; Ghiselli *et al*., 1998; Pellegrini *et al*., 2000). The principle of the determination is that phenolic compounds react with the phosphomolybdic-phosphotungstic reagents in an alkaline medium to produce a coloured product measured at 765 nm (Singleton & Rossi, 1965).

Interferences can be inhibitory, additive or enhancing (Singleton *et al*., 1999). Inhibition is possible when oxidants compete with the F-C reagent to oxidise phenolic compounds. The addition of the F-C reagent before the alkali

is important due to the possibility of phenol oxidation in alkaline medium. Aromatic amines, sugars, ascorbic acid and sulphur dioxide can react with the F-C reagent producing additive effects. Ascorbic acid can have an augmentation effect by reducing quinones and prolonging the reaction. Adjustments for sugar and ascorbic acid content can be made in cases where interference is significant (Singleton *et al*., 1999).

2.8.2 Anthocyanins

Determination of anthocyanins is based on the changes in absorption at different pH levels (Ribéreau-Gayon, 1972). Absorbance at 520 nm at a pH lower than 1 is used to determine total anthocyanins, while absorbance at 520 nm at a pH of 3.5 is used to determine polymeric anthocyanins (Burns *et al*., 2000). The difference in absorbance is due to the content of monomeric anthocyanins. The molar extinction coefficient of the most common anthocyanin occurring in wine, namely malvidin-3-glucoside (ε = 28000 $l.mole^{-1}.cm^{-1}$) is used to calculate the anthocyanin content.

Bleaching of monomeric anthocyanins using excessive amounts of bisulphite can also be used to estimate polymeric anthocyanins (Somers & Evans, 1977). Determination of anthocyanin content by bleaching with sulphite gives higher values than HPLC methods due to incomplete bleaching of some anthocyanins (Bakker *et al*., 1986).

2.8.3 Flavanols

The flavanol content can be estimated by the use of reagents containing aromatic aldehydes such as vannilin and 4-dimethylaminocinnamaldehyde (DAC) resulting in coloured compounds. The reaction is carried out in acidic medium to activate the aldehyde reagent (Treutter, 1989). The aldehyde reagents is not very specific, but the use of DAC are preferred as the maximum absorbance of the reaction product with flavanols is at 640 nm, while reaction of DAC with other phenols and indoles yields products absorbing at other wavelengths (Treutter *et al*., 1994).

McMurrough & McDowell (1978) investigated the reaction of DAC with different flavanols and procyanidins. Maximum absorbance was reached two

minutes after addition of the reagent to a solution containing catechin or epicatechin and the green colour faded to yellow after one to two hours. The specificity of the reagent was also investigated by measuring the reaction of a variety of phenolic compounds with the DAC reagent (McMurrough & McDowell, 1978). The only compounds that gave strong responses were catechin, epicatechin, phloroglucinol and procyanidins. McMurrough & McDowell (1978) concluded that only flavonoids with free meta-oriented hydroxy groups in the A-ring and a single bond in the 2,3-position reacts with the DAC reagent. The procyanidins gave much weaker responses than the monomeric flavanols as the reacting groups is less accesible to the reagent due to steric hindrance.

2.8.4 Flavonols and tartaric acid esters

The characteristic absorption maxima of different phenolic groups can be used to estimate their relative content (Mazza *et al*., 1999). Total phenols can also be estimated in this manner using gallic acid as standard and measuring the absorption of wine in an acidic medium at 280 nm. Phenolic acids, mostly tartaric acid esters in wine, can be estimated as caffeic acid equivalents from the absorption of wine at 320 nm in an acidic medium and flavonols can be estimated from the absorption of wine at 360 nm in an acidic medium as quercetin equivalents. This method of detection is possible as members of certain phenolic groups have the same general chemical structure and thus the same general absorption maxima (Macheix *et al*., 1990). Estimations are accurate relative to each other when comparisons are made between samples of the same general phenolic composition. When different types of samples are used, comparisons are not a good indication of relative content (Mazza *et al*., 1999).

2.9 PHENOLIC COMPOSITION OF WINE

The phenolic composition of wine depends on a variety of parameters including the phenolic composition of the grapes, the extent by which phenolic compounds are mobilised during vinification and the chemical modification of phenolic compounds during maturation (Macheix *et al*., 1990). The phenolic

composition of wine contributes to its sensory qualities such as colour, flavour, astringency and bitterness, as well as its antioxidant potential. Specific combinations of compounds are important in terms of antioxidant activity as synergistic effects may occur (Saucier & Waterhouse, 1999). The average phenolic composition of red and white wines differs substantially (Table 5).

The major types of phenolic compounds found in grapes and wine include flavanols, flavonols, anthocyanins and phenolic acids (Soleas *et al*., 1997a). Other non-flavonoid compounds include stilbenes such as resveratrol (Lamuela-Raventós & Waterhouse, 1993). These compounds exhibit structural and functional diversity.

Phenolic acids are represented in wine by hydroxybenzoic acids and hydroxycinnamic acid derivatives. Hydroxycinnamic acids generally occur in grapes and wine as tartaric acid esters, especially those of caffeic, *p*-coumaric and ferulic acids (Ribéreau-Gayon, 1972). These derivatives could also be acylated and glycosylated in different positions (Macheix *et al*., 1990). Free forms of these compounds occur in wine due to the vinification process (Rice-Evans & Packer, 1998).

Flavanols commonly occurring in wine include (+)-catechin and (-)-epicatechin (Macheix *et al*., 1990). Dimers and polymers containing these flavanols as subunits are called proanthocyanidins and occur in wines due to extraction from grape seeds (Haslam, 1980). Much greater quantities of flavanols and proanthocyanidins are found in red wines than white wines (Arts *et al*., 2000b).

Flavonols such as quercetin, myricetin, kaempferol and glycosides of these compounds with glucose and rutinose are found in wine with rutin being the most abundant (Ribéreau-Gayon, 1972). In white wine only small quantities can be found (Table 5).

Anthocyanins, the glycosylated derivatives of anthocyanidins, occur in grapes and wine (Ribéreau-Gayon, 1972). Acylated anthocyanins, where an organic acid (namely *p*-coumaric acid, caffeic acid or ferulic acid) is attached to the sugar molecule on the 3-position, are also found in grapes and wine (Ribéreau-Gayon, 1972; Wulf & Nagel, 1978). Only the monoglucosides of anthocyanins occur in red cultivars of *Vitis vinifera* grapes. Grapes from other

Table 5 Relative concentrations of phenolic acids and flavonoids in wine[a]

Phenolic group/compound	Concentration (mg/L)	
	Red wine	**White wine**
Non-flavonoids	**240 – 500**	**160 - 260**
Hydroxybenzoic acids	*0 – 260*	*0 - 100*
p-Hydroxybenzoic acid	20.0	-
Gallic acid	63.8 (3.1 - 320)	6.4 (2.8 - 11)
Total gallates	49.0 (38.6 - 58.7)	6.9 (6.8, 7)
Syringic acid	11.5 (4.9, 18)	-
Protocatechuic acid	88.0	-
Hydroxycinnamic acids	*143.1 (74.1 - 226)*	*130 - 154*
p-Coumaroyl tartaric acid	52.2 (21 - 137)	1.8
Caffeoyl tartaric acid	80.9 (13.4 - 178)	5 (3, 7)
Caffeic acid	8.7 (4.7 - 18)	3.171 (1.5 - 5.2)
p-Coumaric acid	4.7 (0.9 - 22)	2.2 (1 - 3.2)
Ferulic acid	10.9 (2.9 - 19)	-
Stilbenes	*11.1 (4 - 19)*	*1.8 (0.04 - 3.5)*
Resveratrol	1.2 (0.09 - 3.2)	0.04 (0 - 0.1)
Flavonoids	**750 - 1060**	**25 - 30**
Flavonols	*127.8 (65.3 - 238.3)*	*traces*
Quercetin	11.5 (0.5 - 28.5)	0.55 (0 - 1.2)
Myricetin	12.3 (0 - 64.5)	0.1 (0 - 0.3)
Kaempferol	1.0 (0.1 - 6)	0.1
Rutin	7.4 (0 - 31.7)	0.3 (0 - 0.9)
Flavanols	*208.8 (27.3 - 557)*	*11.5 (2 - 29)*
Catechin	94.0 (15.3 - 390)	15.4 (1.5 - 46)
Epicatechin	44.3 (9.2 - 62)	8.7 (0.5 - 60)
Procyanidins	215.0 (30.9 - 367.1)	0
Anthocyanins	*270.9 (39.4 - 469)*	*0*
Delphinidin-3-glucoside	10.9 (2.3 - 22)	0
Cyanidin-3-glucoside	38.0	0
Petunidin-3-glucoside	21 (18, 24)	0
Peonidin-3-glucoside	19 (6, 32)	0
Malvidin-3-glucoside	46.7 (0 - 206)	0
Malvidin-3-glucoside-acetate	38.2 (13.2 - 129)	0
Malvidin-3-glucoside-*p*-coumarate	15.1 (8.3 - 44)	0
Total phenolic acids and polyphenols	**1686.4 (700 - 4059)**	**177.6 (96 - 331)**

[a] Values are averages from all values reported in Arts *et al*. (2000b), Carando *et al*. (1999), Ricardo da Silva *et al*. (1990), Fogliano *et al*. (1999), Frankel *et al*. (1995), German & Walzem (2000), Ghiselli *et al*. (1998), Goldberg *et al*. (1998a), Goldberg *et al*. (1998b), Goldberg *et al*. (1999), Lamuela-Raventos & Waterhouse (1993), Mazza (1995), Mazza *et al*. (1999), Pellegrini *et al*. (2000), Ritchey & Waterhouse (1999), Simonetti *et al*. (1997), Soleas *et al*. (1997b). Values in parentheses indicates the range of values reported.

species or hybrids of *Vitis vinifera* with other species can, therefore, be distinguished by the presence of anthocyanin diglucosides (Singleton & Esau, 1969). Copigmentation of anthocyanins with other flavonoids and phenolic acids such as catechin, caffeic acid, ferulic acid and chlorogenic acid occur and contributes to the colour of red wine (Osawa, 1982; Brouillard & Dangles, 1994; Markovíc *et al*., 2000; Darias-Martín *et al*., 2001).

2.9.1 Effect of phenolic composition of grapes

The most important factor in determining the phenolic composition of the grapes used for making wine is related to the specific grape species and cultivar used. Wine grapes from Europe and many other countries are mostly *Vitis vinifera*, the European wine grape, of which many varieties are known, while in some parts of America other *Vitis* spp. such as *Vitis labrusca* and *Vitis rotundiflora* and hybrids of these species with *Vitis vinifera* are commonly used (Ribéreau-Gayon, 1972). Red grape cultivars contain anthocyanins, which are not present in white grape cultivars (Macheix *et al*., 1990). Pinot Noir wines are characterised by high levels of the flavanols, catechin and epicatechin, and low levels of quercetin and *p*-coumaric acid (Goldberg *et al*., 1998a; Goldberg *et al*., 1998b). Generally Merlot wines have a higher content of flavanols than Cabernet Sauvignon wines (Goldberg *et al*., 1998a). Goldberg *et al*. (1999) compared a few white cultivar wines on the basis of flavanol, *p*-coumaric acid and *trans*-resveratrol content. They found very high flavanol contents for wines made from French hybrid grapes. Chardonnay and Sauvignon blanc wines also contained more flavanols than Gewürztraminer and Riesling wines. The *p*-coumaric acid and *trans*-resveratrol content of different white cultivars wines did not differ appreciably (Goldberg *et al*., 1999). Soleas *et al*. (1997b) found that Pinot Noir wines had the highest content of polydatin, but the lowest content of resveratrol amongst five red wine cultivars.

Other factors influencing the phenolic composition of grapes include climatic conditions, soil conditions and exposure to sunlight (Jackson & Lombard, 1993). Low night temperatures, low mean day temperatures or exposure to high-intensity sunlight during grape maturation promote high levels of total phenols and anthocyanins, while excessive soil moisture,

shading of grapes and high crop loads leads to low total phenol and anthocyanin content of the grapes (Jackson & Lombard, 1993). High day temperatures are associated with a lower content of anthocyanins in red grapes (Kliewers, 1970) as evidenced by the higher concentrations of catechin, epicatechin, quercetin and *p*-coumaric acid reported for wines grown in warmer climates such as New World regions (California, Australia and South Africa) (Goldberg *et al*.,1998a; 1998b).

2.9.2 Effect of vinification processes

The major differences in the phenolic composition between red and white wine are mainly due to differences in vinification techniques. The extraction of compounds from the grape is not only influenced by contact time between the grape juice and skins, but parameters such as high temperatures, addition of bisulphite as preservative, crushing of grapes, high ethanol content and homogenisation of wine also increase extraction (Oszmianski *et al*., 1986; Macheix *et al*., 1990; Bakker *et al*., 1998).

The content of phenolic compounds in the skins and seeds are much higher than in the pulp (Macheix *et al*., 1990). Proanthocyanidins such as procyanidin B1, B2, B3 and B4 are mainly present in the seeds, while flavonols, flavanols and anthocyanins are located in the skins. Fermentation on the grape pomace (skins and seeds) causes the extraction of phenolic compounds from the skins and seeds. Red wines are usually produced by fermentation on the grape pomace, while white wines are generally produced by fermentation of the grape juice after removal of pomace. Sun *et al*. (1998) showed the importance of pomace contact during red wine production in terms of extraction of catechins and proanthocyanidins. Increased proanthocyanidin and total phenol contents were reported for an experimental white wine made with pomace contact (Arnold & Noble, 1979; Ricardo da Silva *et al*., 1993; Auw *et al*., 1996). Pomace contact during the making of white wine also increased its ability to inhibit low-density lipoprotein peroxidation (Hurtado *et al*., 1997). The use of carbonic maceration where whole grapes are fermented under a carbon dioxide atmosphere changes the phenolic composition drastically (Ricardo da Silva *et al*., 1993; Pellegrini *et al*., 2000). Carbonic maceration increases the content of procyanidins (Ricardo

da Silva *et al*., 1993), tartaric acid esters (Ricardo da Silva *et al*., 1993) and anthocyanins (Pellegrini *et al*., 2000). No significant difference was, however, found between the antioxidant activity of wines made using carbonic in comparison with traditional maceration (Pellegrini *et al*., 2000).

2.9.3 Effect of maturation

Two stages of maturation can be distinguished after fermentation of grapes to produce wine. The first stage involves maturation of wines in wooden barrels, while the second phase occurs after bottling (Singleton & Esau, 1969).

2.9.3.1 Maturation in wooden barrels

Maturation of wines in wooden barrels causes changes in phenolic composition due to the extraction of phenolic compounds from the wood. Compounds such as ellagic acid, gallic acid, ellagitannins, and aldehydes and acids related to vanillin are commonly extracted during maturation in wooden barrels or during maturation in steel tanks with the addition of wood chips (Singleton & Esau, 1969). Sugars such as glucose, fructose, xylose, arabinose and rhamnose also increase during wood maturation due to deglycosylation of phenolic tannins in the wood (Del Alamo *et al*., 2000). Evaporation losses and extraction effects tend to increase phenolic content during wood maturation, but precipitation reactions of polymeric phenolic compounds generally outweigh these effects (Singleton & Esau, 1969).

Oxidative changes are also important during this maturation phase as oxygen is readily absorbed in the wine (Ribéreau-Gayon & Glories, 1986; Singleton, 1987). Oxidative reactions in white wines generally cause unacceptable browning and are avoided to prevent loss of quality (Singleton, 1987). In red wines, the oxidation of some of the phenolic compounds has a positive effect on the organoleptic quality of wines. These oxidised phenolic compounds can participate more readily in condensation reactions during in-bottle ageing than the parent compounds (Ribéreau-Gayon & Glories, 1986). Oxidation of easily oxidised wine phenolic compounds also contribute to lower levels of these compounds in aged wines (Singleton, 1987).

2.9.3.2 In-bottle ageing

In-bottle ageing of wines is the second stage of maturation and introduces changes to the phenolic composition, especially in the case of red wines. The change is mostly the condensation of simple, monomeric phenolics to yield oligomeric and polymeric forms. Oxidative reactions are not very important during this phase as wine in bottles is protected from oxygen to a considerable extent (Ribéreau-Gayon & Glories, 1986). Despite this fact, Singleton (1987) attributed the decrease of tartaric acid ester content after bottling to oxidation phenomena.

The first change observed in red wines is the decrease of monomeric anthocyanins and the simultaneous increase in more stable polymeric condensation products of anthocyanins and other flavonoids. These changes strongly affect the colour and visual appearance of the wine as the violet-red colour fades to brownish-red. Free anthocyanins may even disappear completely after a time dependent on storage conditions such as temperature and content of sulphur dioxide and acetaldehyde (Nagel & Wulf, 1979). Higher temperatures will promote degradation of anthocyanins. Sulphur dioxide contribute to bleaching of anthocyanins (Bakker *et al*., 1998), while acetaldehyde promote condensation of anthocyanins with flavanols to give polymeric forms (Saucier *et al*., 1997). Nagel & Wulf (1979) found that the total anthocyanin concentration of a Cabernet Sauvignon wine decreased from 255.1 ppm to 22.3 ppm during storage for 7 months at 22ºC after the end of vinification. The disappearance of highly coloured monomeric compounds is the basis for measuring the “chemical age” of a wine (Somers & Evans, 1977). Condensation of anthocyanins with flavanols in the presence of acetaldehyde is one of the most important reactions contributing to colour changes during ageing of red wine (Jurd, 1967; Timberlake & Bridle, 1976; Saucier *et al*., 1997). Condensation reactions such as these causes the formation of complex, polymeric pigments in red wine. Other changes in composition that have been reported include decreases in flavanol and tartaric acid ester contents (Nagel & Wulf, 1979; Gómez-Plaza *et al*., 2000). Cafffeoyltartartaric and *p*-coumaroyltartaric acid content of a young red wine decreased to 54% and 29% respectively of the original content after 12

months, while the catechin and epicatechin content decreased to 26% and 20% of the original content (Gómez-Plaza *et al*., 2000). Procyanidins may undergo self-dissociation reactions producing catechin and epicatechin or higher and lower polymeric forms (Haslam, 1980).

2.10 SUMMARY

Oxidative stress in the cell occurs during disease conditions or when optimal nutrition is absent. In these circumstances, reactive oxygen species are available to initiate lipid peroxidation or damage to other biomolecules. Antioxidants, such as phenolic compounds, can play a role to inactivate harmful reactive oxygen species. Antioxidant assays measure different aspects of antioxidant activity and the necessity exists to use several test systems to fully characterise the antioxidant properties of compounds or foods. The activity of antioxidants depends on their ability to scavenge free radicals and chelate metal ions, which strongly relates to their chemical structure. The many phenolic compounds present in wine have different antioxidant activities and synergistic effects between compounds are likely. This hampers the prediction of antioxidant activity from the phenolic composition alone. Differences in phenolic composition due to cultivar, vinification processes, maturation in wood and in-bottle ageing will affect the antioxidant potential of wines. Factors such as absorption and distribution of antioxidant molecules, as well as structural changes occurring during *in vivo* metabolisation, will influence potential bioactivity.

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CHAPTER 3

ANTIOXIDANT ACTIVITY OF SOUTH AFRICAN RED AND WHITE CULTIVAR WINES: FREE RADICAL SCAVENGING ACTIVITY

Abstract

The free radical scavenging activity of South African red (Cabernet Sauvignon, Ruby Cabernet, Pinotage, Shiraz, Merlot) and white (Sauvignon blanc, Chenin blanc, Chardonnay and Colombard) cultivar wines was determined using the 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) radical cation ($ABTS^{\bullet+}$) and the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging assays. The free radical scavenging activity of red wines was much higher than that of white wines when compared "as-is", as well as on the basis of a similar total phenol content. The total antioxidant activity (TAA) of Ruby Cabernet wines was lower than that of other red cultivar wines. Chardonnay and Chenin blanc wines had the highest and lowest TAA and AP of white cultivar wines respectively. A significant correlation was noticed between the TAA measured using the $ABTS^{\bullet+}$ scavenging assay and total phenol content of both red ($r = 0.94$, $P < 0.001$) and white wines ($r = 0.91$, $P < 0.001$). The main phenolic groups correlating with TAA in the $ABTS^{\bullet+}$ scavenging assay were flavanols for red wines ($r = 0.87$, $P < 0.001$) and tartaric acid esters ($r = 0.73$, $P < 0.001$) for white wines. Similar trends were also observed for the TAA measured using the $DPPH^{\bullet}$ scavenging assay. Canonical discriminant analysis aided differentiation between the red and white cultivar wines based on parameters for phenolic composition and antioxidant characteristics.

3.1 Introduction

Oxidative stress resulting from imbalances in the antioxidant status (reactive oxygen species vs. defence mechanisms) contributes to free radical-mediated diseases such as cancer, atherosclerosis, ischemic heart disease and neurodegenerative diseases (Halliwell & Gutteridge, 1990; Davies, 1995; Leake, 1998). Recently the possible role of dietary antioxidants to protect against reactive oxygen species in addition to endogenous enzymatic defence

mechanisms has been investigated (Rice-Evans & Miller, 1996; Miller & Rice-Evans, 1997; Simonetti *et al*., 1997; Prior & Cao, 1999).

Wine as a source of dietary antioxidants is receiving more and more prominence (Kinsella *et al*., 1993; Soleas *et al*., 1997a; Halliwell, 2000) due to the possible link between a high intake of red wine and the low incidence of coronary heart disease in France (St Leger *et al*., 1979; Renaud & De Lorgeril, 1992). Red wine contains phenolic compounds with high *in vitro* free radical scavenging activity compared to other beverages such as beer, tea and fruit juices (Paganga *et al*., 1999). The intake of red wine and foods containing these phenolic compounds is reported to increase the antioxidant content and status of human blood plasma (Cao *et al*., 1998a; Cao *et al*., 1998b; Sung *et al*., 2000).

The principle phenolic compounds in wine include hydroxybenzoic acids, hydroxycinnamic acid derivatives, flavanols, flavonols and anthocyanins (Macheix *et al*., 1990). Differences in the phenolic content of grape cultivars and vinification techniques contribute to differences in phenolic composition of wines (Macheix *et al*., 1990). Fermentation of red wine on the grape seeds and skins allows more extensive extraction of phenolic components such as flavanols, flavonols and proanthocyanidins than in the case of white wines where pomace contact is generally kept to a minimum (Oszmianski *et al*., 1986; Macheix *et al*., 1990; Sun *et al*., 1998). White wine contains no anthocyanins as these compounds only occur in the skins of red grapes (Singleton & Esau, 1969).

A few studies have evaluated the antioxidant activity of different wines as affected by vinification techniques (Hurtado *et al*., 1997; Manzocco *et al*., 1998; Larrauri *et al*., 1999; Pellegrini *et al*., 2000) and cultivar (Frankel *et al*., 1995; Sato *et al*., 1996; Soleas *et al*., 1997b; Burns *et al*., 2000). Most of these studies only made use of a very small sample size. In the only study up to date on South African wines the activity of fractions of 6 red and 6 white wines were evaluated (Brand, 1998). The antioxidant activity of these wine phenolic fractions was based on the inhibition of low-density lipoprotein peroxidation and did not attempt to differentiate between different cultivar wines.

In most of the studies investigating the modifying effect of vinification or cultivar on the antioxidant activity of wines, synthetic radicals, namely 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) radical cations ($ABTS^{\bullet+}$) (Verhagen *et al*., 1996; Simonetti *et al*., 1997; Soleas *et al*., 1997b; Pellegrini *et al*., 2000), 2,2-diphenyl-1-picrylhydrazyl radicals ($DPPH^{\bullet}$) (Manzocco *et al*., 1998; Sánchez-Moreno *et al*., 1999) and *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride radicals ($DMPD^{\bullet}$) (Fogliano *et al*., 1999) were used. Standardisation of methods has not been introduced yet, which makes comparison of wines from different countries problematic.

The aim of the study was to determine the free radical scavenging activity of a wide selection of South African wines representative of the major red and white wine grape cultivars. Chenin blanc, Colombar, Sauvignon blanc and Chardonnay grapes constituted 34, 24, 6 and 5% respectively of the white wine production during the 2000 season, while Pinotage, the unique South African grape cultivar, Cabernet Sauvignon, Merlot, Shiraz and Ruby Cabernet grapes constituted 21, 18, 10, 8 and 8% respectively of the red wine production (Anonymous, 2001). The two most widely used free radical scavenging methods utilising $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, were chosen to compare the antioxidant activity of South African red and white wines.

3.2 Materials and Methods

Spectrophotometric measurements were performed on a Beckman DU-65 UV/VIS spectrophotometer (Beckman, Cape Town, South Africa) using a 1 cm path length cuvette.

3.2.1 Wines and chemicals

A total of 86 randomly selected cultivar wines comprising of the red wines (1998 vintage), Cabernet Sauvignon (10), Ruby Cabernet (7), Pinotage (10), Shiraz (10) and Merlot (9), and the white wines (1999 vintage), Sauvignon blanc (10), Chenin blanc (10), Chardonnay (10) and Colombar (10) were obtained from wineries in the major wine-producing regions of the Western

Table 1 Red (1998) and white (1999) wines purchased for comparison of antioxidant activity of different red and white cultivar wines

Sample code	Wine	Origin
CS1	Franschhoek Vinyards Cabernet Sauvignon	Franschhoek
CS2	Stony Brook Cabernet Sauvignon	Franschhoek
CS3	Boland Cabernet Sauvignon	Paarl
CS4	Zanddrift Cabernet Sauvignon	Paarl
CS5	Bon Courage Cabernet Sauvignon	Robertson
CS6	Nuy Cabernet Sauvignon 1998	Worcester
CS7	Slanghoek Cabernet Sauvignon	Worcester
CS8	Villiersdorp Cabernet Sauvignon	Villiersdorp
CS9	Zevenrivieren Cabernet Sauvignon (Zevenwacht)	Kuilsriver
CS10	Vlottenburg Cabernet Sauvignon	Stellenbosch
RC1	Ashton Ruby Cabernet	Ashton
RC2	Langverwacht Ruby Cabernet	Bonnievale
RC3	McGregor Ruby Cabernet	McGregor
RC4	Uitvlucht Ruby Cabernet	Montagu
RC5	Robertson Ruby Cabernet	Robertson
RC6	Merwida Ruby Cabernet	Worcester
RC7	Goudini Ruby Cabernet	Worcester
P1	De Wet Pinotage	Worcester
P2	Du Toitskloof Pinotage	Worcester
P3	Landskroon Pinotage	Paarl
P4	Zanddrift Pinotage	Paarl
P5	Rooiberg Pinotage	Robertson
P6	HL Du Preez Pinotage	Worcester
P7	Bellingham Pinotage	Franschhoek
P8	Simonsig Pinotage	Stellenbosch
P9	Vlottenburg Pinotage	Stellenbosch
P10	Simonsvlei Pinotage	Paarl
Sh1	Ashton Shiraz	Ashton
Sh2	Landskroon Shiraz	Paarl
Sh3	Backsberg Shiraz	Paarl
Sh4	Fairview Shiraz	Paarl
Sh5	Rooiberg Shiraz	Robertson
Sh6	Bellingham Shiraz	Franschhoek
Sh7	Simonsig Shiraz	Stellenbosch
Sh8	Hercules Paragon Shiraz (Simonsvlei)	Paarl
Sh9	Zevenwacht Shiraz	Kuilsrivier
Sh10	Kleinbosch Shiraz	Stellenbosch
M1	Dieu Donné Merlot	Franschhoek
M2	Mont Rochelle Merlot	Franschhoek
M3	Vlottenburg Merlot	Stellenbosch
M4	Boland Merlot	Paarl
M5	Landskroon Merlot	Paarl
M6	Jordan Merlot	Stellenbosch
M7	Rooiberg Merlot	Robertson
M8	Graham Beck Merlot	Robertson
M9	Villiera Merlot	Stellenbosch

Table 1 (continued…) Red (1998) and white (1999) wines purchased for comparison of antioxidant activity of different red and white cultivar wines

Sample code	Wine	Area
SB1	Ashton Sauvignon blanc	Ashton
SB2	Villiersdorp Sauvignon blanc	Villiersdorp
SB3	Nuy Sauvignon blanc	Worcester
SB4	Wamakersvallei Sauvignon blanc	Wellington
SB5	Backsberg Sauvignon blanc	Paarl
SB6	Rooiberg Sauvignon blanc	Robertson
SB7	Mont Rochelle Sauvignon blanc	Franschhoek
SB8	Green Door Sauvignon blanc (Delaire)	Stellenbosch
SB9	La Cotte Sauvignon blanc	Franschhoek
SB10	Delheim Sauvignon blanc	Stellenbosch
Chen1	Uitvlucht Chenin blanc	Bonnievale
Chen2	Boland Chenin blanc	Paarl
Chen3	Landskroon Chenin blanc	Paarl
Chen4	Rooiberg Chenin blanc	Robertson
Chen5	Goudini Chenin blanc	Worcester
Chen6	Brandvlei Chenin blanc	Worcester
Chen7	Villiersdorp Chenin blanc	Villiersdorp
Chen8	Porcupine Ridge Chenin blanc	Franschhoek
Chen9	Delheim Chenin blanc	Stellenbosch
Chen10	Simonsig Chenin blanc	Stellenbosch
Char1	Spes Bona Chardonnay (Van Loveren)	Ashton
Char2	Langverwacht Chardonnay	Montagu
Char3	Ashtonvale Chardonnay (Zandvliet)	Ashton
Char4	Wamakersvallei Chardonnay	Wellington
Char5	Bon Courage Chardonnay	Robertson
Char6	Bovlei Chardonnay	Wellington
Char7	Brandvlei Chardonnay	Worcester
Char8	De Doorns Chardonnay	Worcester
Char9	Dassie's Reserve Chardonnay (Botha)	Worcester
Char10	La Cotte Chardonnay	Franschhoek
Col1	Ashton Colombar	Ashton
Col2	Ashtonvale Colombar (Zandvliet)	Ashton
Col3	Van Loveren Colombar	Ashton
Col4	Swartland Colombar	Malmesbury
Col5	Uitvlucht Colombar	Bonnievale
Col6	Robertson Colombar	Robertson
Col7	Rooiberg Colombar	Robertson
Col8	Bon Courage Colombar	Robertson
Col9	Nuy Colombar	Worcester
Col10	Aan de Doorns Colombar	Worcester

Cape, South Africa (Table 1). Red wines were matured in wood for varying times, while none of the white wines were aged in wood.

The following chemicals were used: 2,2'-azino-di-(3-ethylbenzo-thialozine-sulphonate) diammonium salt (ABTS) and ascorbic acid enzymatic test kit (Boehringer Mannheim GmbH, Mannheim, Germany); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Aldrich Chemical Co., Gillingham, Dorset, UK); potassium persulphate ($K_2S_2O_8$), (+)-catechin and gallic acid (Sigma Chemical Co., St. Louis, USA); Folin-Ciocalteau's phenol reagent and quercetin (Merck, Darmstadt, Germany); 4-dimethylamino-cinnamaldehyde (DAC) and caffeic acid (Fluka AG, Buchs, Switzerland); methanol (AR) (Riedel-de Häen, AG Seelze-Hanover, Germany). The water was purified and de-ionised with a Modulab water purification system prior to use (Separations, Cape Town, South Africa).

3.2.2 Sample preparation

Aliquots of each wine were frozen at –18 °C in plastic screw-top sample holders (40 ml) to preserve the phenolic compounds after the bottle was opened until analyses were carried out. The wines were defrosted prior to use and sonicated to dissolve any precipitates present.

3.2.3 Determining the phenolic composition

The total phenol content of wines was determined at 765 nm after reaction of appropriately diluted wine samples (red wines 40 times and white wines 7 times diluted with 10% ethanol) with the Folin-Ciocalteau reagent (Singleton & Rossi, 1965). Gallic acid was used as standard and results were expressed as mg gallic acid equivalents/L (mg GAE/L). Possible interference of sulphur dioxide in the determination of total phenols was investigated before determination of total phenols. A number of samples were analysed before and after addition of acetaldehyde to bind sulphur dioxide (Singleton *et al*., 1999; C. Saucier, Université Victor Segalen, Bordeaux, France, personal communication). The effect of addition of sulphur dioxide to wine on its total phenol content was also investigated (Addendum A).

Anthocyanin content was estimated according to a pH differential method of Burns *et al*. (2000). The absorbance of wine at pH < 1 and pH 3.5 was measured at 520 nm. The absorbance at 700 nm was also measured to correct for turbidity. Total, monomeric and polymeric anthocyanins were quantified as malvidin-3-glucoside (Mv-3-glc) equivalents (the major anthocyanin in red wine) using its molar extinction coefficient, ε = 28 000. The absorbance at pH < 1 was used to calculate the total anthocyanins as all anthocyanins are in the coloured flavylium form. At pH 3.5 monomeric anthocyanins are in the colourless carbinol form and only interfering substances and polymeric anthocyanins absorb. The difference in absorbance was used to calculate the content of monomeric or free anthocyanins.

The flavanol content of wines was measured at 640 nm after reaction of appropriately diluted wines (red wines 40 times diluted with 10% ethanol and white wines undiluted) with DAC reagent (McMurrough & McDowell, 1978). (+)-Catechin was used as a standard and the results expressed as mg catechin equivalents/L (mg CE/L).

The flavonol and tartaric acid ester contents were estimated by measuring the absorbance of the wines at 360 nm and 320 nm after addition of 2% HCl (Mazza *et al*., 1999). Red wines were diluted ten times with 10% ethanol and white wines were used undiluted. Quercetin and caffeic acid were used as standards for the estimation of flavonol and tartaric acid ester contents, respectively. Results were expressed as mg quercetin equivalents/L (mg QE/L) and mg caffeic acid equivalents/L (mg CAE/L) respectively.

3.2.4 Measurement of the 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) radical cation ($ABTS^{\bullet+}$) scavenging activity

The total antioxidant activity (TAA) of wines was determined using the $ABTS^{\bullet+}$ scavenging assay of Re *et al*. (1999) adapted from Miller *et al*. (1993). The effect of addition of sulphur dioxide to wine on its TAA was also investigated (Addendum A). An ABTS solution (7 mM) in water was pre-incubated for at least 12 h with 2.45 mM (final concentration) $K_2S_2O_8$ to produce the radical

cation, $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of ca. 0.7 (± 0.02) at 734 nm. After addition of 1 ml $ABTS^{\bullet+}$ solution to 50 µl of appropriately diluted wine samples (red wines 50 times and white wines 5 times diluted with 10% ethanol), standard Trolox solution (0 – 400 µM) in ethanol or 10% ethanol (control) the reaction mixture was vortexed (Scientific Industries, South Africa) and incubated immediately in a water bath at 37 °C. The absorbance of the mixture was determined 4 min. after initiation of the reaction. All determinations were carried out in triplicate and results averaged.

The concentration of $ABTS^{\bullet+}$ in the control and samples was calculated using the absorbance readings and the molar extinction coefficient of $ABTS^{\bullet+}$, ε = 16 000 (Re *et al*., 1999):

$$[ABTS^{\bullet+}](\mu M) = \frac{Abs_{734nm}}{\varepsilon (ABTS^{\bullet+})} \times 1000\,000$$

where Abs_{734nm} = absorbance of reaction mixture at 734 nm

$\varepsilon (ABTS^{\bullet+})$ = molar extinction coefficient of $ABTS^{\bullet+}$ in ethanol

The amount of $ABTS^{\bullet+}$ scavenged (relative to the control containing no antioxidant) was determined for each sample:

$$\text{nanomoles } ABTS^{\bullet+} \text{ scavenged} = ([ABTS^{\bullet+}]_C - [ABTS^{\bullet+}]_S) \times RV$$

where $[ABTS^{\bullet+}]_C$ = $ABTS^{\bullet+}$ concentration in control (in µM)

$[ABTS^{\bullet+}]_S$ = $ABTS^{\bullet+}$ concentration in sample (in µM)

RV = reaction volume (in ml) = 1.05 ml

A plot (Figure 1) of nanomoles $ABTS^{\bullet+}$ scavenged against concentration of Trolox in the standard samples was used to calculate the TAA_{ABTS} of the wines. The TAA is the concentration of Trolox needed to obtain a free radical

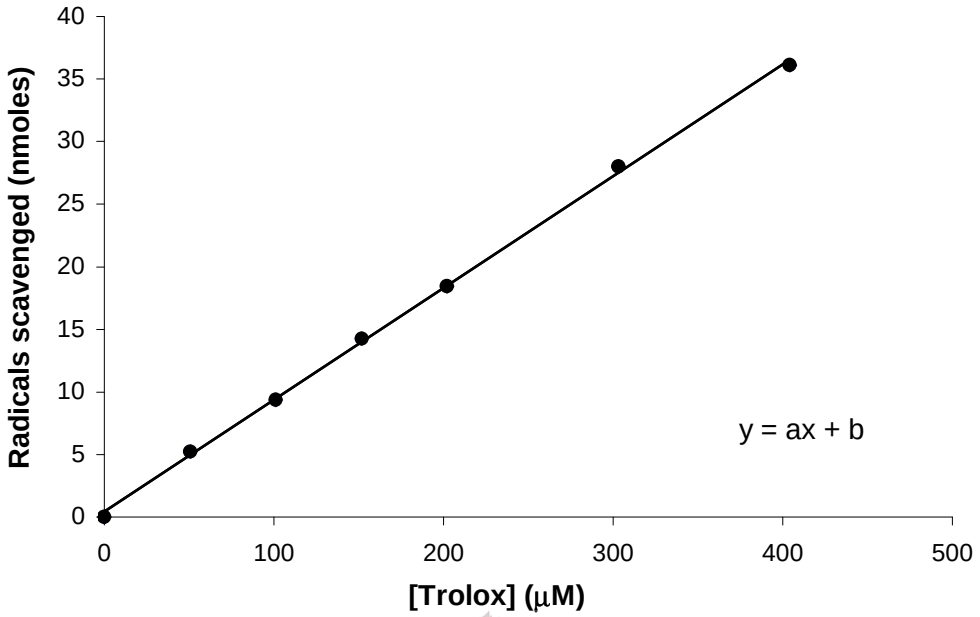

Figure 1 A graphical illustration of a plot of ABTS radical cation or DPPH radical scavenging activity as a function of Trolox concentration. The plot is used to calculate the total antioxidant activity of wine samples.

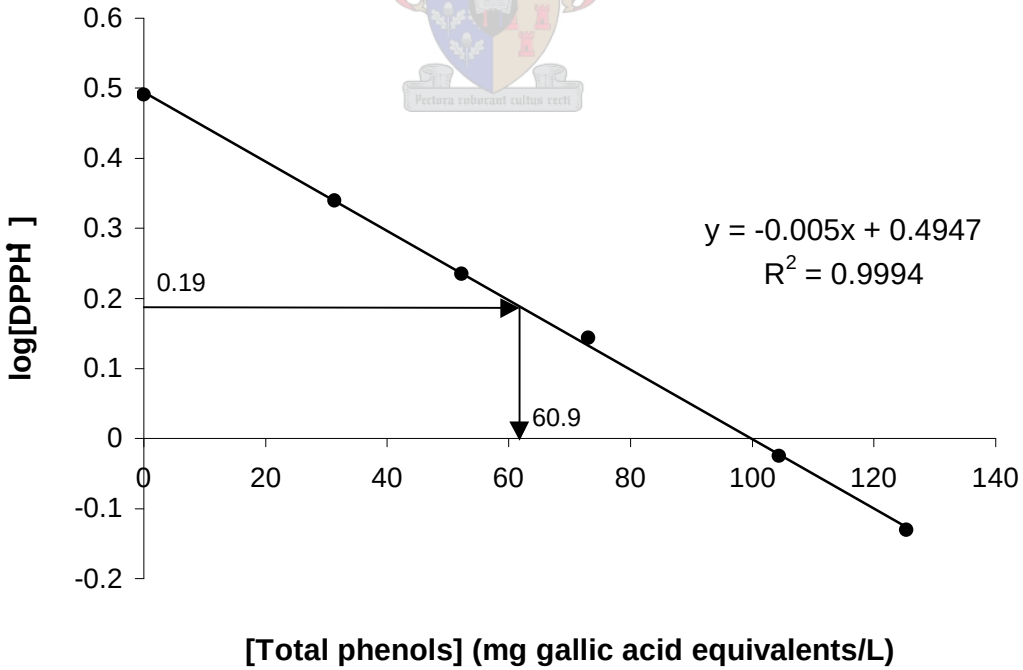

Figure 2 A semilogarithmic plot of the DPPH radical scavenging activity against total phenol content for the estimation of EC_{50} values for wines.

scavenging activity equivalent to that of the wine under investigation on an "as-is" basis. Antioxidant potency (AP) of wine phenolic fractions were calculated as the ratio of TAA to total phenols:

$$\text{Antioxidant potency (AP)} = \frac{\text{TAA}}{\text{Total phenols}} \times 1000$$

where TAA = Total antioxidant activity (mM Trolox)
Total phenols = total phenol content (mg GAE/L)

3.2.5 Measurement of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activity

The free radical scavenging ability of the wines was quantified in terms of their ability to scavenge the stable free radical, DPPH•. Scavenging of DPPH• was determined according to a modified version of the method of Brand-Williams *et al*. (1995). The effect of addition of sulphur dioxide to wine on its total phenol content was also investigated (Addendum A). An appropriate dilution series was prepared for each red (0 - 150 mg GAE/L) and white (0 - 250 mg GAE/L) wine in 10% ethanol and 50 µl of the diluted samples or 10% ethanol (control) was added to 2 ml of a 3.04×10^{-5} M methanolic solution of DPPH•, followed by vortexing (Scientific Industries, South Africa). References for each sample containing 50 µl of diluted sample and 2 ml of methanol were also incubated to correct for any inherent absorbance interference by wines. The reaction was carried out in the dark for 2 h (at room temperature) until steady state conditions were reached and the absorbance determined at 515 nm in order to calculate the concentration of remaining DPPH•. A DPPH• concentration calibration curve with the following equation (as determined by linear regression) was prepared to estimate the remaining DPPH• concentration:

$Abs_{515nm} = 0.1198 \times [DPPH^{\bullet}] - 0.0119$ (r = 0.977) (n = 4)

where Abs_{515nm} = the absorbance at 515 nm

$[DPPH^{\bullet}]$ = the concentration of $DPPH^{\bullet}$

The concentration of the remaining $DPPH^{\bullet}$ at steady state conditions was calculated and the values plotted on a graph showing $[DPPH^{\bullet}]$ or $\log([DPPH^{\bullet}])$ as a function of the total phenol concentration of the wine sample (Figure 2). The EC_{50} value (the total phenol concentration of wine required to scavenge 50% of the initial $DPPH^{\bullet}$ in the reaction mixture) was estimated. All determinations were carried out in duplicate and the results averaged.

The initial scavenging rate for all the wines was determined during unsteady state conditions. Absorbance was read at 515 nm every 6 seconds for the first 5 min. of the reaction. The initial scavenging rate was expressed as the absolute value of the gradient of the line from a plot of the data for the first minute.

The Radical Scavenging Efficiency (RSE), a new parameter combining scavenging activity in terms of the amount of radicals scavenged and the initial scavenging rate, is defined as follows:

$$RSE = \frac{\text{initial scavenging rate}}{EC_{50}} \times 1000$$

where RSE = Radical Scavenging Efficiency

initial scavenging rate = absolute value of the gradient for the plot of $DPPH^{\bullet}$ concentration as a function of time for the first min. of the reaction

EC_{50} = total phenol concentration required to scavenge 50% of the initial $DPPH^{\bullet}$ concentration

The TAA_{DPPH} of the wines was calculated like the TAA_{ABTS} using a plot of the nanomoles of $DPPH^{\bullet}$ scavenged against the concentration of Trolox in a series of standard samples (Figure 1). This parameter gives the TAA as mM Trolox with activity equivalent to that of the wine under investigation on an "as-is" basis. The AP_{DPPH} of wine phenolic fractions was also calculated as described for AP_{ABTS}.

3.2.6 Determination of the contribution of ascorbic acid to the free radical scavenging activity

The ascorbic acid content of white wines was determined with an enzymatic test kit from Boehringer Mannheim, Mannheim, Germany.

The contribution of ascorbic acid to the total antioxidant activity of wines was calculated using the TEAC (Trolox Equivalent Antioxidant Capacity) of ascorbic acid (Rice-Evans *et al*., 1996). The TEAC of ascorbic acid is 0.99, which means that a 0.99 mM Trolox solution has an equivalent antioxidant activity to a 1 mM ascorbic acid solution. The ascorbic acid contribution to the TAA was calculated as follows:

$$\text{Ascorbic acid contribution} = [AA] \times 0.99$$

where [AA] = ascorbic acid concentration in mM

3.2.7 Statistical analysis

All tests were carried out in duplicate (EC_{50} values) or triplicate (all other tests) with red and white wines randomised. One way ANOVA was performed on the means to determine whether they differed significantly. Statistical comparisons between means for cultivars were made using the Student's t-LSD test ($P < 0.05$). Canonical discriminant analysis was used to differentiate between cultivars on the basis of phenolic composition (total phenol, anthocyanin (red wine only), flavanol and flavonol tartaric acid ester contents) and antioxidant activity (TAA_{ABTS}, EC_{50}, initial scavenging rate, RSE and TAA_{DPPH}). The SAS version 6.12 software package was used for statistical analysis.

3.3 Results and Discussion

3.3.1 Phenolic composition

The mean concentrations of total phenols, monomeric anthocyanins (for red wines), flavanols, flavonols and tartaric acid esters in wines for each grape cultivar are summarised in Table 2, while the ratio of each phenolic group to the total phenol content is reported in Table 3. Total phenol content of wines was determined without the addition of acetaldehyde to bind free sulphur dioxide. A preliminary test on 10 white and 10 red wine samples showed no difference in results with and without the addition of acetaldehyde (data not shown). Red wine contained approximately 8 times more total phenols than white wine. A contributing factor is the presence of anthocyanins in red wine. Apart from anthocyanins, the content of all other phenolic groups was far greater in the red wines than in white wines. The monomeric anthocyanins, polymeric anthocyanins, flavanols, flavonols and tartaric acid esters contributed 5.87, 3.67, 10.21, 3.16 and 10.10% of total phenols respectively in red wine (Table 3). In the case of white wine flavanols, flavonols and tartaric acid esters constituted 2.27, 2.94 and 15.3% of total phenols respectively (Table 3).

The total phenol content of Ruby Cabernet (2016.0 mg GAE/L) was significantly ($P < 0.05$) lower than that of Shiraz (2412.4 mg GAE/L) and Merlot (2498.8 mg GAE/L) (Table 2). However, the monomeric anthocyanin content of Ruby Cabernet (168.21 mg Mv-3-glc equivalents/L) wines was significantly ($P < 0.05$) higher than the other red cultivar wines (115.28 – 138.31 mg Mv-3-glc equivalents/L). The relative contribution of anthocyanins to total phenols (8.67% of total phenols) was significantly ($P < 0.05$) higher for Ruby Cabernet than is the case for the other red cultivar wines (4.97 – 5.68% of total phenols). In addition, the contribution of polymeric anthocyanins to total phenols for Ruby Cabernet (4.18% of total phenols) was higher than that of other red cultivar wines (3.54 – 3.62% of total phenols). On the other hand, its flavanol content (189.14 mg CE/L) was the lowest of all red cultivar wines (239.07 – 265.82 mg CE/L) and contributed only 9.39% to the total phenol content in contrast to 10.04 – 10.49% for other red cultivar wines. Shiraz

Table 2 Phenolic composition of different South African cultivar wines[a]

Wine	Total phenols[b]	Monomeric antho-cyanins[c]	Polymeric antho-cyanins[c]	Flavanols[d]	Flavonols[e]	Tartaric acid esters[f]
Red						
Cabernet Sauvignon	2344.4 ab[g] (± 262.8)[h]	115.28 b (± 31.74)	82.65 a (± 14.98)	247.63 a (± 54.51)	50.78 c (± 27.98)	188.19 c (± 33.09)
Ruby Cabernet	2016.0 b (± 364.2)	168.21 a (± 39.85)	85.17 a (± 27.12)	189.14 b (±38.49)	66.52 bc (± 33.89)	214.75 bc (± 47.36)
Pinotage	2342.4 ab (± 411.4)	128.12 b (± 39.16)	83.59 a (± 17.51)	239.07 ab (± 62.41)	66.62 bc (± 24.02)	240.64 ab (± 32.28)
Shiraz	2412.4 a (± 450.6)	138.31 b (± 23.06)	85.01 a (± 15.82)	253.16 a (± 60.75)	99.58 a (± 37.45)	271.81 a (± 48.54)
Merlot	2498.8 a (± 410.7)	130.62 b (± 15.02)	90.24 a (± 24.03)	265.82 a (± 70.02)	88.60 ab (24.84)	252.63 ab (± 46.64)
Average	**2339.0**	**133.48**	**85.24**	**241.63**	**74.63**	**234.42**
White						
Sauvignon blanc	266.7 ab (± 31.2)	N/A	N/A	6.98 ab (± 2.76)	6.72 b (± 2.92)	46.11 a (± 8.02)
Chenin blanc	242.0 b (± 52.4)	N/A	N/A	3.59 c (± 1.76)	6.34 b (± 2.94)	35.27 b (± 8.39)
Chardonnay	292.7 a (± 28.7)	N/A	N/A	9.52 a (± 4.37)	12.11 a (± 3.30)	46.36 a (± 4.97)
Colombar	268.4 ab (± 53.8)	N/A	N/A	5.51 bc (± 2.30)	7.85 b (± 5.84)	39.29 ab (± 13.90)
Average	**273.8**			**6.23**	**8.22**	**41.77**

N/A Not applicable.

[a] Detailed results for each wine are reported in Addendum B.

[b] Total phenol content expressed as mg gallic acid equivalents/L.

[c] Anthocyanin content expressed as mg malvidin-3-glucoside equivalents/L.

[d] Flavanol content expressed as mg catechin equivalents/L.

[e] Flavonol content expressed as mg quercetin equivalents/L.

[f] Tartaric acid ester content expressed as mg caffeic acid equivalents/L.

[g] Averages in a column followed by different letters, differ significantly ($P < 0.05$). Data for red and white wines were analysed separately.

[h] Standard deviation.

Table 3 Phenolic composition of different South African cultivar wines as a percentage of the total phenol content[a]

Wine	% Monomeric antho-cyanins[b]	% Polymeric antho-cyanins[b]	% Flavanols[b]	% Flavonols[b]	% Tartaric acid esters[b]
Red					
Cabernet Sauvignon	4.97 b[c] (± 1.50)[d]	3.56 ab (± 0.72)	10.49 a (± 1.33)	2.09 c (± 1.03)	8.02 b (± 1.08)
Ruby Cabernet	8.67 a (± 2.95)	4.18 a (± 0.71)	9.36 b (± 0.61)	3.18 abc (± 1.31)	10.63 a (± 0.79)
Pinotage	5.46 b (± 1.54)	3.62 ab (± 0.73)	10.04 ab (± 1.14)	2.92 bc (± 1.13)	10.43 a (± 1.37)
Shiraz	5.68 b (± 0.74)	3.54 b (± 0.36)	10.42 a (± 0.90)	4.11 a (± 1.42)	11.40 a (± 1.55)
Merlot	5.34 b (± 0.98)	3.60 ab (± 0.62)	10.52 a (± 1.28)	3.54 ab (± 0.85)	10.20 a (± 1.68)
Average	**5.87**	**3.67**	**10.21**	**3.16**	**10.10**
White					
Sauvignon blanc	N/A	N/A	2.65 ab (± 1.04)	2.48 b (± 0.96)	17.29 a (± 2.28)
Chenin blanc	N/A	N/A	1.47 c (± 0.58)	2.55 b (± 0.87)	14.57 b (± 1.98)
Chardonnay	N/A	N/A	3.23 a (± 1.39)	4.20 a (± 1.36)	15.90 ab (± 1.64)
Colombar	N/A	N/A	2.04 bc (± 0.71)	2.72 b (± 1.73)	14.40 b (± 3.26)
Average			**2.27**	**2.94**	**15.30**

N/A Not applicable.

[a] Detailed results for each wine are reported in Addendum B.

[b] % Phenolic group = g phenolic group per 100 g total phenols.

[c] Averages in a column followed by different letters, differ significantly ($P < 0.05$). Data for red and white wines were analysed separately.

[d] Standard deviation.

exhibited the highest flavonol content (99.58 mg QE/L) contributing 4.11% to the total phenol content, significantly ($P < 0.05$) higher than that of Cabernet Sauvignon (50.78 mg QE/L and 2.09% of total phenols), Ruby Cabernet (66.52 mg QE/L and 3.18% of total phenols) and Pinotage (66.62 mg QE/L and 2.92% of total phenols). In terms of tartaric acid ester content, Cabernet Sauvignon (188.19 mg CAE/L) had a significantly ($P < 0.05$) lower value than Pinotage (240.64 mg CAE/L), Shiraz (271.81 mg CAE/L) and Merlot (252.63 mg CAE/L). Cabernet Sauvignon (8.02% of total phenols) also represented the lowest ($P < 0.05$) tartaric acid ester to total phenol ratio of all the red cultivar wines (10.20 – 11.40% of total phenols). Shiraz (271.81 mg CAE/L) has the highest tartaric acid ester contents, significantly ($P < 0.05$) higher than Ruby Cabernet (214.75 mg CAE/L) and Cabernet Sauvignon (188.19 mg CAE/L).

The phenolic composition of Chardonnay and Chenin blanc wines represents the highest and lowest values for white wines respectively (Table 2). The total phenol content of Chardonnay (292.7 mg GAE/L) wines was significantly ($P < 0.05$) higher than that of Chenin blanc (242.0 mg GAE/L) wines. The flavanol (9.53 mg CE/L) and flavonol (12.11 mg QE/L) contents of Chardonnay wines were also significantly ($P < 0.05$) higher than that of the other white cultivar wines (3.59 – 6.98 mg CE/L and 6.34 – 7.85 mg QE/L). Chardonnay (46.35 mg CAE/L) and Sauvignon blanc (46.11 mg CAE/L) had significantly ($P < 0.05$) higher contents of tartaric acid esters than Chenin blanc (35.27 mg CAE/L).

The large differences in phenolic composition between red and white wines can be attributed to the extraction of flavanols, flavonols and proanthocyanidins from the grape pomace during the fermentation of red wine (Oszmianski *et al*., 1986; Macheix *et al*., 1990; Sun *et al*., 1998). In the vinification of white wine, pomace contact is usually kept to a minimum and only the free run juice fermented.

High standard deviations for values of the same cultivar were observed for all phenolic group contents (Table 2 and 3). Consequently, differentiation between red and white wines of different cultivars was poor. Several factors

such as climate, soil type, vinification techniques and time of wood maturation (only for red wines), would differ between wines as they were purchased from different wineries around the Western Cape. The climatic conditions of the different regions where wine was purchased also varied from cool to warm during the growth and ripening seasons (Jackson & Lombard, 1993). The Liquor Products Act (Act No. 60 of 1989) of South Africa states that when only one cultivar is noted on the label of the wine at least 75% of the wine should be from the stated cultivar. This aspect could, therefore, interfere with the differentiation between cultivar wines with respect to specific parameters.

3.3.2 Total antioxidant activity measured using the ABTS$^{\bullet+}$ scavenging assay

The total antioxidant activity (TAA) of red and white wines is reported in Table 4. When compared to white wines (0.939 mM Trolox), the average TAA_{ABTS} value for red wines (14.916 mM Trolox) was significantly ($P < 0.05$) higher.

Except for Ruby Cabernet (13.177 mM Trolox) with a significantly lower ($P < 0.05$) average TAA_{ABTS} value than Merlot (15.757 mM Trolox), TAA_{ABTS} values were not significantly ($P \geq 0.05$) different for the other red cultivar wines (13.177 - 15.757 mM Trolox). The high concentration of monomeric and polymeric anthocyanins (168.21 mg Mv-3-glc/L) in Ruby Cabernet did not compensate for its low flavanol (189.14 mg CE/L) and total phenol (2016.0 mg GAE/L) contents in terms of total antioxidant activity. This can be explained by the relative effectivity of flavanols and anthocyanins in the ABTS$^{\bullet+}$ scavenging assay (Rice-Evans *et al*., 1996). In this regard, (+)-catechin and (-)-epicatechin, the major flavanols in wine, have TEAC values of 2.4 and 2.5 mM Trolox respectively, while malvidin-3-glucoside, the major anthocyanin in red wine, has a TEAC value of 1.8 mM Trolox. Although the anthocyanidins (without glycoside moieties) has high TEAC values (2.1 to 4.4 mM Trolox) compared to flavanols, the anthocyanins occur in greater quantities in red wine (Macheix *et al*., 1990).

Amongst the white cultivar wines, Chardonnay (1.060 mM Trolox) has a significantly ($P < 0.05$) higher TAA_{ABTS} value than Chenin blanc (0.800 mM

Table 4 Free radical scavenging activity of different South African red and white cultivar wines[a]

Wine	TAA_{ABTS}[b]	AP_{ABTS}[c]	EC_{50}[d]	Initial scavenging rate[e]	RSE[f]	TAA_{DPPH}[b]	AP_{DPPH}[c]
Red							
Cabernet Sauvignon	15.073 ab[g] (± 1.392)[h]	6.45 a (± 0.38)	63.56 b (± 14.52)	0.0321 a (± 0.0070)	0.516 a (± 0.096)	12.390 a (± 3.243)	5.24 a (± 0.92)
Ruby Cabernet	13.177 b (± 2.742)	6.53 a (± 0.57)	73.86 ab (± 9.96)	0.0341 a (± 0.0031)	0.466 a (± 0.051)	9.510 b (± 2.058)	4.73 a (± 0.72)
Pinotage	15.286 ab (± 2.964)	6.52 a (± 0.32)	67.88 ab (± 12.90)	0.0340 a (± 0.0058)	0.507 a (± 0.072)	11.913 ab (± 2.355)	5.11 a (± 0.72)
Shiraz	14.851 ab (2.617)	6.18 a (± 0.31)	78.09 a (± 10.88)	0.0349 a (±0.0056)	0.455 a (± 0.095)	11.517 ab (± 2.586)	4.76 a (± 0.52)
Merlot	15.757 a (± 2.412)	6.32 a (± 0.30)	70.58 ab (± 11.08)	0.0336 a (± 0.0043)	0.480 a (± 0.043)	12.133 a (± 2.462)	4.84 a (± 0.37)
Average	**14.916**	**6.39**	**70.60**	**0.0337**	**0.486**	**11.608**	**4.95**

Table 4 (continued …) Free radical scavenging activity of different South African red and white cultivar wines[a]

Wine	TAA_{ABTS}[b]	AP_{ABTS}[c]	EC_{50}[d]	Initial scavenging rate[e]	RSE[f]	TAA_{DPPH}[b]	AP_{DPPH}[c]
White							
Sauvignon blanc	0.919 ab (± 0.175)	3.43 ab (± 0.43)	133.52 b (± 14.40)	0.0231 b (0.0023)	0.175 ab (± 0.027)	0.631 ab (± 0.171)	2.37 a (± 0.59)
Chenin blanc	0.800 b (± 0.251)	3.25 b (± 0.39)	157.54 a (± 28.27)	0.0232 b (± 0.0039)	0.150 b (± 0.026)	0.544 b (± 0.200)	2.22 ab (± 0.54)
Chardonnay	1.060 a (± 0.140)	3.62 a (± 0.30)	127.15 b (± 7.22)	0.0243 ab (± 0.0033)	0.192 a (± 0.028)	0.719 a (± 0.096)	2.46 a (± 0.27)
Colombard	0.896 ab (± 0.255)	3.30 ab (± 0.44)	160.81 a (± 15.39)	0.0268 a (± 0.0038)	0.169 ab (± 0.033)	0.532 b (± 0.191)	1.95 b (± 0.34)
Average	**0.939**	**3.40**	**143.84**	**0.0240**	**0.170**	**0.626**	**2.27**

[a] Detailed results for each wine are reported in Addendum C.

[b] Total antioxidant activity as mM Trolox equivalents measured using the 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) radical cation or 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay.

[c] Antioxidant potency (AP) = TAA (mM Trolox) X 1000 / total phenols (mg gallic acid equivalents/L).

[d] EC_{50} = concentration of wine in mg gallic acid equivalents/L required to obtain 50% scavenging.

[e] The initial scavenging rates of wines were estimated as the negative of the slope of the line for [DPPH] against time for the first minute of the reaction.

[f] RSE (Radical Scavenging Efficiency) = initial scavenging rate $\times 1000 / EC_{50}$.

[g] Averages in a column followed by different letters, differ significantly ($P < 0.05$). Data for red and white wines were analysed separately.

[h] Standard deviation.

Trolox), which exhibited the lowest TAA_{ABTS} value, although the latter was not significantly ($P \geq 0.05$) lower than Sauvignon blanc and Colombar (Table 4). As discussed for the red wine the higher content of total phenols, flavanols, flavonols and tartaric acid esters in Chardonnay explains this difference.

AP_{ABTS} was significantly ($P < 0.05$) different for red (6.39) and white (3.40) wines, indicating that the red wine phenolic fractions were more effective free radical scavengers (Table 4). This could be attributed to a concentration effect of specific phenolic groups, the presence of individual phenolic compounds with high potency or synergistic interactions of specific combinations of phenolic compounds. No significant ($P \geq 0.05$) differences were observed between the red wines. Considering white wines, the AP_{ABTS} was significantly ($P < 0.05$) higher for Chardonnay (3.62) than for Chenin blanc (3.25). Presumably, this can be attributed to the higher flavanol, flavonol and tartaric acid ester contents of Chardonnay.

TAA values of South African wines analysed in this study compared well to that of wines from other countries (Table 5). Differences between TAA values reported for wines from different countries could not only be due to environmental factors and technological aspects related to the vinification process, but could be the result of differences in the experimental procedure used for measuring the antioxidant activity (Table 5). These include differences in radical generation methods and reaction time. Radical generation strategies include generation of radicals in the presence of antioxidant samples using ferrylmyoglobin (Miller *et al*., 1993) and other peroxidases (Arnao *et al*., 1996) or pre-generation of radicals using 2,2'-azinobis-(2-amidinopropane) (ABAP), a thermolabile compound (Campos & Lissi, 1996; Van den Berg *et al*., 1999), manganese dioxide (Miller *et al*., 1996) or potassium persulphate (Re *et al*., 1999). When the radical cations are generated enzymatically in the presence of the antioxidant sample, more than one effect could account for antioxidant activity measured (Strube *et al*., 1997; Van den Berg *et al*., 1999). In this case, antioxidant molecules could scavenge radical cations or inhibit the generation of radical cations by inhibition of the enzyme, causing overestimation of antioxidant activity. Some investigators

Table 5 Reported total antioxidant activity of various red and white wines from studies in different countries

Type of wine	Total phenols[a]	Vintage	TAA[b] (number of wines)	AP[c]	Reaction time	Reference
Red, France	1360 - 3545	1991 - 1999	9.6 – 29.2[d] (20)	7.6 – 9.8	4 min.	Landrault *et al*., 2001
Red, Merlot, France	1783 - 2698	1993 - 1999	16.2 – 22.2[d] (7)	7.5 – 9.9	4 min.	Landrault *et al*., 2001
Red, Cabernet Sauvignon, France	1842 - 2532	1993 - 1999	16.5 – 29.9[d] (7)	7.0 – 16.2	4 min.	Landrault *et al*., 2001
Red, Chile	N/A	1991 – 1992	25.1 – 29.9[e] (3)	N/A	6 min.	Campos & Lissi, 1996
Red, Canada	N/A	1991 – 1994	7.5 - 28.6[d] (10)	N/A	3 min.	Soleas *et al*., 1997b
Vini Novelli, red, Italy		1997	10.9 - 22.9[d] (8)	9.7 - 10.9	3 min.	Pellegrini *et al*., 2000
Red, Spain	N/A	1992	14.1[f] (1)	N/A	6 min.	Verhagen *et al*., 1996
Red, Italy	1365 – 2347	1991 – 1994	7.8 - 14.1[d] (9)	5.2 – 6.3	3 min.	Simonetti *et al*., 1997
Red, Italy	700 - 2300	1989 – 1996	6.1 - 11.6[d] (3)	3.5 – 8.9	1 min.	Fogliano *et al*., 1999
Cabernet Sauvignon, red, Chile	N/A	1990 – 1993	27.8 – 33.3[e] (7)	N/A	6 min.	Campos & Lissi, 1996
Cabernet Sauvignon, red, Italy	3326	1994	19.8[d] (1)	6.0	3 min.	Simonetti *et al*., 1997
Cabernet Sauvignon, red, Canada	N/A	1993 – 1994	16.1 - 16.8[d] (2)	N/A	3 min.	Soleas *et al*., 1997b
Merlot, red, Canada	N/A	1994 – 1994	14.2 - 14.4[d] (2)	N/A	3 min.	Soleas *et al*., 1997b
Rose, Spain	N/A	1993	2.4[f] (1)	N/A	6 min.	Verhagen *et al*., 1996
Rose, Chile	N/A	1994	5.0[e] (1)	N/A	6 min.	Campos & Lissi, 1996
White, Italy	110 - 150	1996	1.4 - 1.9[d] (4)	10.0 – 15.8	1 min.	Fogliano *et al*., 1999
White, Spain	N/A	1993	0.8[f] (1)	N/A	6 min.	Verhagen *et al*., 1996
White, Italy	96 - 146	1994 – 1995	0.0 - 3.6[d] (3)	0 – 24.7	3 min.	Simonetti *et al*., 1997
White, Chile	N/A	1991 – 1994	2.9 – 5.2[e] (3)	N/A	6 min.	Campos & Lissi, 1996

N/A **data not available**

[a] Total phenol content as mg gallic acid equivalents/L.

[b] Total antioxidant activity as mM Trolox equivalents.

[c] Antioxidant potency (AP) = TAA (mM Trolox) * 1000 / total phenols (mg gallic acid equivalents/L).

[d] Generation of $ABTS^{\bullet+}$ with ferrylmyoglobin during assay.

[e] Generation of $ABTS^{\bullet+}$ with 2,2'-azobis(2-amidinopropane) before assay.

[f] Generation of $ABTS^{\bullet+}$ with manganese dioxide before assay.

prefer pre-generation of radicals to prevent these complications (Strube *et al*., 1997; Re *et al*., 1999; Van den Berg *et al*., 1999). These factors stress the importance of a standardised method for the measurement of the antioxidant activity of wines. This would allow reliable comparison of wines from different countries. When such a method exists, the antioxidant activity of wines could become a quality parameter influencing consumer choice in addition to the sensory quality.

3.3.3 Antioxidant activity measured using the DPPH• scavenging assay

DPPH• scavenging activity of wine phenolic compounds, expressed in terms of EC_{50} values, initial scavenging rates, RSE, TAA_{DPPH} and AP_{DPPH} is summarised in Table 4.

The average EC_{50} value (Table 4) for the red wines (70.60 mg GAE/L) was approximately 50% less than that of the white wines (143.84 mg GAE/L) indicating that the phenolic compounds in red wine is more effective DPPH• scavengers (low EC_{50} values ≈ high antioxidant activity). Cabernet Sauvignon (63.56 mg GAE/L) exhibited the lowest and Shiraz (78.09 mg GAE/L) the highest EC_{50} value of the red cultivar wines. Amongst the white wines, Chardonnay (127.15 mg GAE/L) and Sauvignon blanc (133.52 mg GAE/L) were significantly ($P < 0.05$) more effective DPPH• scavengers than Chenin blanc (157.54 mg GAE/L) and Colombar (160.81 mg GAE/L).

Red wine phenolic constituents not only scavenged more DPPH• than that of white wine, but the initial scavenging rate (0.0337) of DPPH• was also significantly ($P < 0.05$) higher than that of white wine (0.0240) (Table 4). This is in agreement with a study evaluating the DPPH• scavenging activity of red, rosé and white wines indicating that red wines scavenge DPPH• faster than white wine (Sánchez-Moreno *et al*., 1999). In the present study, initial scavenging rates of DPPH• did not differ significantly ($P \geq 0.05$) amongst the red cultivar wines. Colombar (0.0268) displayed a significantly faster ($P < 0.05$) initial scavenging rate than Sauvignon blanc (0.0231) and Chenin blanc (0.0232) when the white

cultivar wines are considered. This difference could not be explained by the content of their phenolic groups, which was similar. The contribution of individual phenolic compounds of high potency or interactions of combinations of compounds is thus of importance in this case. The scavenging rates of Chardonnay (0.0243) and Chenin blanc (0.0232) did not differ significantly ($P \geq 0.05$), even though they represent the respective high and low values for the different phenolic groups, and the ratios of the phenolic groups, flavanols and flavonols to total phenols were much lower for Chenin blanc (1.46 and 2.55% of total phenols) than for Chardonnay (3.23 and 4.20% of total phenols).

The average RSE of red wine (0.486) was almost three times as much as that of white wine (0.170) (Table 4). The higher activity and initial scavenging rate for red wines, mentioned above, gave them a higher RSE than the white wines. Sánchez-Moreno *et al*. (1999) also found that red wine has a much higher antiradical efficiency than white wine. Antiradical efficiency was expressed as the ratio of EC_{50} to the time required to completely scavenge $DPPH^{\bullet}$ at the EC_{50} concentration. Although the RSE value in the present study takes into account both the amount of phenolic compounds necessary to scavenge 50% of $DPPH^{\bullet}$ and the initial rate of scavenging, no significant differences in RSE could be found between the different red cultivar wines. However, for white wines, Chardonnay (0.192) exhibited a significantly ($P < 0.05$) higher RSE value than Chenin blanc (0.150). This pattern is similar to that observed for EC_{50} values, indicating that the amount of phenolic compounds needed to scavenge 50% of $DPPH^{\bullet}$ are the most important factor determining the RSE and not the initial scavenging rate.

The TAA_{DPPH}, representing the antioxidant activity of the wines on an “as-is” basis, of red wines (11.608 mM Trolox) was much higher ($P < 0.05$) than for white wines (0.626 mM Trolox). This can be expected due to their substantially higher ($P < 0.05$) content of total phenols. The TAA_{DPPH} values for Ruby Cabernet wines (9.510 mM Trolox) were significantly ($P < 0.05$) lower than those for Cabernet Sauvignon (12.390 mM Trolox) and Merlot (12.133 mM Trolox). Amongst the white wines, Chardonnay wines (0.719 mM Trolox) had significantly

higher activity than Chenin blanc (0.544 mM Trolox) and Colombar (0.532) wines. When expressing antioxidant activity on a similar total phenol basis, no significant ($P \geq 0.05$) differences were observed between AP_{DPPH} of red cultivar wines. Colombar wines (1.95) exhibited a significantly ($P < 0.05$) lower AP_{DPPH} than both Chardonnay (2.46) and Sauvignon blanc wines (2.37).

The free radical scavenging activity for red and white wines was similar using the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays when expressed in terms of the same reference compound, namely Trolox (Table 4). However, some minor differences existed that could be of importance in the application of the two methods in subsequent studies. When compared to the TAA_{DPPH}, the TAA_{ABTS} could not differentiate significantly ($P < 0.05$) between antioxidant activity of Cabernet Sauvignon and Ruby Cabernet. No difference exists between the assays when considering the antioxidant potency of the red wine phenolic fractions. In the case of white wines, both the TAA and antioxidant potency determined using the $DPPH^{\bullet}$ scavenging assay distinguished between Chardonnay and Colombar, while no effect could be observed using the $ABTS^{\bullet+}$ scavenging assay. In this case, therefore, the $DPPH^{\bullet}$ scavenging assay could discriminate more effectively between red and white cultivars than the $ABTS^{\bullet+}$ scavenging assay.

3.3.4 Contribution of ascorbic acid to the free radical scavenging activity

The ascorbic acid content of white wines was determined as it is often added to prevent unacceptable oxidative browning reactions in white wines. As ascorbic acid has a high antioxidant activity in both the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays, the TAA of these wines could not be solely attributed to their phenolic content. Most of the white wines (75%) used in the present study tested negative for the presence of ascorbic acid. The ascorbic acid content of the remaining wines and its effect on their TAA is reported in Table 6. One Sauvignon blanc and one Colombar wine contained high amounts of ascorbic acid (0.348 and 0.261 mM). To assess the contribution of phenolic compounds alone to the TAA,

Table 6 Ascorbic acid content and contribution to total antioxidant activity of white wines

Sample code[a]	**Ascorbic acid content[b]**	**Ascorbic acid contribution[c]**	**% of total activity[d]**
Char 1	2.05	0.012	1.31
Char 7	2.91	0.017	1.41
Col 1	2.91	0.017	1.69
Col 3	7.64	0.046	5.33
Col 8	46.01	0.274	19.59
Chen 2	16.95	0.101	8.15
Chen 6	1.5	0.011	1.34
Chen 10	14.07	0.084	6.87
SB 3	10.36	0.062	5.76
SB 5	2.60	0.016	1.31
SB 8	61.34	0.366	29.87

[a] Sample codes for wines is as described in Table 1.
[b] mg/L.
[c] Total antioxidant activity as mM Trolox equivalents.
[d] % of total antioxidant activity of wine contributed by ascorbic acid.

the TAA_{ABTS} and TAA_{DPPH} values were adjusted by subtracting the contribution of ascorbic acid as was done by Fogliano *et al*. (1999). Ascorbic acid contributed to 1.3 – 29.8% of the TAA of wines containing ascorbic acid. Adjustments to the TAA values of white wines only had a small influence on the average values for cultivar wines (data not shown). Although Saucier & Waterhouse (1999) found no synergistic effect when ascorbic acid was added to a (+)-catechin solution, the contribution of ascorbic acid to the total phenol content might not be additive as wine is a complex mixture of phenolic compounds and other constituents. For the purpose of this study, therefore, the data pertaining to the Sauvignon blanc and Colombar wine containing a high amount of ascorbic acid were removed from the data set and TAA values for other wines used unadjusted as ascorbic acid content of other wines contributed less than 10% of total antioxidant activity.

3.3.5 Correlation analysis

The correlation coefficients for TAA_{ABTS} and TAA_{DPPH} values in relation to the phenolic group contents of red and white cultivar wines is reported in Table 7. TAA_{ABTS} values of red wines ($r = 0.94$, $P < 0.001$) and white wines ($r = 0.91$, $P < 0.001$) correlated well with the total phenol content (Figures 3A and B). With respect to the phenolic groups, the flavanol ($r = 0.87$, $P < 0.001$) content of red wines (Figure 4) and the tartaric acid ester ($r = 0.77$, $P < 0.001$) content of white wines (Figure 5) appears to have the most effective predictive value for TAA_{ABTS}. These phenolic groups were also present in the highest quantities in red and white wines respectively. Correlations of polymeric anthocyanins ($r = 0.54$, $P < 0.001$) and tartaric acid esters ($r = 0.50$, $P < 0.001$) in red wine and flavonols ($r = 0.62$, $P < 0.001$) in white wine with TAA_{ABTS} were, however, also significant, but weaker. In previous antioxidant studies, the activity of wine utilising the low-density lipoprotein (Frankel *et al*., 1995; Meyer *et al*., 1997) and free radical scavenging (Simonetti *et al*., 1997; Fogliano *et al*., 1999) assays was investigated and correlates with total phenol and flavanol content. The monomeric anthocyanin content of red wines, which was lower than that of flavanols, showed no correlation with TAA_{ABTS} values ($r = 0.06$, $P = 0.71$) although these compounds are effective free radical scavengers (Rice-Evans *et al*., 1996). Other investigators found that

Table 7 Correlation of free radical scavenging activity according to the content of phenolic groups of wine

	TAA_{ABTS}[a]		TAA_{DPPH}[b]	
	r	**P**	**r**	**P**
Red wine				
Total phenols	0.94	< 0.001	0.79	< 0.001
Monomeric anthocyanins	0.06	0.71	-0.08	0.60
Polymeric anthocyanins	0.54	< 0.001	0.49	< 0.001
Flavanols	0.87	< 0.001	0.82	< 0.001
Flavonols	0.38	0.009	0.31	0.04
Tartaric acid esters	0.50	< 0.001	0.35	0.019
White wine				
Total phenols	0.91	< 0.001	0.73	< 0.001
Flavanols	0.54	0.009	0.55	< 0.001
Flavonols	0.62	< 0.001	0.52	0.009
Tartaric acid esters	0.77	< 0.001	0.63	< 0.001

[a] Total antioxidant activity as mM Trolox equivalents determined according to the ABTS radical cation scavenging assay.

[b] Total antioxidant activity as mM Trolox equivalents determined according to the DPPH radical scavenging assay.

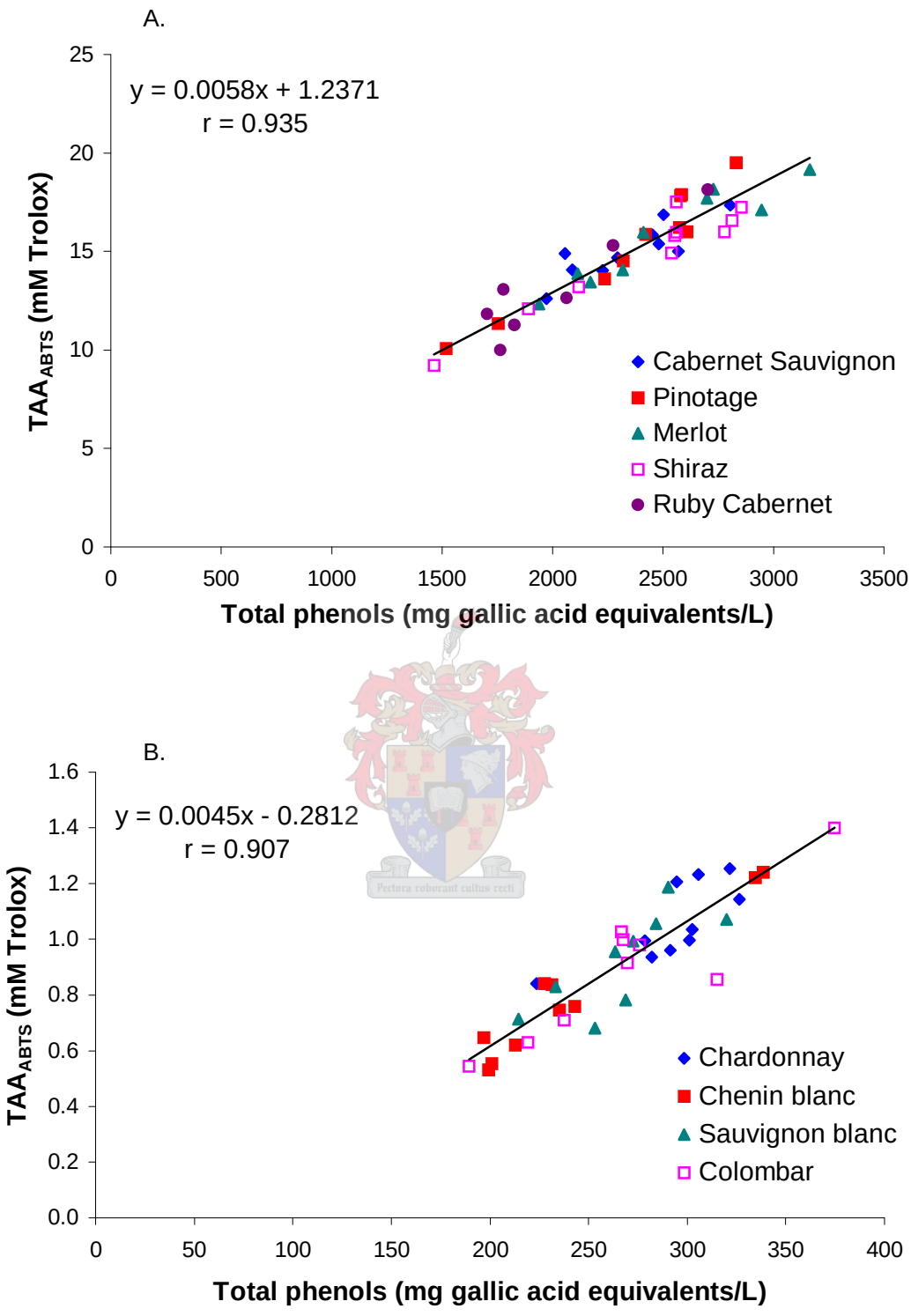

Figure 3 Total antioxidant activity as measured as a function of total phenol content of red (A) and white (B) wines. Total antioxidant activity was measured using the ABTS radical cation scavenging assay (Re *et al*., 1999).

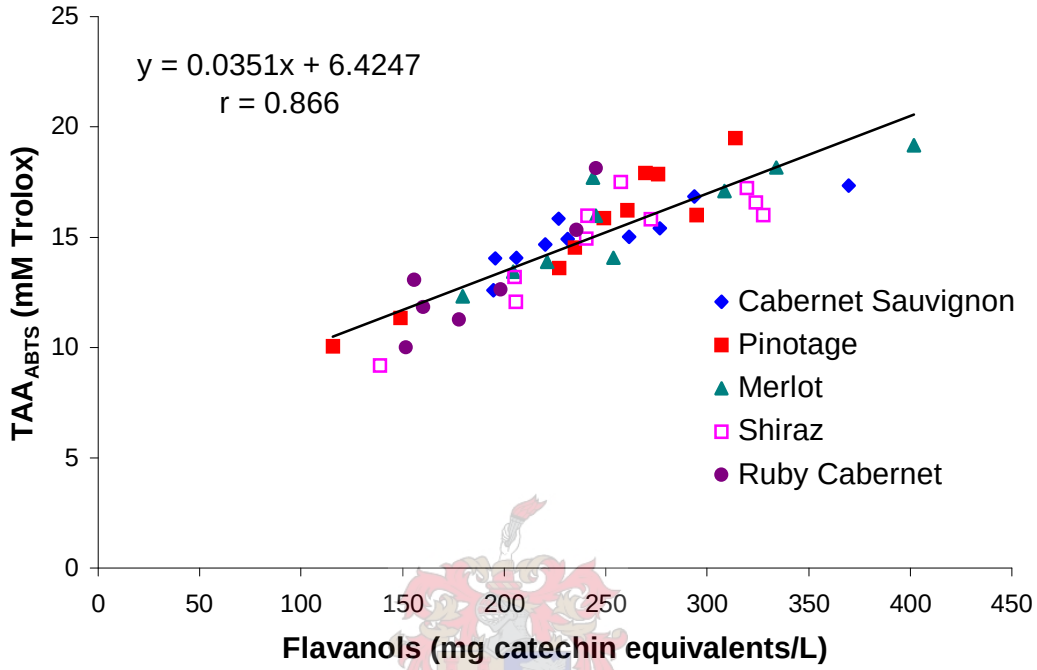

Figure 4 Total antioxidant activity as measured as a function of flavanol content of red wines. Total antioxidant activity was measured using the ABTS radical cation scavenging assay (Re *et al*., 1999).

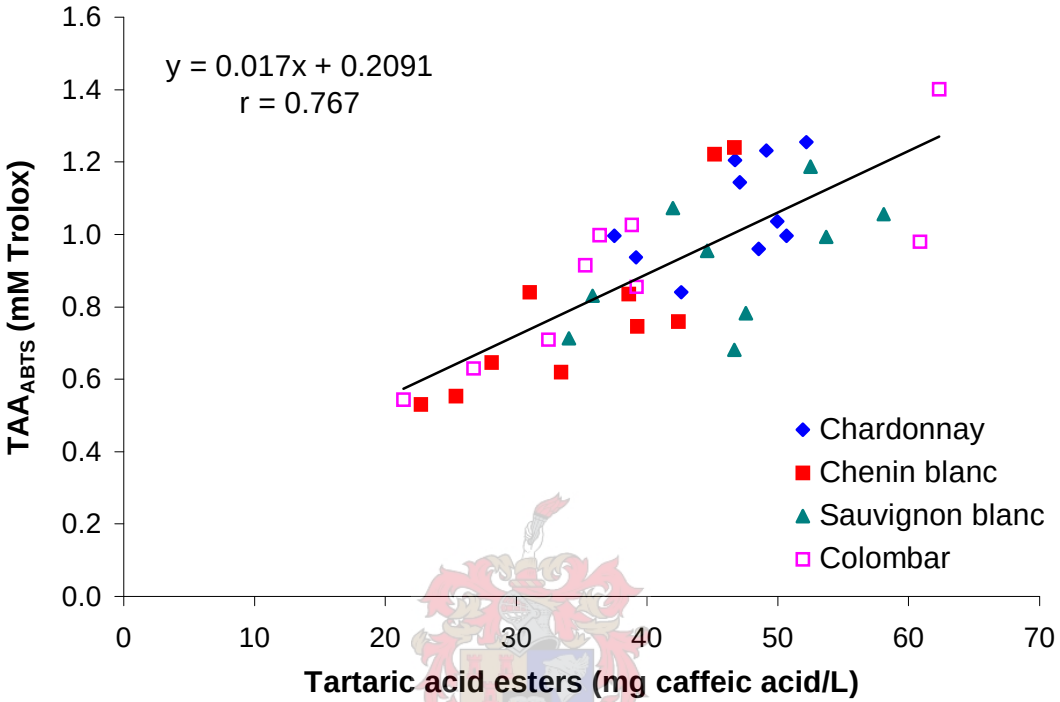

Figure 5 Total antioxidant activity as measured as a function of tartaric acid ester content of white wines. Total antioxidant activity was measured using the ABTS radical cation scavenging assay (Re *et al*., 1999).

anthocyanins contributed considerably to the inhibition of low-density lipoprotein oxidation of red wines (Frankel *et al*., 1995; Ghiselli *et al*., 1998) and grape extracts (Meyer *et al*., 1997). One French study, however, also found no correlation ($r < 0.3$, $P < 0.099$) of antioxidant activity measured using the $ABTS^{\bullet+}$ scavenging assay with the content of several anthocyanins (Landrault *et al*., 2001). The same relative trends were observed for TAA_{DPPH} values as in the case of TAA_{ABTS} values (Table 7).

The lack of correlation of monomeric anthocyanin content with TAA value and the importance of flavanols in contributing to the TAA for red wines, explain the low TAA values for Ruby Cabernet. Based on the correlations obtained, the difference in TAA_{ABTS} between Chardonnay and Chenin blanc could be mostly attributed to the contribution of flavonols ($r = 0.62$, $P < 0.001$) and tartaric acid esters ($r = 0.77$, $P < 0.001$) with a lesser contribution by flavanols ($r = 0.54$, $P = 0.009$). The low correlation coefficients for flavanols and flavonols in white wines could be attributed to a concentration effect as low amounts were found in white wines. Another factor to consider, is that the estimation of phenolic group contents, for example flavonol content, is based on their similar basic structure, although their substitution patterns may differ (Chapter 2). The specific substitution patterns of individual compounds are closely related to antioxidant activity (Rice-Evans *et al*., 1996), but does not affect the estimation of phenolic group content.

The TAA_{DPPH} values correlated well with the TAA_{ABTS} values ($r = 0.77$, $P < 0.001$ for red wines and $r = 0.77$, $P < 0.001$ for white wines) (Figures 6A and B), although two different free radicals were scavenged. This shows that these two methods measure the same type of activity. $DPPH^{\bullet}$ scavenging activity is not usually expressed as a total antioxidant activity in terms of Trolox, but this parameter was calculated to permit comparison with $ABTS^{\bullet+}$ scavenging activity for wine on an “as-is” basis.

3.3.6 Canonical discriminant analysis

Canonical discriminant analysis was conducted to attempt better differentiation of cultivar wines on the basis of the parameters measured for phenolic composition and antioxidant activity. Figures 7A and B show plots of

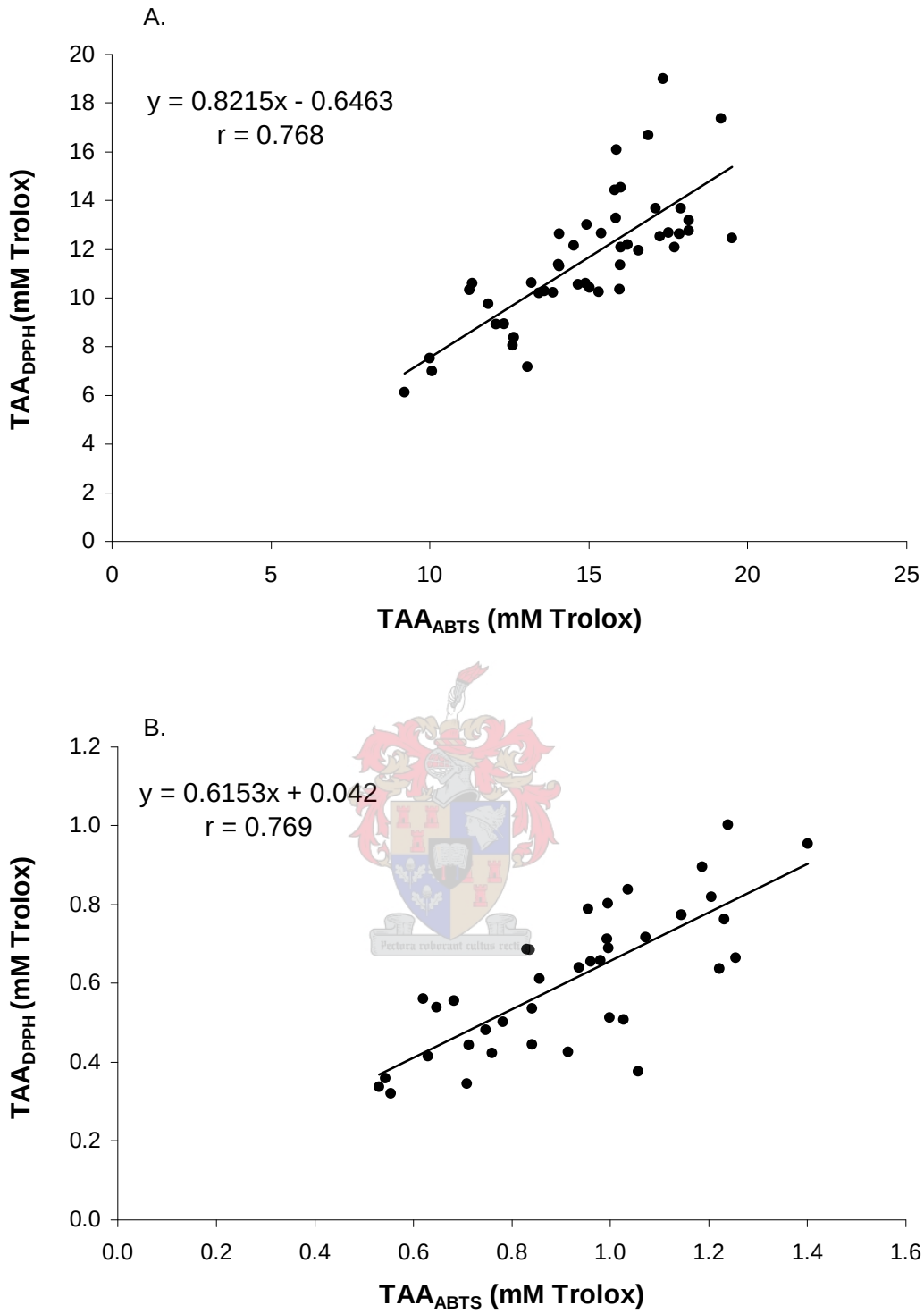

Figure 6 Total antioxidant activity measured using the DPPH radical scavenging assay (Brand-Williams *et al*., 1995) as a function of total antioxidant activity measured using the ABTS radical cation scavenging assay (Re *et al*., 1999) of red (A) and white (B) wines.

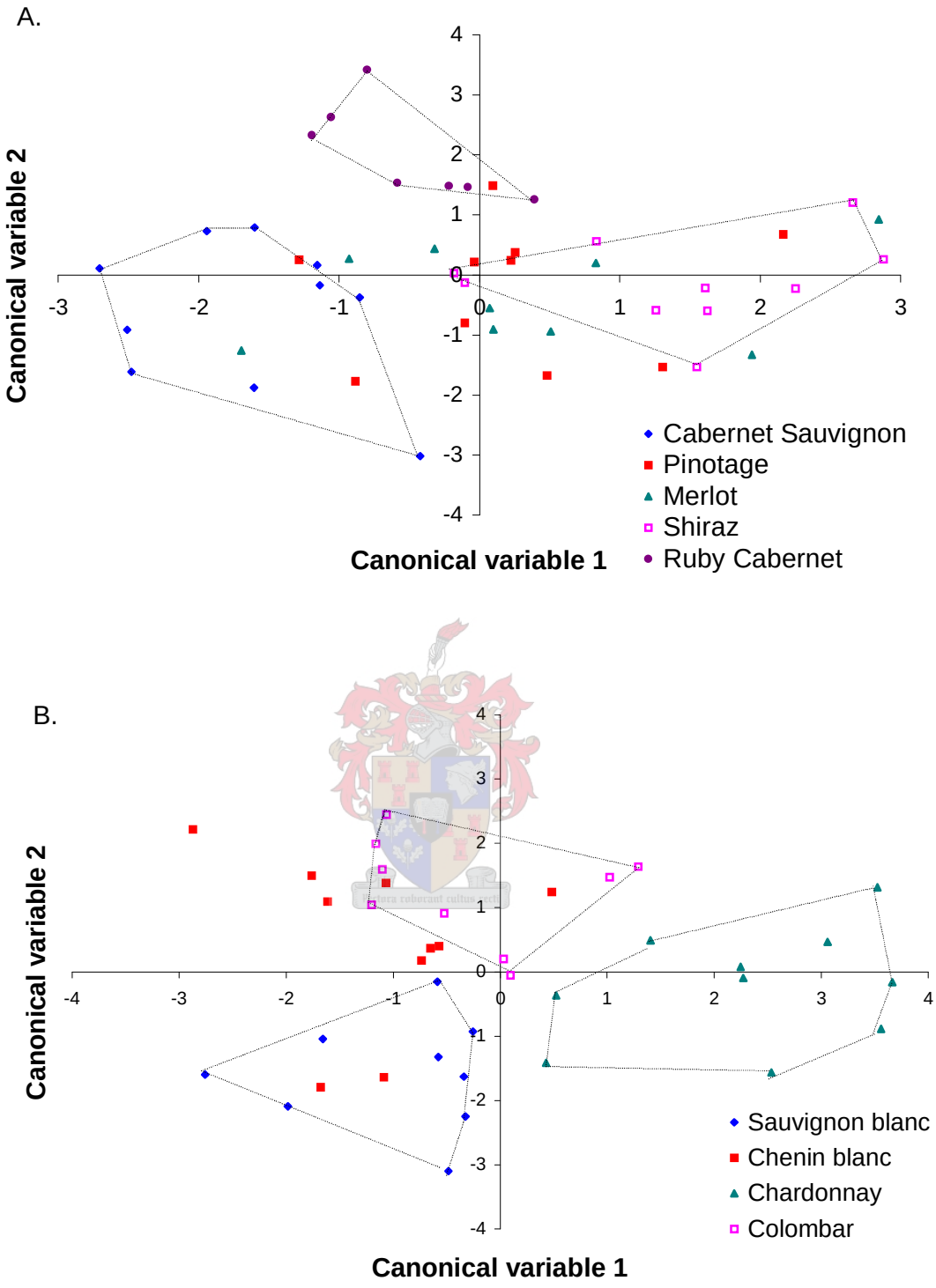

Figure 7 Canonical discriminant analysis plots for red wine (A) and white wine (B) using parameters for total phenol, anthocyanin (red wine only), flavanol and flavonol tartaric acid ester contents, as well as TAA_{ABTS}, EC_{50}, initial scavenging rate, RSE and TAA_{DPPH}.

the first two canonical variables for the red and white wines respectively. Only Ruby Cabernet wines can be differentiated from the other red wine cultivars although two data points of Pinotage wines overlap. Addition of up to 25% of another cultivar, as allowed by the Liquor Products Act (Act No. 60 of 1989) of South Africa, when labelling a wine as a single cultivar wine, could contribute to this poor differentiation. The Chardonnay, Sauvignon blanc and Colombar wines could be differentiated from each other, while data points for Chenin blanc wines were scattered between those of other white cultivar wines. When data for Chenin blanc wines were removed from the data set, good differentiation of the three remaining white cultivar wines was still obtained. Data points for Chardonnay and Chenin blanc were also separated as can be expected from their different phenolic composition and antioxidant behaviour. This type of discriminant analysis could be useful in future in determining adulteration of cultivar wines with wine from another cultivar not stated on the label.

3.4 Conclusions

The total antioxidant activity of wines correlated with its phenolic content and composition. Different phenolic groups did not contribute to the same degree to the total antioxidant activity and different combinations and/or synergistic interactions between phenolic compounds are likely to affect the outcome when comparing the free radical scavenging activity of different cultivar wines. The practice of ascorbic acid addition to prevent browning of white wines further complicates this issue.

Different cultivar wines could not be totally differentiated on the basis of their phenolic composition or free radical scavenging activity. The use of canonical discriminant analysis using multiple parameters for phenolic composition and antioxidant activity effectively discriminates between white cultivar wines, except for Chenin blanc, while a far weaker effect was obtained with red cultivar wines.

Comparison of wines evaluated in the present study with wines produced elsewhere and analysed in other laboratories was complicated by differences in assay protocols. The use of a standardised method to compare

wines from different countries is urgently needed before the use of antioxidant activity as a quality parameter for wines can be feasible.

The two free radical scavenging methods used to measure the antioxidant activity of wines gave similar results for red wines although only the TAA_{DPPH} could distinguish between Cabernet Sauvignon and Ruby Cabernet. Both methods lack any differentiation between red cultivar wines when considering the antioxidant potency of the phenolic fractions. Using the $DPPH^{\bullet}$ scavenging assay, Chardonnay and Colombar could be differentiated on the basis of total antioxidant activity and antioxidant potency, while this was not possible using the $ABTS^{\bullet+}$ scavenging assay. The $DPPH^{\bullet}$ scavenging assay were, therefore, more effective for the differentiation of different cultivar wines in this study. Due to practical implications, however, more researchers use the $ABTS^{\bullet+}$ scavenging assay to determine the free radical scavenging activity of wines and other foods. This assay would, therefore, be best suited to standardisation for use in screening large amounts of samples for comparison across countries and laboratories.

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CHAPTER 4

ACTIVITY OF SOUTH AFRICAN RED AND WHITE CULTIVAR WINES AND SELECTED WINE PHENOLIC COMPOUNDS IN A MICROSOMAL LIPID PEROXIDATION AND DEOXYRIBOSE ASSAY

Abstract

The ability of South African red (Cabernet Sauvignon, Ruby Cabernet, Pinotage, Shiraz, Merlot) and white (Sauvignon blanc, Chenin blanc, Chardonnay and Colombar) cultivar wines to inhibit lipid peroxidation in a membrane system was evaluated using rat liver microsomes. Red wines were more effective inhibitors of microsomal lipid peroxidation (MLP) than white wines when evaluated on an equal total phenol basis. This was reflected in the IC_{50} values, of 4.44 and 11.84 mg gallic acid equivalents/L, determined for a red and white wine, respectively and by the antioxidant potency (AP) of red (14.25) and white (4.19) wine phenolic fractions. The red wines, Ruby Cabernet and Pinotage exhibited the lowest inhibition of MLP, while Merlot was the most effective. The AP was significantly ($P < 0.05$) lower for Pinotage than for other red cultivar wines. Amongst the white wines, Chenin blanc and Colombar exhibited the lowest % MLP inhibition and AP, and Chardonnay the highest % MLP inhibition and AP. Ascorbic acid present in some wines modified the activity of white wines as lipid peroxidation inhibitors. The antioxidant activity in the MLP assay was correlated with total phenol content of red ($r = 0.90$, $P < 0.001$) and white ($r = 0.78$, $P < 0.001$) wines. The flavanol ($r = 0.88$, $P < 0.001$) content of the red wines and flavanol ($r = 0.85$, $P < 0.001$) and tartaric acid ester ($r = 0.71$, $P < 0.001$) contents of the white wines exhibited the strongest correlations of the individual phenolic groups with % MLP inhibition. No correlation ($P > 0.01$) was obtained between % MLP inhibition and monomeric anthocyanin content, while other phenolic groups exhibited weaker correlations. In the deoxyribose assay, red wines exhibited a higher pro-oxidant effect than white wines. The hydroxyl radical scavenging and metal chelating abilities, however, were higher for white wines than red wines at the same total phenol concentration.

4.1 Introduction

Polyunsaturated fatty acids (PUFA) are important constituents of cellular structures such as liposomes, erythrocytes and membranal fractions. They are susceptible to undergo lipid peroxidation by the action of reactive oxygen species (ROS) (O'Brien, 1987; Luc & Fruchart, 1991; Frei, 1995). Damage to membranes, as well as DNA and protein, is causal factors in ageing and chronic diseases such as cancer and atherosclerosis (Halliwell & Gutteridge, 1989; Cutler, 1991; Keli *et al*., 1996; Rice-Evans & Packer, 1998; Lindsay, 2000). Reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), superoxide anion radical ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) are the result of endogenous metabolic processes (Davies, 1995). Although H_2O_2 is not a free radical species, it can be converted to the very reactive $OH^{\bullet}$ during the Fenton reaction involving iron (Halliwell & Gutteridge, 1990). *In vivo*, iron is usually bound to transport proteins such as transferrin and ferritin, but during disease conditions or tissue damage iron can be mobilised (Halliwell & Gutteridge, 1990). Some antioxidants may have pro-oxidant activity in the presence of mobilised iron by reducing Fe^{3+} to Fe^{2+} (Aruoma, 1994).

Recently the role of phenolic compounds in the diet has become more important in the prevention of free radical-mediated diseases (Frankel *et al*., 1995; Kinsella *et al*., 1995; Wiseman, 1996; Rice-Evans & Packer, 1998; Lindsay, 2000). Chain-breaking antioxidants such as phenolic compounds can prevent peroxidation of PUFA's by scavenging free radical species involved in the initiation and chain propagation reactions, while secondary antioxidants can chelate metal ions preventing the formation of alkoxyl and peroxyl radicals from lipid hydroperoxides (Halliwell & Gutteridge, 1990). The latter radicals can initiate or further propagate lipid peroxidation in membranes (Halliwell & Gutteridge, 1990). Although the current knowledge about the absorption and bioavailability of phenolic compounds is incomplete, *in vitro* studies have shown an increased antioxidant status of human blood plasma after intake of red wine and other sources of phenolic compounds (Cao *et al*., 1998; Duthie *et al*., 1998; Prior & Cao, 1999).

Low-density lipoprotein (LDL) has been used extensively as an oxidisable substrate due to the link between peroxidation of LDL and coronary

heart disease (St Leger *et al*., 1979; Renaud & De Lorgeril, 1991; Luc & Fruchart, 1991). The protective effect of antioxidants (Myara *et al*., 1993; Meyer *et al*., 1998a; Meyer *et al*., 1998b; Moon & Terao, 1998) and foods (Frankel *et al*., 1995; Hurtado *et al*., 1997; Meyer *et al*., 1997; Teissedre *et al*., 1997; Pearson *et al*., 1998) against atherosclerosis have, therefore, been studied using this method. Other substrates such as erythrocytes, hepatocytes, micelles and microsomal membrane preparations can be utilised to evaluate the activity of antioxidants in biological membranes due to the association of lipid peroxidation in membranes with ageing and age-related diseases. The latter has been applied to evaluate the antioxidant activity of plant extracts (Ohta *et al*., 1994; Haraguchi *et al*., 1996; Boveris *et al*., 1998; Yen & Hsieh, 1998; Yen & Wu, 1999), foods (Plumb *et al*., 1997; Daglia *et al*., 2000) and several phenolic compounds (Osawa *et al*., 1987; Afanas'ev *et al*., 1989; Mora *et al*., 1990; Van Acker *et al*., 1996). At present, no information is available about the ability of red and white wines to inhibit microsomal lipid peroxidation.

The present study investigated the antioxidant activity of a large selection of South African red and white cultivar wines in the microsomal lipid peroxidation assay. The efficacy of red and white wine phenolic compounds to scavenge $OH^{\bullet}$, promote its formation, or act as metal-chelators were also investigated.

4.2 Materials and methods

4.2.1 Wines and chemicals

A total of 86 randomly selected cultivar wines were obtained from wineries in the major wine-producing regions of the Western Cape, South Africa (3.2.1, Table 1).

The following chemicals were used: ascorbic acid enzymatic test kit (Boehringer Mannheim GmbH, Mannheim, Germany); iron (III) chloride ($FeCl_3$) (BDH Laboratory Supplies, Poole, UK); 2-thiobarbituric acid (TBA) (Aldrich Chemical Co., Gillingham, Dorset, UK); bovine serum albumin, 2,[6]-butylated hydroxytoluene (BHT), 2-deoxy-D-ribose, (+)-catechin,

(-)-epicatechin and gallic acid (Sigma Chemical Co., St. Louis, USA); Folin-Ciocalteau's phenol reagent, sodium carbonate (AR) (Na_2CO_3), sodium hydroxide (NaOH), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$) , iron (II) sulphate heptahydrate ($Fe_2SO_4.7H_2O$) and quercetin (Merck, Darmstadt, Germany); ethylene-diamine-tetraacetic acid (EDTA) (AR), hydrogen peroxide, potassium hydroxide (AR) (KOH) and trichloroacetic acid (TCA) (Saarchem, Johannesburg, South Africa); 4-dimethylaminocinnamaldehyde (DAC) and caffeic acid (Fluka AG, Buchs, Switzerland); and malvidin chloride, cyanidin chloride and delphinidin chloride (Extrasynthese, Genay, France). Procyanidin B1 were kindly supplied by Prof Ferreira (University of the Free State, South Africa). The water was de-ionised with a Modulab water purification system prior to use (Separations, Cape Town, South Africa).

4.2.2 Sample preparation

Sample preparation was as described in 3.2.2. Wines used for the deoxyribose assay were also de-alcoholised by vacuum evaporation of water and alcohol which is known to be a good hydroxyl radical scavenger (Aruoma, 1994). Dried wine samples were reconstituted in de-ionised water.

4.2.3 Determining the phenolic composition and ascorbic acid content

Spectrophotometric determination of the total phenol (Singleton & Rossi, 1965), anthocyanin (Burns *et al*., 2000), flavanol (McMurrough & McDowell, 1978), flavonol (Mazza *et al*., 1999) and tartaric acid ester (Mazza *et al*., 1999) contents of red and white wines, as well as the ascorbic acid content of white wine, is described in 3.2.3.

4.2.4 Microsomal lipid peroxidation (MLP) assay

Microsomes were prepared as described in Van Acker *et al*. (1996). The livers of male Fischer (F344) rats weighing between 200 and 250 g were rinsed in 1.15% (m/v) ice-cold KCl and homogenised in a Polytron PT300 (Kinematica, Switzerland) homogeniser. The mixture was centrifuged at 9000 x g for 10 min. and the microsomal fractions obtained by

ultra-centrifugation of the supernatant for 1 h at 40 000 x g in a Sorvall RC 2-B centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut). The microsomal pellet was washed by resuspension in 0.1 M K-phosphate buffer (pH 7.4) in a glass dounce and re-centrifuged for 1 h at 40 000 x g. The final microsomal pellet was resuspended and stored in the phosphate buffer at –80 ºC. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Microsomal lipid peroxidation (MLP) was performed according to a method modified from Yen & Hsieh (1998). Glass test tubes were soaked in 0.5% (m/v) EDTA for at least 24 h to remove traces of iron (Farag *et al*., 1989) and rinsed thoroughly with purified, de-ionised water prior to use. The reaction mixture contained 400 µl rat liver microsomes (final concentration = 0.4 mg protein/L), 200 µl diluted wine samples or solutions of phenolic compounds, 200 µl $FeCl_2$ (final concentration = 0.5 mM) and 200 µl H_2O_2 (final concentration = 0.5 mM) in a reaction volume of 1 ml. Sample blanks, containing all reagents, except for the microsomal fraction, were included to compensate for possible absorbance of wine samples at 532 nm. The preparation of the reaction mixture was carried out on ice. The reaction mixture was incubated in a water bath at 37 ºC for 1 h. The oxidation reaction was terminated by addition of 2 ml of cold TCA/BHT/EDTA mixture ([TCA] = 10% (m/v), [BHT] = 0.006% (m/v), [EDTA] = 0.15 mM) to precipitate the microsomal protein and to prevent any non-specific oxidation (Esterbauer & Cheeseman, 1991). This mixture was centrifuged for 25 min. at 4200 x g (Hettich Universal 16 centrifuge, Centrolab, Cape Town) and 1 ml of the supernatant mixed with 1 ml of a 0.67% (m/v) TBA solution. After incubation at 95 ºC for 15 min. the mixture was cooled to room temperature and the amount of malondialdehyde (MDA) formed during the oxidation determined at 532 nm using the molar extinction coefficient of 1.56×10^5 $M^{-1}.cm^{-1}$ according to the following formula (Beuge & Aust, 1978):

$$\text{Amount of MDA formed (moles)} = \frac{V \times A_{532}}{\varepsilon}$$

where V = final volume of test solution in ml = 2 ml

A_{532} = absorbance at 532 nm

ε = molar extinction coefficient = 1.56 x 10^5 $M^{-1}.cm^{-1}$

Antioxidant activity of wines were reported as % MLP inhibition:

$$\%\,\text{Inhibition} = \frac{MDA_C - MDA_S}{MDA_C} \times 100$$

where MDA_C = amount of MDA formed in control

MDA_S = amount of MDA formed when the sample is added to the reaction mixture

Antioxidant potency (AP) of wine phenolic fractions were calculated as the ratio of % MLP inhibition to total phenols:

$$\text{Antioxidant potency (AP)} = \frac{\%\ \text{MLP inhibition}}{\text{Total phenols}}$$

where % MLP inhibition = % inhibition of microsomal lipid peroxidation

Total phenols = content of total phenols in the reaction mixture (mg GAE/L)

The linearity of the inhibitory activity of wine in the MLP assay were determined using one red (Pinotage) and one white (Chardonnay) experimental wine. These wines were prepared according to the Nietvoorbij standardised procedure for small-scale winemaking at the experimental winery of Nietvoorbij (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). This was done by analysis of wine samples diluted with 10% ethanol in a

range of concentrations giving 20 - 80% MLP inhibition. The ethanol concentration in the reaction mixture for all determinations were, therefore, 2%. The IC_{50} values, expressed as mg GAE/L of phenolic compounds in the reaction mixture required for 50% MLP inhibition, were determined from a plot of the % MLP inhibition against the concentration of wine phenolic compounds.

The inhibitory activity of different red and white cultivar wines on MLP was carried out on diluted red (100 times) and white (5 times) wine samples. A 10% ethanol in water mixture was used for diluting the wine samples to ensure that the ethanol concentration in the reaction mixture for all samples were 2%. Activity in the microsomal lipid peroxidation assay was quantified as % MLP inhibition.

A selection of known wine phenolic compounds [(+)-catechin, (-)-epicatechin, procyanidin B1, malvidin chloride, cyanidin chloride and delphinidin chloride] was tested at a concentration range giving between 20% and 80% MLP inhibition. (+)-Catechin, (-)-epicatechin and procyanidin B1 were dissolved in ethanol, while malvidin chloride, cyanidin chloride and delphinidin chloride were dissolved in acidified ethanol (pH 1.3). Stock solutions of the phenolic compounds were diluted before addition to the reaction mixture to give no more than 1% of ethanol in the reaction mixture to ensure minimum interference of ethanol (Kagan *et al*., 1990). The IC_{50} value (μM or mg/L) of each compound was determined graphically.

4.2.5 Deoxyribose assay

Two red (Pinotage and Cabernet Sauvignon) and two white (Chardonnay and Chenin blanc) cultivar wines, prepared according to the Nietvoorbij standardised procedure for small-scale winemaking at the experimental winery of the ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa), were used to determine the differences in antioxidant/pro-oxidant behaviour of red and white wine using the deoxyribose assay.

Four variations of the deoxyribose assay were used to determine different types of activity in a Fenton system. The hydroxyl radical scavenging activity was measured by adding ascorbic acid and EDTA to the reaction

mixture, while the pro-oxidant activity was measured in the absence of ascorbic acid (Aruoma, 1994). The metal chelating ability was measured in the absence of EDTA (with and without the addition of ascorbic acid) (Aruoma, 1994). The phenolic composition of wines used in the deoxyribose assay was also determined as described above.

The deoxyribose assay was performed according to the method of Zhao *et al*. (1994) and Zhao & Jung (1995) with minor modifications. In addition to 500 µl of diluted red (0 - 160 mg GAE/L) and white (0 - 40 mg GAE/L) wine samples, the reaction mixture (1.5 ml) in a centrifuge tube (2 ml) contained the following reagents (added in the order mentioned): 500 µl potassium phosphate buffer (pH 7.4) (final concentration = 24 mM), 500 µl deoxyribose (final concentration = 3 mM), 500 µl H_2O_2 (final concentration = 1.4 mM), 500 µl ascorbic acid (final concentration = 1.5 mM), 250 µl $FeSO_4.7H_2O$ (final concentration = 30 µM) and 250 µl EDTA (final concentration = 45 µM). Iron and EDTA (when appropriate) were pre-mixed before addition to the reaction mixture. In the assays where either ascorbic acid or EDTA was omitted, the reaction volume was adjusted by adding purified, de-ionised water. Sample references, containing all reagents except for deoxyribose, were included to compensate for possible absorbance of samples while a sample without deoxyribose and wine was included for the calibration of the spectrophotometer. The reaction mixtures were incubated in a water bath at 37 °C for 1 h. The degradation of deoxyribose was monitored by vortexing the reaction mixture (1 ml) with 1 ml of 2.8% (m/v) cold TCA and 1 ml of 1% (m/v) TBA (in 0.05 M NaOH). After incubation at 95 °C for 15 min. and cooling on ice, the absorbance was measured at 532 nm. The antioxidant/pro-oxidant activity of wine samples was expressed as % change (positive values correspond to pro-oxidant activity and negative values correspond to antioxidant activity) relative to a control:

$$\% \text{ Change} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

where $\text{Abs}_{\text{Control}}$ = Absorbance of control at 532 nm

$\text{Abs}_{\text{Sample}}$ = Absorbance of sample at 532 nm

4.2.6 Statistical analysis

All tests were carried out in triplicate and the results averaged. Microsomal lipid peroxidation assays for wines were carried out in a random manner in triplicate on two separate days and the results averaged. Analysis of phenolic compounds was carried out on two separate days. One way ANOVA was performed on the means to determine significant differences between cultivars and the different phenolic compounds. Statistical comparisons between cultivars were made using the Student's t-LSD test ($P < 0.05$). The SAS version 6.12 software package was used for statistical analysis.

4.3 Results and discussion

4.3.1 Phenolic composition and ascorbic acid content

Phenolic composition of the red and white cultivar wines is described in Chapter 3 (3.3.1, Table 2 and 3). For the purpose of this discussion, a brief summary will be given. The total phenol and the individual phenolic group contents, were much higher in red wines than in white wines. The flavanols were the most prevalent phenolic group in the red wines, while the tartaric acid esters were the dominant phenolic group in the white wines. Ruby Cabernet wines contained significantly ($P < 0.05$) less total phenols and flavanols than Shiraz and Merlot wines, but more monomeric anthocyanins than the other red wine cultivars. Pinotage wines, made from the unique South African wine grape cultivar of the same name, exhibited a phenolic composition comparable to the other red wines investigated. Amongst the white wines, Chardonnay and Chenin blanc represented the highest and lowest content of total phenols and all phenolic groups determined. The phenolic content within cultivars varied considerably in terms of all phenolic groups determined. As the wines were purchased at different wineries around

the Western Cape differences in climate, soil type, vinification techniques and time of wood maturation were likely to contribute to this variation.

Eleven of the forty white wines investigated contained a measurable amount of ascorbic acid (3.2.6, Table 6). The presence of ascorbic acid is relevant as it acts as a free radical scavenger (Rice-Evans *et al*., 1996), as well as a pro-oxidant, by recycling Fe^{3+} to Fe^{2+} (Sevanian & Ursini, 2000).

4.3.2 Inhibition of microsomal lipid peroxidation (MLP)

Inhibition of microsomal lipid peroxidation exhibited a linear response to an increase in total phenol content in the reaction mixture at a range of 0 – 7 mg GAE/L for a Pinotage and 0 – 20 mg GAE/L a Chardonnay (Figure 1). The red wine exhibited an IC_{50} value of 4.44 mg GAE/L, while for the white wine it was 11.84 mg GAE/L suggesting that the combination of phenolic compounds found in red wine was more effective as inhibitor of lipid peroxidation in a microsomal membrane system than that of white wine.

For comparison of cultivar wines, all wines within a group (red or white wine) were diluted on the same volumetric basis to allow easy comparison of their inhibitory activity. Red wines exhibited a higher inhibitory effect on MLP than white wines, although the total phenol content for individual wines in the reaction mixture ranged from 2.93 – 6.33 mg GAE/L for red wines and from 7.57 – 17.59 mg GAE/L for white wines (Table 1). Considering the red cultivar wines, Merlot (74.8%) had a significantly ($P < 0.05$) higher % MLP inhibition than Ruby Cabernet (58.2%) and Pinotage (61.8%), while the inhibitory effect of Cabernet Sauvignon (67.4%) and Shiraz (69.4%) was similar ($P \geq 0.05$) to that of Merlot (74.8%). Ruby Cabernet wines contained significantly ($P < 0.05$) less total phenols and flavanols, but significantly ($P < 0.05$) more monomeric anthocyanins than the Merlot wines (3.3.1, Table 2). Subsequently the ratio of flavanols and monomeric anthocyanins to the total phenols were also significantly ($P < 0.05$) lower and higher for Ruby Cabernet than Merlot respectively (3.3.1, Table 3). The AP of Merlot (15.07), Ruby Cabernet (14.46), Cabernet Sauvignon(14.40) and Shiraz (14.29), however, did not differ significantly ($P < 0.05$) although the phenolic composition of Ruby Cabernet differed from that of other red cultivar wines. The AP for

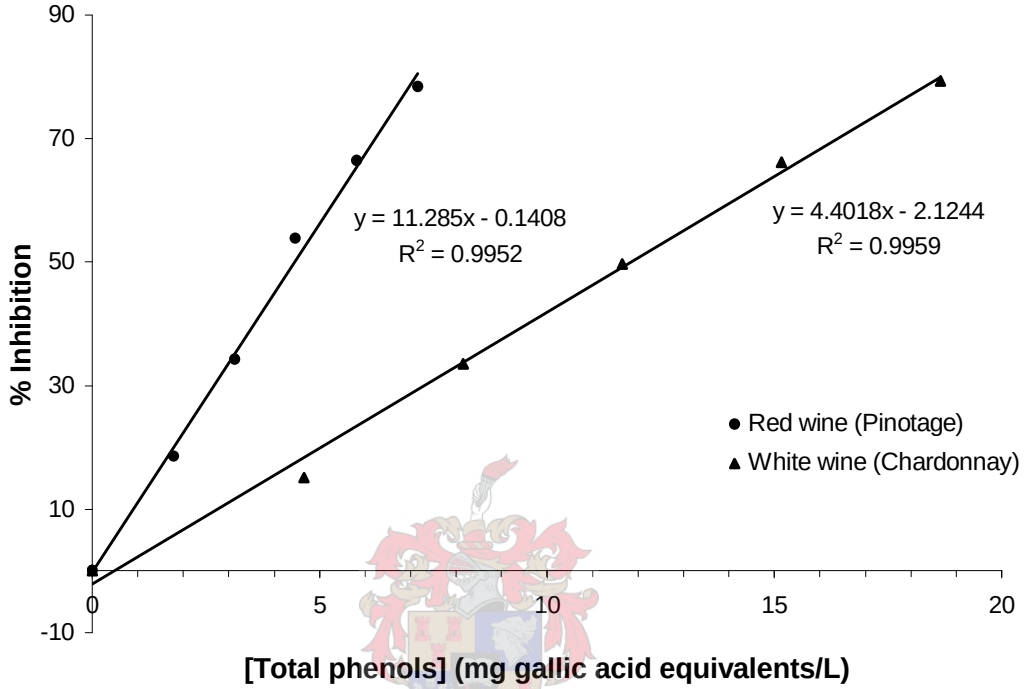

Figure 1 Inhibition of microsomal lipid peroxidation by a red (Pinotage) and a white wine (Chardonnay) at different total phenol concentrations in the reaction mixure.

Table 1 Inhibition of microsomal lipid peroxidation by South African red and white cultivar wines[a]

Wine	Total phenols[b]	% Inhibition	AP[c]
Red			
Cabernet Sauvignon	4.69 ab[d] (± 0.53)[e]	67.4 abc (± 7.6)	14.40 a (± 0.97)
Ruby Cabernet	4.03 b (± 0.73)	58.2 c (± 9.8)	14.46 a (± 0.81)
Pinotage	4.68 ab (± 0.82)	61.8 bc (± 12.1)	13.16 b (± 1.06)
Shiraz	4.82 a (± 0.90)	69.4 ab (± 15.5)	14.29 a (± 1.19)
Merlot	5.00 a (± 0.82)	74.8 a (± 10.3)	15.07 a (± 1.22)
Average (Red)	**4.67**	**66.7**	**14.25**
White			
Sauvignon Blanc	11.00 ab (± 1.58)	49.4 ab (± 15.2)	4.54 ab (± 1.45)
Chenin Blanc	9.68 b (± 2.10)	33.8 b (± 9.4)	3.79 b (± 0.96)
Chardonnay	11.71 a (± 1.15)	59.5 a (± 16.6)	4.89 a (± 1.22)
Colombar	11.42 ab (± 2.97)	35.4 b (± 13.1)	3.55 b (± 1.10)
Average (White)	**10.95**	**44.5**	**4.19**

[a] Detailed results for each wine are reported in Addendum D.

[b] Total phenols expressed as mg gallic acid equivalents/L in the reaction mixture.

[c] Antioxidant potency (AP) = % inhibition / total phenol content in the reaction mixture (mg gallic acid equivalents/L).

[d] Averages in a column followed by different letters, differ significantly ($P < 0.05$). Data for red and white wines were analysed separately.

[e] Standard deviation.

Pinotage (13.16) was significantly ($P < 0.05$) lower than for the other red cultivar wines (14.29 - 15.07) (Table 1). Differences in the phenolic composition as estimated spectrophotometrically in this study could not explain this phenomenon. The importance of individual phenolic compounds, which may be present in higher amounts in Pinotage than in other red cultivar wines, is therefore a possible explanation. The contribution of individual phenolic compounds and specific combinations of compounds is again highlighted in this case as discussed in 3.3.

Amongst the white cultivar wines, Chardonnay (59.5%) was a significantly ($P < 0.05$) more effective MLP inhibitor than Chenin blanc (33.8%) and Colombar (35.4%). Chardonnay contained a significantly ($P < 0.05$) higher total phenol content than Chenin blanc (3.3.1, Table 2), while its total phenol content was similar to that of Colombar. The difference in efficacy to inhibit MLP between Chardonnay, Colombar and Chenin blanc could be attributed to the flavanol and flavonol contents (3.3.1, Table 2) and their ratios with respect to total phenols (3.3.1, Table 3). In addition, AP was significantly ($P < 0.05$) higher for Chardonnay (4.89) than for Chenin blanc (3.79) and Colombar (3.55) (Table 1). The contribution of flavanols to the MLP inhibition appears to be prominent for white wine.

4.3.3 Correlation analysis

Correlation analysis of data obtained for phenolic composition and inhibition of MLP are reported in Table 2.

The total phenol ($r = 0.90$, $P < 0.001$) (Figure 2) and flavanol ($r = 0.88$, $P < 0.001$) (Figure 3A) contents of red wine exhibited the strongest correlations with inhibition of MLP. The other phenolic groups, namely tartaric acid esters ($r = 0.69$, $P < 0.001$), polymeric anthocyanins ($r = 0.58$, $P < 0.001$) and flavonols ($r = 0.50$, $P < 0.001$), exhibited a weaker correlation to MLP inhibitory activity. No correlation between the monomeric anthocyanin content and inhibition of MLP ($r = 0.06$, $P = 0.68$) was observed. Anthocyanidins (the aglycones of anthocyanins) have, however, been reported as effective inhibitors of low-density lipoprotein and liposome oxidation (Tsuda *et al*., 1994; Satué-Gracia *et al*., 1997). In red wines, the glycoside derivatives of

Table 2 Correlation of the inhibition of microsomal lipid peroxidation with the contents of phenolic groups of red and white wines

	% Inhibition (all wines)[a]		% Inhibition (wines without ascorbic acid)[a]	
	r	**P**	**r**	**P**
Red wine				
Total phenols	0.90	< 0.001	N/A	N/A
Monomeric anthocyanins	0.06	0.68	N/A	N/A
Polymeric anthocyanins	0.58	< 0.001	N/A	N/A
Flavanols	0.88	< 0.001	N/A	N/A
Flavonols	0.50	< 0.001	N/A	N/A
Tartaric acid esters	0.60	< 0.001	N/A	N/A
White wine				
Total phenols	0.53	< 0.001	0.73	< 0.001
Flavanols	0.79	< 0.001	0.85	< 0.001
Flavonols	0.54	< 0.001	0.50	0.009
Tartaric acid esters	0.73	< 0.001	0.71	< 0.001

[a] % Inhibition of microsomal lipid peroxidation.
N/A Not applicable.

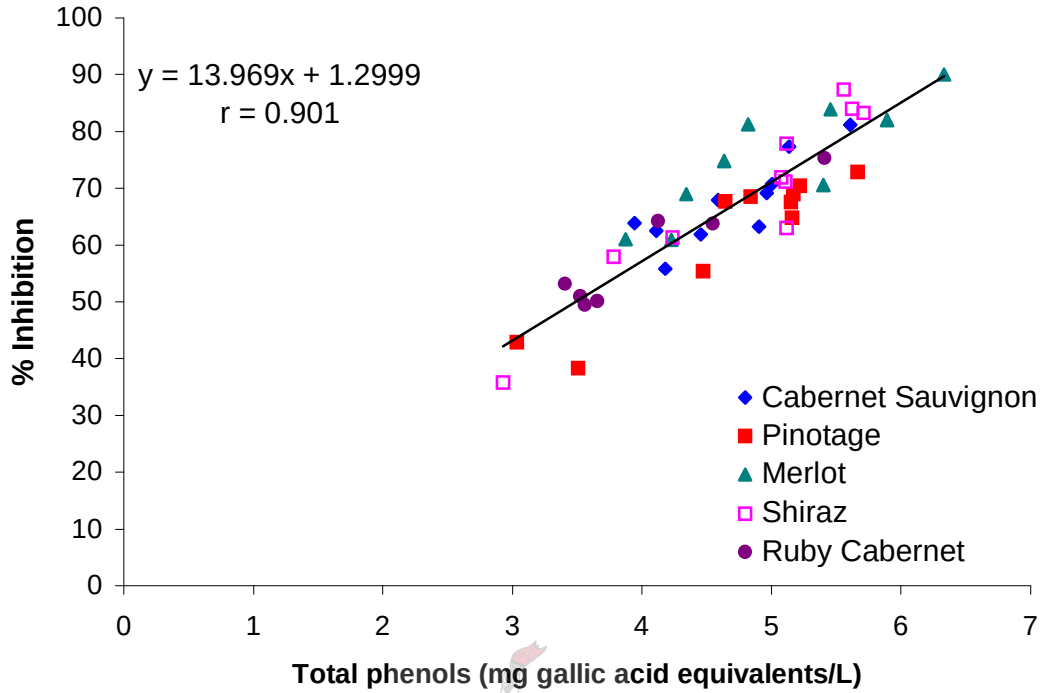

Figure 2 Inhibition of microsomal lipid peroxidation by different red cultivar wines as a function of total phenol content in the reaction mixture.

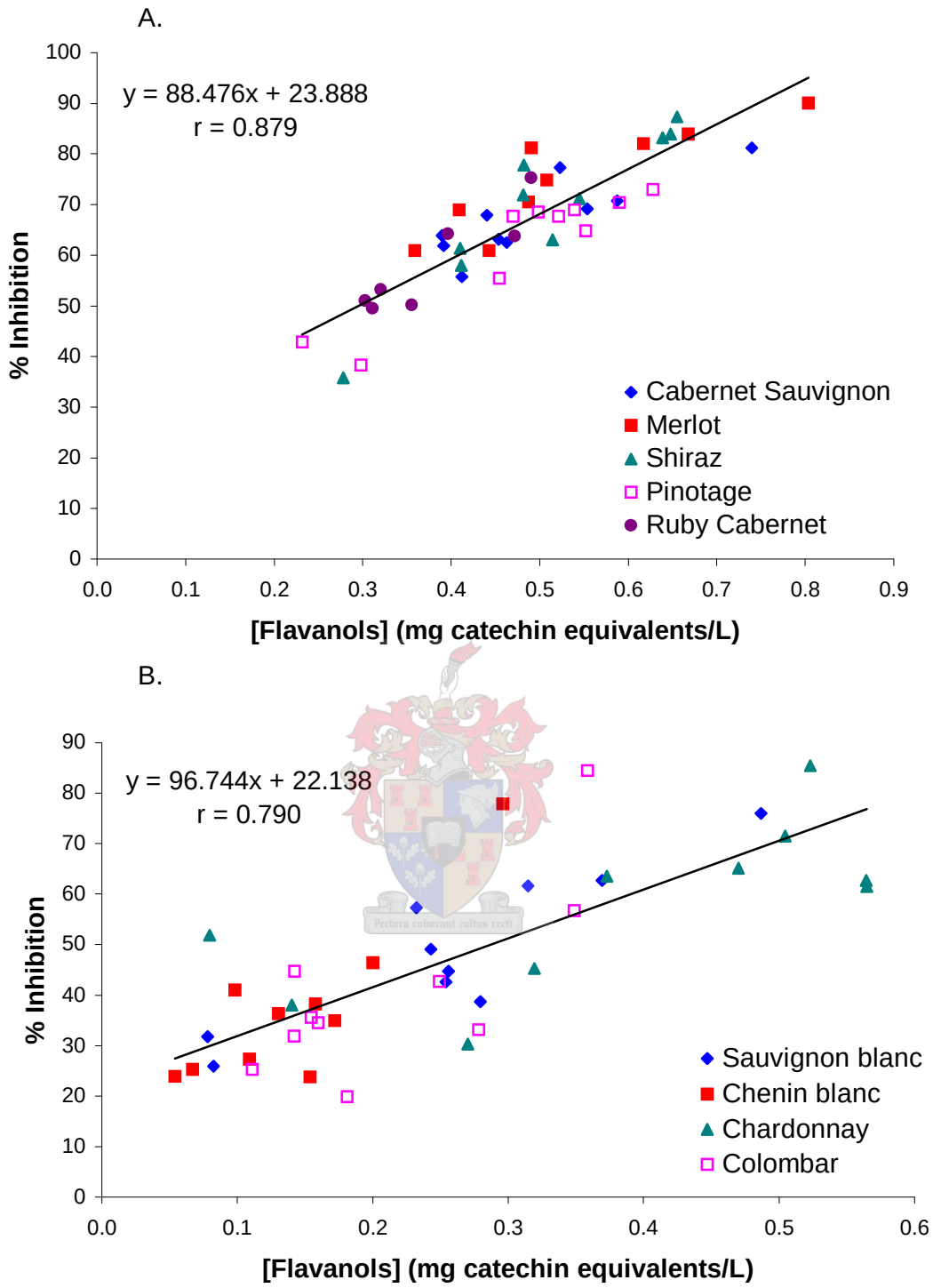

Figure 3 Inhibition of microsomal lipid peroxidation by different red (A) and white (B) cultivar wines as a function of flavanol content in the reaction mixture.

anthocyanidin, namely the anthocyanins, are generally found (Macheix *et al*., 1990). Several studies indicated that quercetin can penetrate artificial membranes with a concomitant fluidifying effect on the membrane due to its planar structural conformation (Saija *et al*., 1995; Van Dijk *et al*., 2000). This effect would also be possible with anthocyanidins as they also have no chiral centres and, therefore, have a planar conformation. Anthocyanins on the other hand, would be incorporated into the membrane to a smaller extent due the lowered lipophilicity and the bulky glucoside constituent (Saija *et al*., 1995). At the pH in the reaction medium, anthocyanidins and anthocyanins would be present in the quinoidal base form, which is less active as antioxidant (Lapidot *et al*., 1999) and is less stable than at the lower pH expected in wine (Cabrita *et al*., 2000).

In the case of white wines including those containing ascorbic acid, the flavanol ($r = 0.79$, $P < 0.001$) (Figure 3B) and tartaric acid ester ($r = 0.73$, $P < 0.001$) (Figure 4) contents strongly correlated with inhibition of MLP. Flavonols ($r = 0.54$, $P < 0.001$) exhibited a weaker correlation with MLP inhibitory activity. When wines containing ascorbic acid were omitted, the total phenol ($r = 0.73$, $P < 0.001$) (Figure 5), flavanol ($r = 0.85$, $P < 0.001$) and tartaric acid ester ($r = 0.71$, $P < 0.001$) contents showed good correlation with the MLP inhibitory activity with flavanols exhibiting a weaker correlation ($r = 0.50$, $P = 0.009$). Correlations for total phenols and flavanols improved after ommission of wines containg ascorbic acid. Wines containing ascorbic acid had the same or less inhibitory activity than wines containing no ascorbic acid at the same total phenol concentration (Figure 5). This became evident for the two wines (one Sauvignon blanc and one Colombar) containing 46.01 and 61.34 mg/L ascorbic acid. Using the regression analysis for % MLP inhibition as a function of total phenol content for wines in the absence of ascorbic acid the estimated % MLP inhibition for these wines is 106.9 and 76.4% respectively. The measured values were, however, only 44.8 and 25.9% for the Sauvignon blanc and Colombar respectively. This discrepancy could be ascribed to the ability of ascorbic acid, a good antioxidant, to recycle Fe^{3+} to Fe^{2+}, thereby promoting lipid peroxidation (Sevanian & Ursini, 2000). In the nonpolar membrane matrix where the propagation reactions are fast,

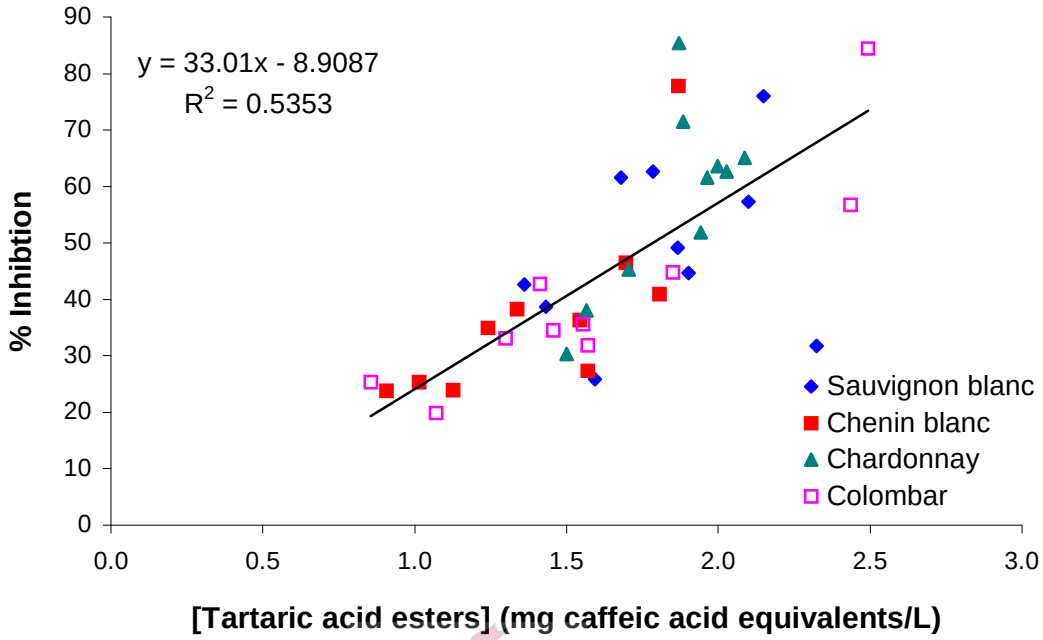

Figure 4 Inhibition of microsomal lipid peroxidation by different white cultivar wines as a function of tartaric acid ester content in the reaction mixture.

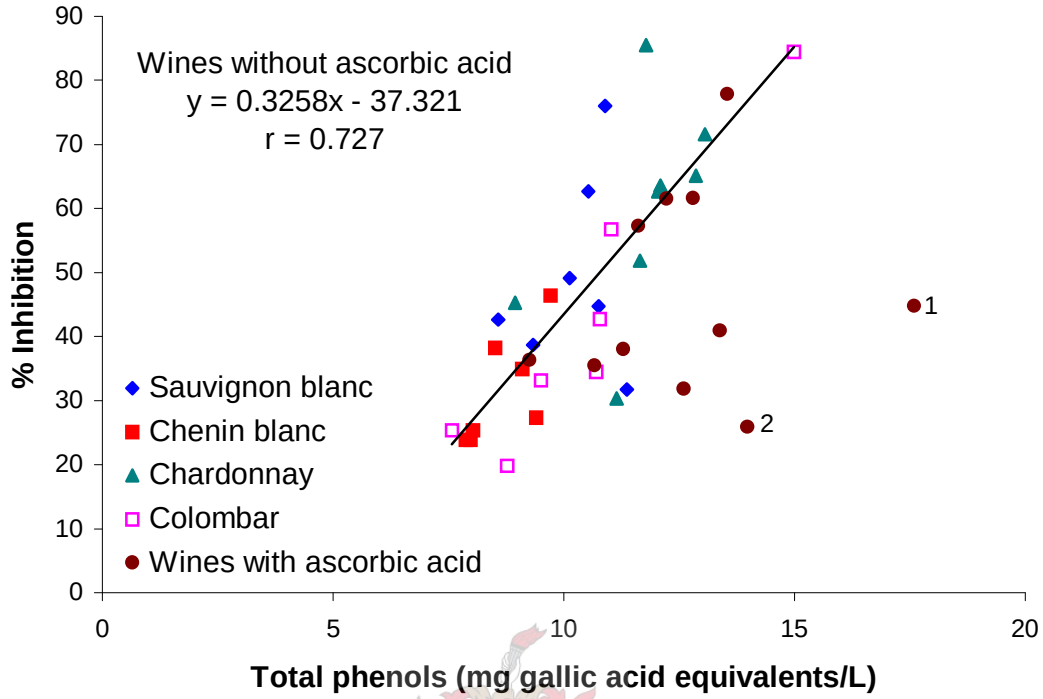

Figure 5 Inhibition of microsomal lipid peroxidation by different white cultivar wines as a function of total phenol content in the reaction mixture. Wines containing ascorbic acid were not shown according to cultivar, but separately. Data points marked 1 and 2 represents two wines with the highest ascorbic acid content, namely 46.01 and 61.34 mg/L, respectively.

ascorbic acid cannot efficiently compete with lipids for the reduction of peroxyl radicals and, therefore, its antioxidant action cannot overcome the pro-oxidant action (Sevanian & Ursini, 2000). This phenomenon could increase the lipid peroxidation resulting in underestimation of the protective effect of wines containing ascorbic acid in a membrane system.

4.3.4 Determination of IC_{50} values for selected wine phenolic compounds

Selected wine phenolic compounds were tested for their ability to inhibit microsomal lipid peroxidation (Table 3). The graph used to calculate the IC_{50} values for (+)-catechin and (-)-epicatechin is shown in Figure 6. The IC_{50} values (μM) for the compounds increased in the order: quercetin < procyanidin B1 ≈ malvidin < cyanidin ≈ (-)-epicatechin < (+)-catechin ≈ delphinidin. When the IC_{50} values were expressed as mg/L the order was as follows: quercetin < (-)-epicatechin ≈ malvidin ≈ cyanidin < procyanidin B1 < (+)-catechin < delphinidin. Liao & Yin (2000) determined the activity of several phenolic compounds, including (+)-catechin, (-)-epicatechin and quercetin, in human erythrocyte membrane ghosts and found the same trends in the protective activity. Liao & Yin (2000) also showed that the less lipophilic compounds exhibited a higher antioxidant activity. When considering membrane systems, the partitioning of antioxidants between the lipid and aqueous phases becomes important. Partitioning would depend on lipophilicity of the molecule, as well as three-dimensional conformation of the molecule (Liao & Yin, 2000; Saija *et al*., 1995; Van Dijk *et al*., 2000). Hydrophilic antioxidants can localise on the surface of the membrane where they are accessible to chain-initiating peroxyl radicals which can be easily trapped (Rice-Evans & Packer, 1998). Lipophilic antioxidants, on the other hand, would be able to partition into the lipid phase to a greater extent and could prevent propagation reactions or regenerate endogenous antioxidants such as α-tocopherol (Van Acker *et al*., 1996; Rice-Evans & Packer, 1998). Phenolic compounds, which are more hydrophilic, could, therefore, mainly act as scavengers of chain-initiating peroxyl radicals and metal chelators

Table 3 Antioxidant activity of selected wine phenolic compounds

Phenolic compound	**IC_{50}[a] (μM)**	**IC_{50}[a] (mg/L)**
Quercetin	1.27 a[b]	0.43 a
(+)-Catechin	6.54 d	1.90 d
(-)-Epicatechin	4.57 c	1.33 b
Malvidin	3.46 b	1.27 b
Cyanidin	4.40 c	1.42 b
Delphinidin	6.91 d	2.34 e
Procyanidin B1	4.83 b	2.79 c

[a] Concentration needed in reaction mixture to obtain 50% inhibition of microsomal lipid peroxidation.

[b] Averages in a column followed by different letters, differ significantly ($P < 0.05$).

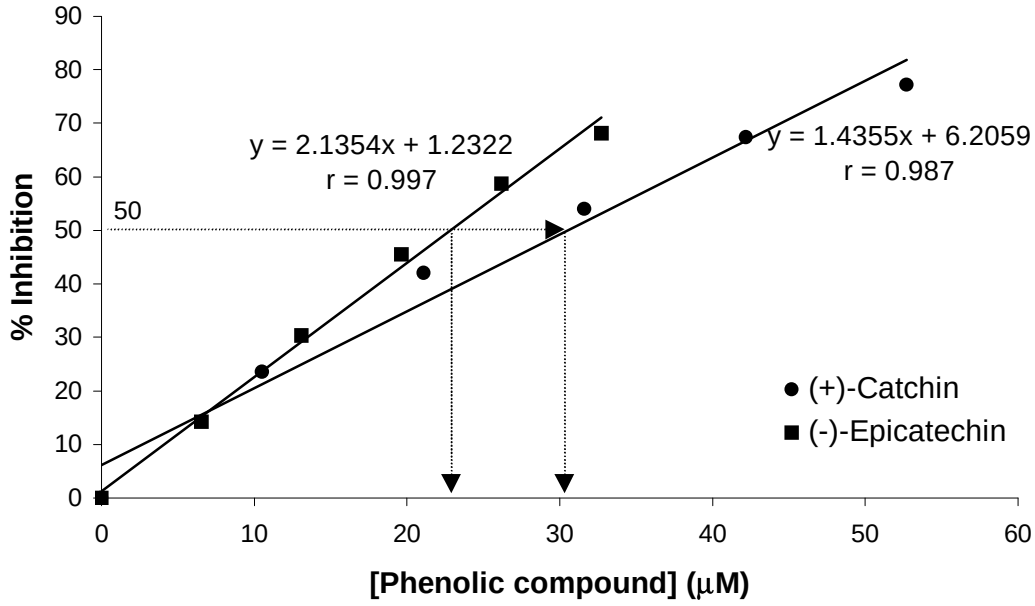

Figure 6 Graphic determination of IC_{50} values for (+)-catechin and (-)-epicatechin.

on the surface between the lipid and aqueous phase. A planar structural conformation (e.g. flavonols and anthocyanidins) will promote incorporation of molecules into the membrane, whereas a twisted conformation (e.g. flavanols) or substituents out of the plane of the flavone skeleton (e.g. tartaric acid esters or glycoside derivatives of flavonols and anthocyanidins) will obstruct incorporation of molecules into the membrane (Saija *et al*., 1995; Van Dijk *et al*., 2000). The binding of iron to microsomes (Vile & Winterbourne, 1987) enables the immediate reaction of iron-mediated free radical species with available PUFA's. Metal chelators could, therefore, in theory prevent iron-dependent lipid peroxidation in membranes. A study by Van Acker *et al*. (1998), however, reported that metal chelation did not play a role in the antioxidant activity of a number of phenolic compounds using a microsomal lipid peroxidation assay. On the other hand, a study by Sugihara *et al*. (1999) showed that activity of phenolic compounds to inhibit lipid hydroperoxide dependent peroxidation in cultured hepatocytes differ depending on the metal ion used. This was attributed to differring abilities of phenolic compounds to chelate different metal ions.

4.3.5 Activity in the deoxyribose assay

The phenolic composition of wines used in the deoxyribose assay is summarised in Table 4. The Pinotage had a higher total phenol content than the Cabernet Sauvignon, while the Chardonnay had a higher total phenol content than the Chenin blanc. The contributions of phenolic groups to total phenols were similar for the two red wines except for the tartaric acid ester to total phenol ratio. The Cabernet Sauvignon wine has less tartaric acid esters per total phenols than the Pinotage wine. For the white wines, the ratios of phenolic groups to total phenols were also the same except for the flavanol to total phenol ratio, which were lower in Chenin blanc than in Chardonnay.

The activity of the phenolic compounds of the wines in the deoxyribose assay depended on whether ascorbic acid and/or EDTA were present in the reaction mixture. The activity of the wines are shown in Figure 7A and B (Pinotage), Figure 8A and B (Cabernet Sauvignon), Figure 9A and B (Chardonnay) and Figure 10A and B (Chenin blanc). Changes in absorbance

Table 4 Phenolic composition of wines used in the deoxyribose assay

	Pinotage	Cabernet Sauvignon	Chardonnay	Chenin blanc
Total phenols[a]	1965.5	1645.2	233.4	166.1
Anthocyanins[b]	433.23	360.24		
% Anthocyanins[c]	22.04	21.90		
Flavanols[d]	162.92	139.42	9.46	2.87
% Flavanols[c]	8.29	8.47	4.05	1.73
Flavonols[e]	145.56	113.33	19.78	13.89
% Flavonols[c]	7.41	6.89	8.47	8.36
Tartaric acid esters[f]	308.26	193.86	57.57	38.70
% Tartaric acid esters[c]	15.68	11.78	24.67	23.30

[a] expressed as mg gallic acid equivalents/L.
[b] anthocyanins expressed as mg Malvidin-3-glucosides/L.
[c] % Phenolic group = g phenolic group per 100g total phenols.
[d] expressed as mg catechin equivalents/L.
[e] expressed as mg quercetin equivalents/L.
[f] expressed as mg caffeic acid equivalents/L.

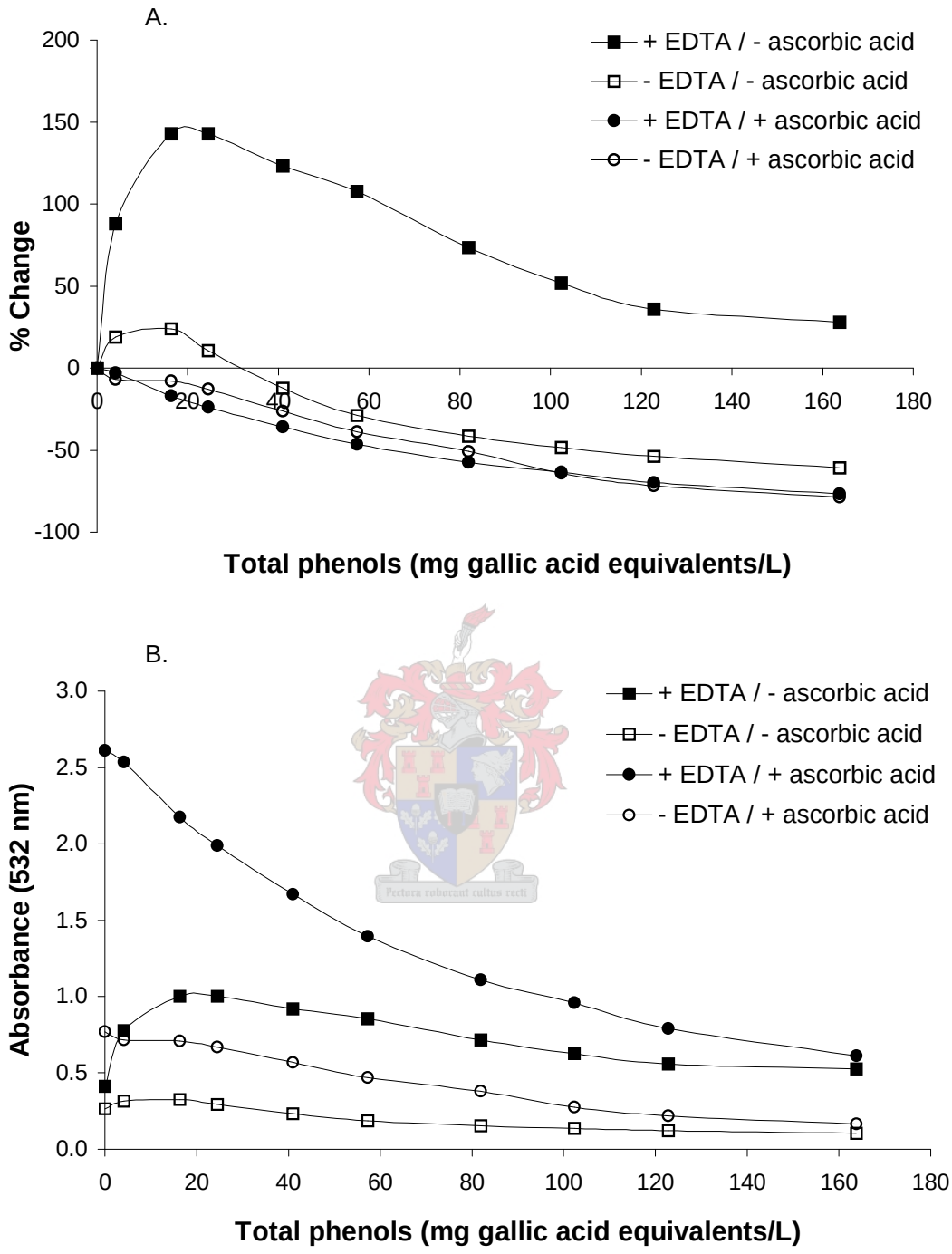

Figure 7 Activity of Pinotage wine in the deoxyribose assay using different assay conditions expressed as % change relative to the control (A) and absorbance (532 nm) (B) as a function of total phenol content in the reaction mixture (+ = presence and - = absence).

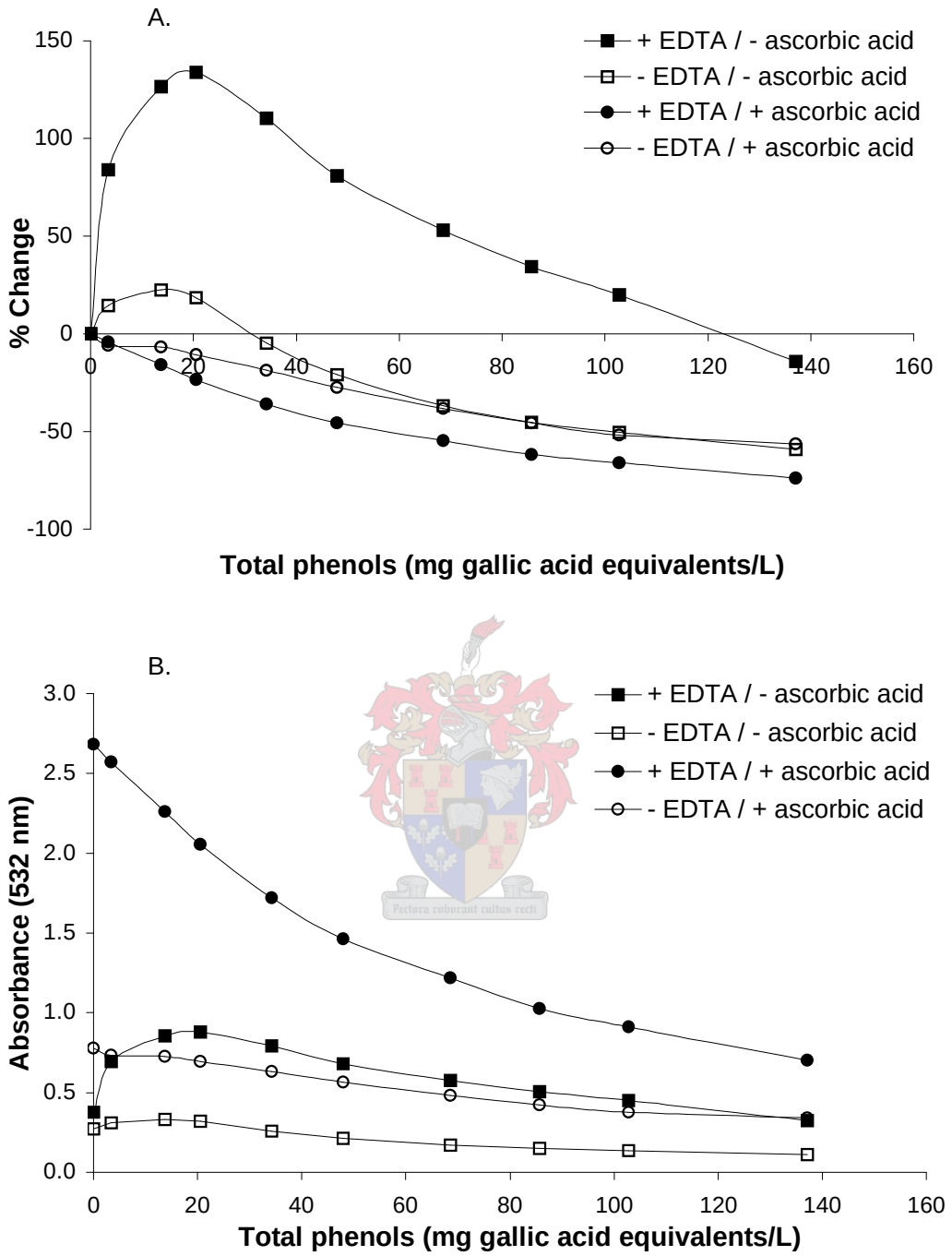

Figure 8 Activity of Cabernet Sauvignon wine in the deoxyribose assay using different assay conditions expressed as % change relative to the control (A) and absorbance (532 nm) (B) as a function of total phenol content in the reaction mixture (+ = presence and - = absence).

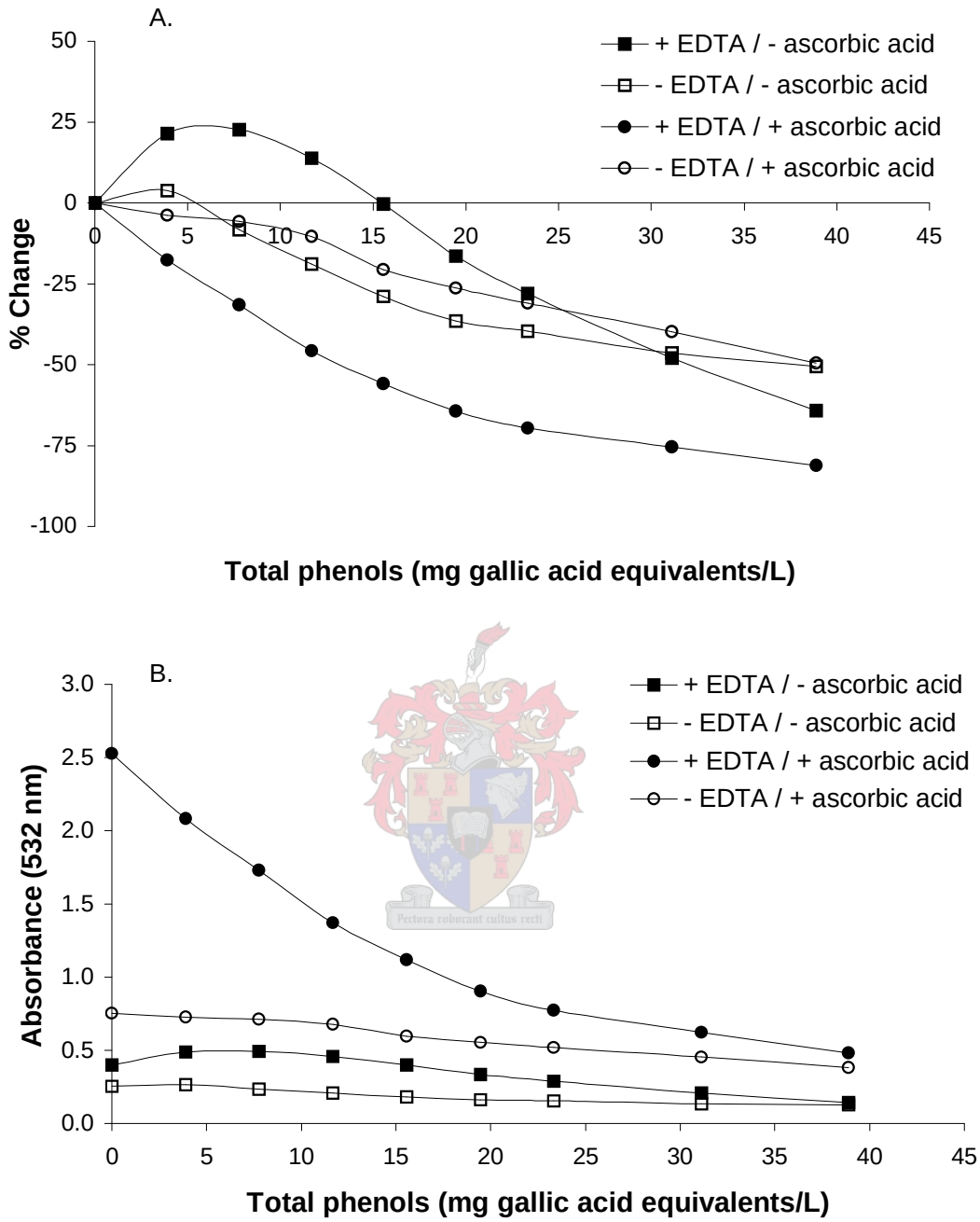

Figure 9 Activity of Chardonnay wine in the deoxyribose assay using different assay conditions expressed as % change relative to the control (A) and absorbance (532 nm) (B) as a function of total phenol content in the reaction mixture (+ = presence and - = absence).

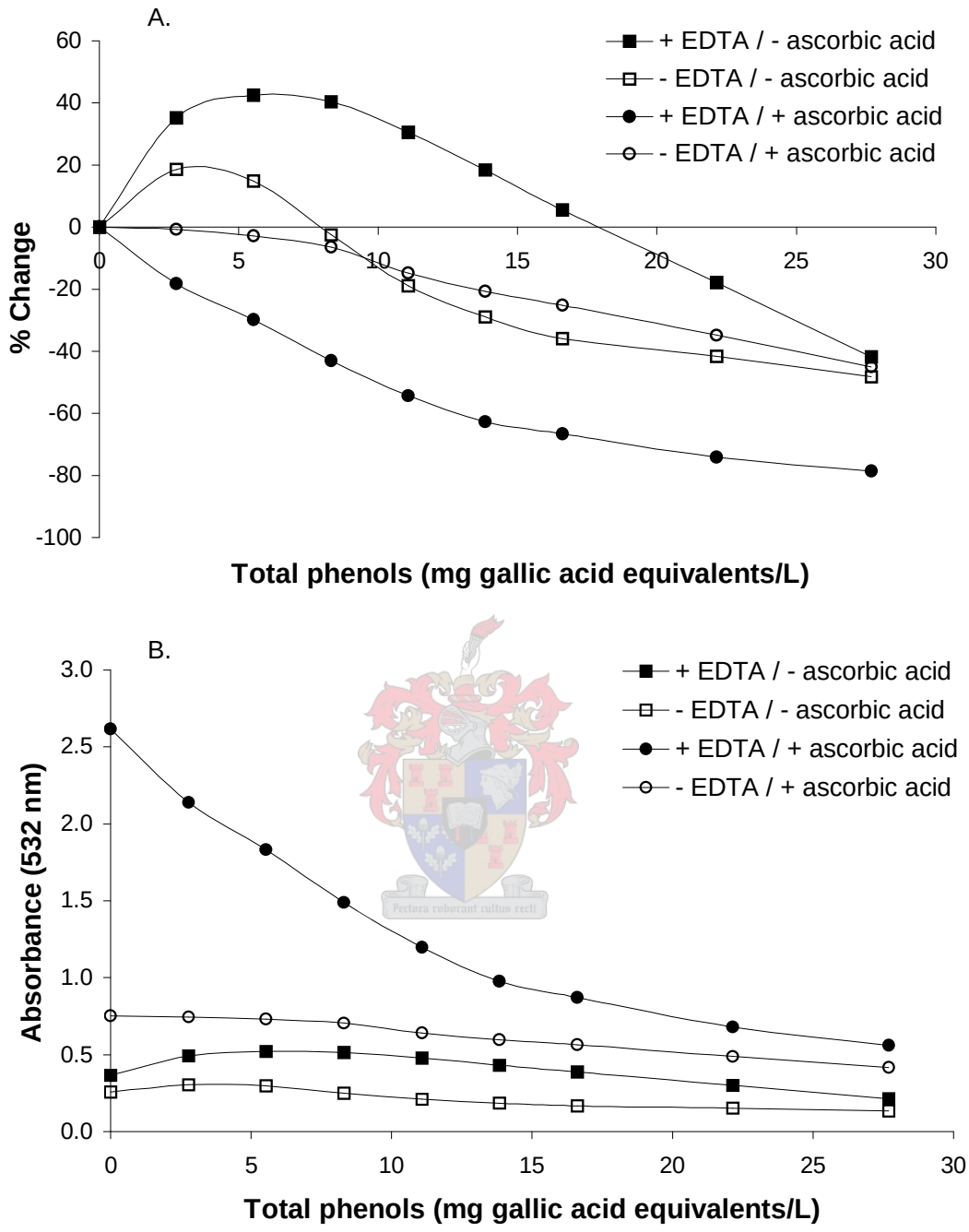

Figure 10 Activity of Chenin blanc wine in the deoxyribose assay using different assay conditions expressed as % change relative to the control (A) and absorbance (532 nm) (B) as a function of total phenol content in the reaction mixture (+ = presence and - = absence).

values are shown in Figure 7A, 8A, 9A and 10A, while the % change from the intial absorbance is shown in Figure 7B, 8B, 9B and 10B. A positive % change indicates pro-oxidant activity, while a negative value indicates antioxidant activity.

The possible pro-oxidant activity of samples is evaluated in the presence of EDTA without ascorbic acid (Aruoma, 1994). Pro-oxidant activity is exhibited if antioxidant compounds are able to reduce Fe^{3+} to Fe^{2+} (Zhao *et al*., 1994). All four wines (both red and white) exhibited pro-oxidant activity with the phenolic compounds of red wines having a higher maximum pro-oxidant activity. Maximum pro-oxidant activity (ca. 143%) for both red wines occurred at approximately 20 mg GAE/L, while for both white wines maximal pro-oxidant activity (ca. 22% for Chardonnay and ca. 42% for Chenin blanc) occurred at approximately 6 mg GAE/L. This indicates the higher efficiency of red wine phenolic compounds to reduce Fe^{3+} to Fe^{2+}.

The hydroxyl radical scavenging activity of the wines was determined in the presence of both ascorbic acid and EDTA. This is due to maximum iron recycling by ascorbic acid (Aruoma *et al*., 1994). All the wines scavenged hydroxyl radicals under these reaction conditions. The phenolic compounds of both white wines were more effective hydroxyl radical scavengers than those of red wines. At a total phenol concentration of 20 mg GAE/L, red wines exhibited approximately 20% inhibition, while white wines exhibited approximately 70% inhibition. Red wines only reached a level of 70% inhibition at approximately 120 mg GAE/L. White wine phenolic compounds were, therefore, much better hydroxyl radical scavengers than those of the red wines.

Metal chelating ability of wines was determined in the absence of EDTA. In this instance, iron ions loosely associate with deoxyribose molecules resulting in the formation of hydroxyl radicals close to the deoxyribose molecules. Damage to deoxyribose is, therefore, 'site-specific' (Aruoma *et al*., 1994). When ascorbic acid was also omitted, the wines still exhibited some pro-oxidant activity at very low total phenol concentration. The reason for this is that at these concentrations iron chelation was not effective, allowing some recycling of iron by phenolic compounds. The red wines exhibited pro-oxidant activity between 0 and 30 mg GAE/L, while for white wines this behaviour was

only evident between 0 and 8 mg GAE/L. Chardonnay and Chenin blanc exhibited 40% inhibition at a total phenol content of 23 and 22 mg GAE/L respectively, while Pinotage and Cabernet Sauvignon exhibited 40% inhibition only at 80 and 67 mg GAE/L respectively. When EDTA was omitted with ascorbic acid present in the reaction mixture, however, no pro-oxidant activity was detected and metal chelating ability was demonstrated at all concentrations. At a total phenol concentration of 20 mg GAE/L red wines inhibited deoxyribose damage up to 10%, while white wines at the same total phenol concentration inhibited deoxyribose degradation up to 27%. This reduction in 'site-specific' deoxyribose damage could be ascribed to metal chelating ability of phenolic compounds. The metal chelating ability of white wine phenolic compounds is, therefore, greater than that of red wine phenolic compounds.

4.4 Conclusions

The phenolic content of wines and their composition in terms of different phenolic groups were important determinants of their ability to inhibit microsomal lipid peroxidation. Significant differences in the activity of red and white wines were observed as a result of differences in their phenolic composition. Total phenol and flavanol content of red wines correlated well with inhibitory activity while other phenolic groups exhibited a weaker correlation. Monomeric anthocyanin content was not correlated to inhibition of microsomal lipid peroxidation. Total phenols, flavanols and tartaric acid esters in white wines exhibited strong correlations with inhibition of microsomal lipid peroxidation while flavonols in white wines exhibited a weaker correlation. The presence of ascorbic acid in some white wines modified their antioxidant behaviour by its pro-oxidant action in the microsomal membrane system, counteracting their protective effect.

Red wine phenolic fractions which exhibited higher antioxidant potency than white wines in the microsomal lipid peroxidation assay, also exhibited higher pro-oxidant effects in the deoxyribose assay. The metal chelating ability of red wines was lower than that of white wines, confirming the notion

that metal chelating ability does not play a major role during inhibition of microsomal lipid peroxidation.

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CHAPTER 5

THE EFFECT OF IN-BOTTLE AGEING ON THE ANTIOXIDANT ACTIVITY OF RED AND WHITE WINES

Abstract

The effect of in-bottle ageing on the antioxidant activity of South African red (Pinotage and Cabernet Sauvignon) and white (Chardonnay and Chenin blanc) cultivar wines was determined during storage at 0, 15 and 30 ºC for 12 months using the 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) radical cation ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging assays. Storage resulted in a significant ($P < 0.05$) decrease in both the total antioxidant activity (TAA_{ABTS} and TAA_{DPPH}) and the total phenol content of the red and white wines. The TAA_{ABTS} of Cabernet Sauvignon, as well as the TAA_{ABTS} and total phenol content Chardonnay stored at 30 ºC, were significantly ($P < 0.05$) lower than at 0 ºC. The antioxidant potency of the phenolic fraction of most wines also decreased during storage. The $ABTS^{\bullet+}$ scavenging assay seems to be a better method to differentiate wines with subtle differences in their phenolic composition from each other. The total phenol content correlated with the TAA_{ABTS} for the red ($r = 0.938$, $P < 0.001$) and white ($r = 0.913$, $P < 0.001$) wines. The decrease in the TAA_{ABTS} of wines could, therefore, be attributed to a decrease in their total phenol content. Although the monomeric anthocyanins in red wines also decreased significantly ($P < 0.05$) over 12 months at 15 and 30 ºC, they only correlated weakly ($r = 0.50$, $P < 0.001$) with TAA_{ABTS} suggesting a less important role in the reduction of TAA_{ABTS}. The flavanol content of wines increased up to 9 months with a subsequent decrease to 12 months, while only minor changes in the flavonol and tartaric acid ester content of both red and white wines were observed. These phenolic groups also exhibited significant ($P < 0.001$) correlation with antioxidant activity. After 3 months of storage at 30 ºC, a significant ($P < 0.05$) decrease in total phenol content of Cabernet Sauvignon, monomeric and polymeric anthocyanin content of both red wines, as wel as a significant ($P < 0.05$) increase in flavanol content of Pinotage, Cabernet Sauvignon and Chardonnay and tartaric acid ester content of Cabernet Sauvignon, were observed. The importance of monitoring changes in the levels of individual phenolic compounds during ageing to explain the changes in the accompanied antioxidant status of wines, became apparent.

5.1 Introduction

Increased interest in the ability of wines and other foods to prevent free radical-mediated diseases was observed in recent years (Halliwell & Gutteridge, 1990; Davies, 1995; Leake, 1998). The possible health-promoting properties of wine are related in part to phenolic compounds and their ability to act as antioxidants (Kinsella *et al*., 1993). The influence of various technological factors relating to vineyard and vinification practices on the antioxidant activity of wines have been investigated recently (Hurtado *et a*l., 1997; Pellegrini *et al*., 2000). In-bottle ageing is critical in determining the quality of wines resulting in the improvement of red wine, while for white wine it could have detrimental effects such as non-enzymatic browning. Conflicting reports in terms of the effect of ageing on the antioxidant activity of red wines have appeared. In a small study, Manzocco *et al*. (1998) reported a decreased 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging rate for a red wine produced in 1973 compared to that of two red wines of the same cultivar produced in 1995 and 1996 respectively. Larrauri *et al*. (1999), however, showed an increase in antiradical efficiency of red wines with increased ageing status when using the same $DPPH^{\bullet}$ scavenging assay. The antiradical efficiency is a parameter combining the amount of total phenols needed and the time it takes to scavenge 50% of the initial free radicals (Larrauri *et al*., 1999). In both these studies, wines of different vintages were used for analysis and the differences in the original composition of the wines, as well as storage temperature, were not taken into account. In the study of Larrauri *et al*. (1999), wines of different ages were also matured in wood for different lengths of time. These factors, as well as differences in methodology used, contributed to the outcome of these studies.

Changes in phenolic composition of red wine occur during wood maturation and in-bottle ageing, depending on the phenolic composition, temperature, the presence of oxygen and sulphur dioxide concentration of the wine (Ribéreau-Gayon & Glories, 1986). Wood maturation results in extraction of phenolic compounds from wood and increased oxidative changes due to aeration (Del Alamo *et al*., 2000). In-bottle ageing results in

mostly anaerobic processes involving the copigmentation and polymerisation of anthocyanins although some oxygen is included in the headspace of the bottle (Somers & Pocock, 1990; Dallas & Laureano, 1994).

Copigmentation is the hydrophobic stacking of anthocyanins and other phenolic compounds such as (+)-catechin, (-)-epicatechin and a variety of phenolic acids. It is reported to be the first step in the formation of covalent bonds during condensation reactions (Brouillard & Dangles, 1994). Acetaldehyde formed during fermentation of wine or oxidation of phenolic compounds can also play a role in condensation reactions although these reactions are less important as free sulphur dioxide limits the amount of acetaldehyde available (Wildenradt & Singleton, 1974; Timberlake & Bridle, 1976; Saucier *et al*., 1997a; Saucier *et al*., 1997b). Formation of these complexes are favoured by low temperatures (Mazza, 1995). Hydroxyl groups, which confer antioxidant activity to phenolic compounds, are generally not involved during condensation reactions although after condensation they might be less available for reaction with free radical species due to steric hindrance (Yoshida *et al*., 1989). A large number of new compounds formed as products of condensation reactions has been elucidated (Jurd, 1967; Castagnino & Vercauteren, 1996; Fulcrand *et al*., 1997; Fulcrand *et al*., 1998; Es-Safi *et al*., 1999a; Es-Safi *et al*., 1999b; Remy *et al*., 2000).

Reactions in white wines after fermentation mostly involve non-enzymatic, oxidative browning (Ribéreau-Gayon *et al*., 2000). Oxidation of *ortho*-dihydroxyphenolic compounds such as (+)-catechin, (-)-epicatechin, caffeic acid and other hydroxycinnamic acids leads to the formation of yellow or brown products from the polymerisation of *ortho*-quinones (Singleton, 1987; Guyot *et al*., 1996). Other constituents of wine such as metal ions and sulphur dioxide can play a role in the reaction (Singleton, 1987; Cilliers & Singleton, 1990). Sulphur dioxide added to wine is able to reduce the *ortho*-quinones back to their original forms, while metal ions can catalyse the oxidation reaction (Singleton, 1987). Ascorbic acid is added to some white wines to prevent browning due to its antioxidant action. However, it has been shown that ascorbic acid can increase oxidative browning of (+)-catechin in a model solution. This phenomenon is reportedly caused by dehydroascorbic acid by an unknown mechanism (Bradshaw *et al*., 2001). No information is

available about the effect of changes in phenolic composition during in-bottle ageing of white wines on their antioxidant activity.

The aim of the study was to determine the effect of in-bottle ageing on the antioxidant activity of South African red (Pinotage and Cabernet Sauvignon) and white (Chardonnay and Chenin blanc) cultivar wines in relation to changes in the phenolic composition. Storage of wines at 0, 15 and 30 ºC enabled evaluation of accelerated storage conditions (30 ºC) and normal cellar conditions (15 ºC) compared to changes that occur at very low temperature storage (0 ºC) over the period of one year.

5.2 Materials and methods

5.2.1 Wines and chemicals

Wines were prepared according to the Nietvoorbij standardised procedure for small-scale winemaking at the experimental winery of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa). No ascorbic acid was added to white wines during vinification. Three batches each of grapes from two red wine grape cultivars (Pinotage and Cabernet Sauvignon) and two white wine grape cultivars (Sauvignon blanc and Chenin blanc) were prepared and bottled during the 2000 harvesting season. The wines were stored at 0, 15 and 30 ºC for 12 months in temperature-controlled rooms in the dark. One bottle of each batch and cultivar/temperature combination was sampled every three months and aliquots frozen at –18 °C in plastic screw-top sample holders (40 ml) until analysed.

Chemicals used were the same as described in 3.3.1.

5.2.2 Determining the phenolic composition

Spectrophotometric determination of the total phenol (Singleton & Rossi, 1965), anthocyanin (Burns *et al*., 2000), flavanol (McMurrough & McDowell, 1978), flavonol (Mazza *et al*., 1999) and tartaric acid ester (Mazza *et al*., 1999) contents of red and white wines is described in 3.2.3.

5.2.3 Measurement of the 2,2'-azino-di-(3-ethylbenzothialozine-sulphonic acid) radical cation ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging activity

The antioxidant activity of wines was measured using the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays as described in 3.2.4 and 3.2.5. Antioxidant potency (AP) of wine phenolic fractions was calculated from the total antioxidant activity and total phenol content as described in 3.2.4.

5.2.4 Statistical analysis

All tests were carried out in triplicate directly after sampling. Treatment combinations (cultivar and temperature) within the same storage period were randomised. One way ANOVA was used to determine whether the means for different treatment combinations differed significantly. Statistical comparisons between different treatment combinations were made using the Student's t-LSD test ($P < 0.05$). The SAS version 6.12 software package was used for statistical analysis.

5.3 Results and discussion

5.3.1 Phenolic composition

5.3.1.1 Total phenol content

The total phenol content of the red wines decreased between 10.2 and 16.5% as a function of time at the different storage temperatures (Figure 1A). The total phenol content of Pinotage wines stored at 30 °C was significantly ($P < 0.05$) lower than the initial values after 3 months, while storage at 0 and 15 °C caused a significant ($P < 0.05$) reduction after 6 months. Cabernet Sauvignon wines at 15 °C and 30 °C for 6 months caused a significant ($P < 0.05$) decrease in total phenol content, while a significant ($P < 0.05$) reduction was only observed after 9 months of storage at 0 °C. The final total phenol content of Pinotage wines stored at 0 and 30 °C, as well as Cabernet Sauvignon wines stored at 0, 15 and 30 ºC, were significantly ($P < 0.05$) lower than the initial values (Table 1). Although storage temperature affected the

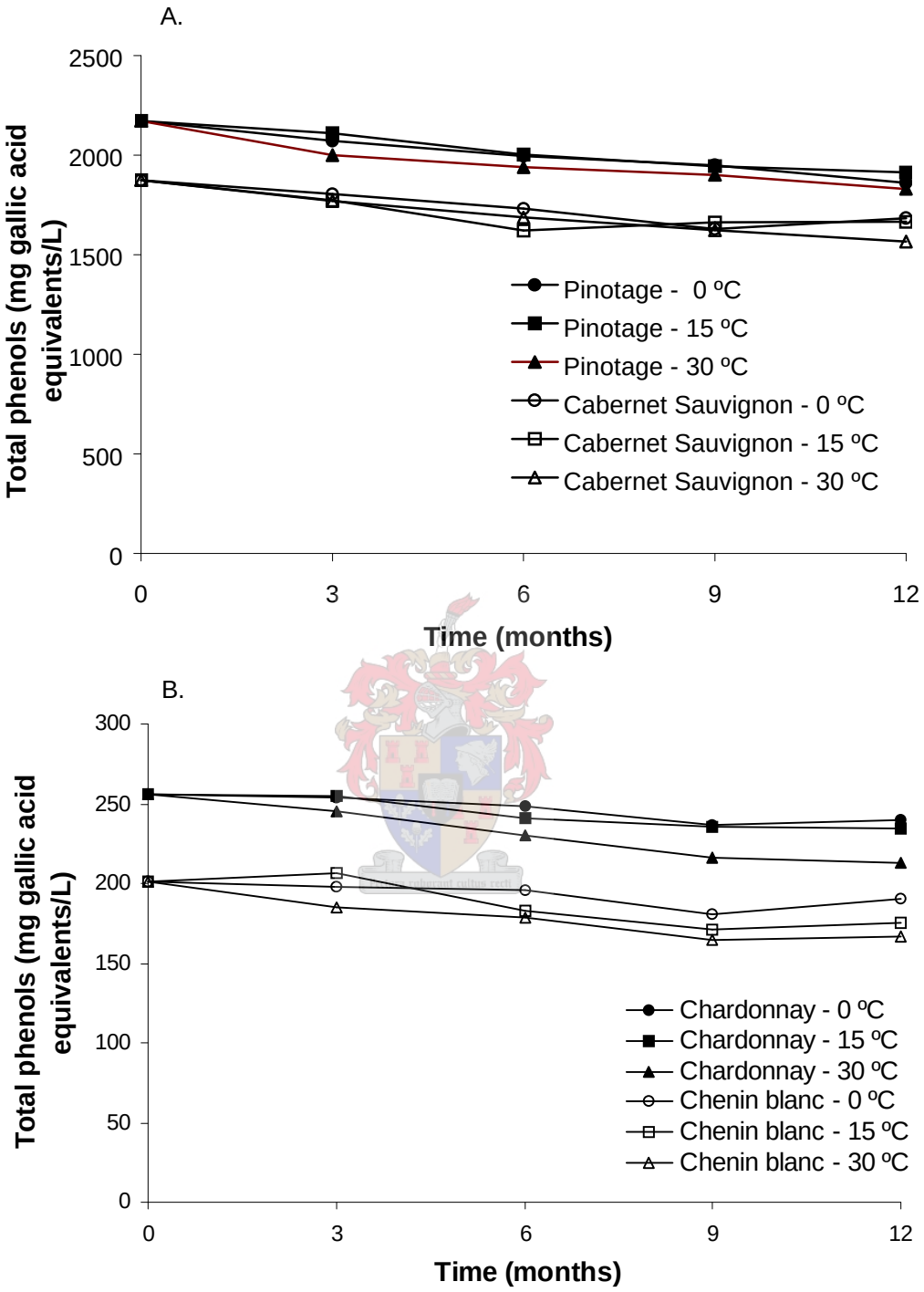

Figure 1 Change in the total phenol content of red (A) and white (B) wines during storage at 0, 15 and 30 °C.

Table 1 Initial and final phenolic composition of red and white wines stored at different temperatures[a]

Phenolic group	Initial[b]	Final[c] (0 ºC)	Final[c] (15 ºC)	Final[c] (30 ºC)
Pinotage				
Total phenols[d]	2171.99 a[e] (± 172.80)[f]	1861.45 b (± 174.81)	1913.46 ab (± 132.48)	1830.86 b (± 148.26)
Total anthocyanins[g]	452.67 a (± 68.64)	426.84 ab (± 59.97)	315.58 b (± 48.09)	113.52 c (± 21.94)
Monomeric anthocyanins[g]	411.58 a (± 59.86)	363.06 b (± 51.79)	273.45 c (± 38.73)	62.12 d (± 16.22)
Polymeric anthocyanins[g]	62.67 ab (± 8.78)	63.78 a (± 8.22)	62.45 ab (± 9.37)	48.26 b (± 6.08)
Flavanols[h]	176.35 ab (± 15.38)	148.36 a (± 19.72)	171.49 ab (± 19.30)	195.32 b (± 25.12)
Flavonols[i]	144.70 a (± 14.48)	113.96 bc (± 20.50)	121.37 abc (± 14.50)	107.30 c (± 23.40)
Tartaric acid esters[j]	300.82 a (± 21.12)	300.82 a (± 23.88)	311.21 a (± 21.13)	305.89 a (± 27.14)
Cabernet Sauvignon				
Total phenols[d]	1874.31 a (± 80.95)	1682.96 b (± 38.63)	1665.47 b (± 71.63)	1565.47 b (± 58.67)
Total anthocyanins[g]	474.25 a (± 26.23)	407.22 b (± 25.43)	335.90 c (± 22.09)	110.38 d (± 7.11)
Monomeric anthocyanins[g]	380.39 a (± 22.73)	335.61 b (± 20.49)	247.44 c (± 19.13)	58.61 d (± 7.17)
Polymeric anthocyanins[g]	72.28 a (± 4.61)	71.84 a (± 5.24)	68.14 a (± 4.91)	54.91 b (± 2.81)
Flavanols[h]	153.41 bc (± 5.90)	141.13 a (± 3.88)	146.69 ab (± 6.37)	157.59 c (± 3.37)
Flavonols[i]	123.59 a (± 6.11)	103.22 ab (± 9.62)	95.44 b (± 8.39)	99.52 b (± 8.91)
Tartaric acid esters[j]	203.24 a (± 6.53)	204.93 a (± 7.57)	206.62 a (± 7.95)	220.14 b (± 5.02)

Table 1 (continued…) Initial and final phenolic composition of red and white wines stored at different temperatures[a]

Phenolic group	Initial[a]	Final[b] (0 ºC)	Final[b] (15 ºC)	Final[b] (30 ºC)
Chardonnay				
Total phenols[d]	256.32 a (± 14.66)	239.92 b (± 3.49)	234.76 b (± 8.06)	212.87 c (± 2.60)
Flavanols[h]	8.31 a (± 1.16)	8.44 a (± 1.58)	9.49 a (± 0.21)	8.32 a (± 0.48)
Flavonols[i]	19.69 a (± 1.55)	17.95 b (± 0.23)	18.51 ab (± 0.36)	17.69 b (± 0.23)
Tartaric acid esters[j]	52.52 a (± 4.27)	55.23 a (± 1.20)	55.78 a (± 1.05)	55.83 a (± 1.13)
Chenin blanc				
Total phenols[d]	201.42 a (± 10.08)	190.01 ab (± 11.24)	175.39 bc (± 14.03)	166.80 c (± 10.50)
Flavanols[h]	2.48 a (± 0.08)	2.75 a (± 0.12)	2.66 a (± 0.24)	2.50 a (± 0.20)
Flavonols[i]	13.77 a (± 0.54)	11.43 b (± 0.95)	12.14 b (± 0.51)	12.25 b (± 0.71)
Tartaric acid esters[j]	39.65 a (± 1.91)	39.00 a (± 2.77)	40.44 ab (± 1.78)	43.20 b (± 1.85)

[a] Detailed results for each wine are reported in Addendum E.
[b] Values of parameters at 0 months of storage.
[c] Values of parameters at 12 months of storage.
[d] Total phenol content expressed as mg gallic acid equivalents/L.
[e] Averages in a row followed by different letters, differ significantly ($P < 0.05$). Data for each parameter for red and white wines were analysed separately.
[f] Standard deviation.
[g] Anthocyanin content expressed as mg malvidin-3-glucoside equivalents/L.
[h] Flavanol content expressed as mg catechin equivalents/L.
[i] Flavonol content expressed as mg quercetin equivalents/L.
[j] Tartaric acid ester content expressed as mg caffeic acid equivalents/L.

initial rate of decrease in total phenol content up to 9 months (Figure 1A), storage temperature did not affect the final total phenol content of the red wines significantly ($P \geq 0.05$) after 12 months (Table 1).

White wines exhibited a decrease of between 5.7 to 17.2% in total phenol content during storage at the different temperatures (Figure 1B). The total phenol content of Chardonnay wines stored at 15 and 30 °C was significantly ($P < 0.05$) lower after 6 months, while storage at 0 °C caused a significant ($P < 0.05$) decrease only after 9 months. Storage of Chenin blanc wines at 15 and 30 °C caused a significant ($P < 0.05$) decrease in total phenol content after 6 and 3 months respectively. All white wine temperature treatments exhibited a significantly ($P < 0.05$) lower final total phenol content compared to the initial values, except Chenin blanc stored at 0 °C (Table 1). The total phenol content of Chardonnay and Chenin blanc wines stored at 30 °C was significantly ($P < 0.05$) lower than for storage at 0 °C, but no significant ($P \geq 0.05$) difference could be observed for Chardonnay wines between 0 and 15 °C and for Chenin blanc wines between 0 and 15 °C or 15 and 30 °C (Table 1).

The decrease in total phenol content of red and white wines as measured with the Folin-Ciocalteau assay indicates a decrease in hydroxyl groups (Singleton & Rossi, 1965). During in-bottle ageing of wines oxidation of phenolic compounds results in the loss of their hydroxyl moieties (Singleton, 1987). Condensation of anthocyanins with other phenolic compounds with the involvement of their hydroxyl moieties can contribute to this phenomenon (Timberlake & Bridle, 1976). Incorporation of monomeric phenolic compounds into polymeric structures can reduce their reactivity with the Folin-Ciocalteau reagent due to steric hindrance.

5.3.1.2 Anthocyanin content

The most distinct change in red wine phenolic composition during ageing was observed for anthocyanin content. The changes in total anthocyanin content are shown in Figure 2. The decrease in total anthocyanin content was more pronounced for both red wines at 15 and 30 °C giving a significant ($P < 0.05$) reduction after only 3 months compared to 12 months for Cabernet Sauvignon

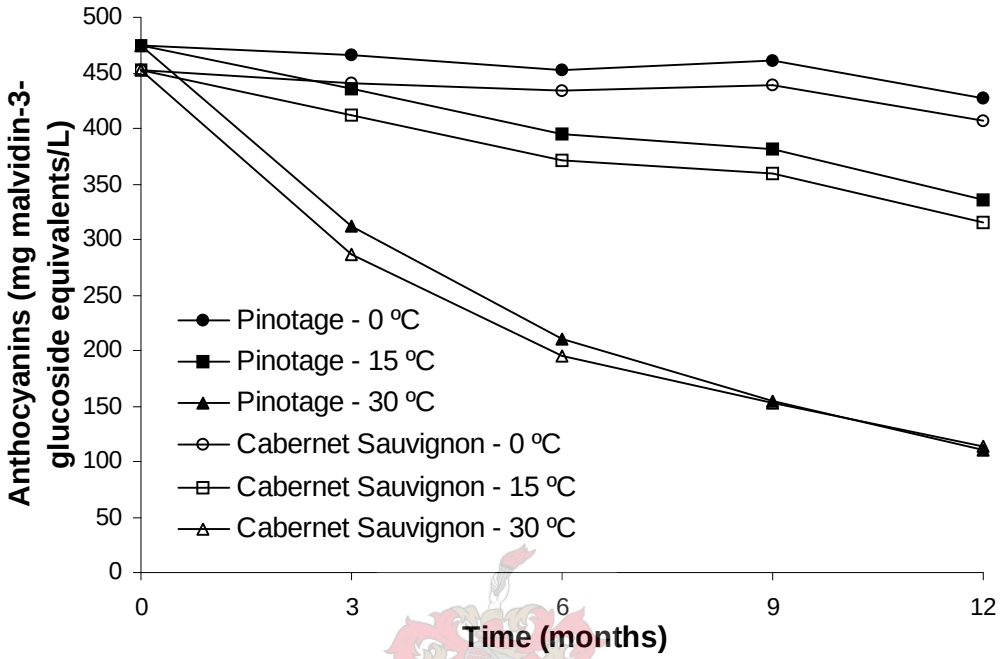

Figure 2 Change in the total anthocyanin content of red wines during storage at 0, 15 and 30 °C.

at 0 °C. Storage at 30 °C significantly ($P < 0.05$) reduced the total anthocyanin content of Pinotage after 3 months, while storage at 0 and 15 °C caused a significant ($P < 0.05$) decrease only after 6 months. Temperature affected the final total anthocyanin content of Cabernet Sauvignon wines with significantly ($P < 0.05$) lower contents observed at higher temperatures (Table 1). The final total anthocyanin content of Pinotage stored at 0 °C was not significantly ($P \geq 0.05$) different to those stored at 15 °C, but significant ($P < 0.05$) differences were observed between 0 and 30 °C, as well as between 15 and 30 °C. Storage of red wines at 0, 15 and 30 °C caused an average reduction in total anthocyanin content of 9.9, 29.8 and 75.8% respectively.

The general trend observed for the monomeric anthocyanin content (Figure 3A) was similar to that for the total anthocyanin content. The reduction in monomeric anthocyanin content was significant ($P < 0.05$) after 3 months of storage for Pinotage at 15 and 30 °C and Cabernet Sauvignon at 30 °C. Six months of storage at 15 °C was required for Cabernet Sauvignon wines to achieve significant ($P < 0.05$) reduction of its monomeric anthocyanin content, while this period was prolonged to 12 months for both Pinotage and Cabernet Sauvignon wines at 0 °C. The final monomeric anthocyanin content differed significantly ($P < 0.05$) from the initial values for both Pinotage and Cabernet Sauvignon (Table 1). With increasing storage temperature, an average reduction of 11.8, 34.3 and 84.8% at 0, 15 and 30 °C respectively, was obtained. The polymeric anthocyanin content (Figure 3B) did not change significantly ($P \geq 0.05$) over the 12 months storage period at 0 and 15 °C for both Pinotage and Cabernet Sauvignon wines. At 30 °C a significant ($P < 0.05$) decrease of approximately 21.3% was observed for both Pinotage and Cabernet Sauvignon wines (Table 1). When monomeric and polymeric anthocyanins were expressed as a percentage of the total anthocyanin content the monomeric (Figure 4A) and polymeric (Figure 4B) anthocyanin contribution significantly ($P < 0.05$) decreased and increased respectively, over the 12 month storage period at 30°C only.

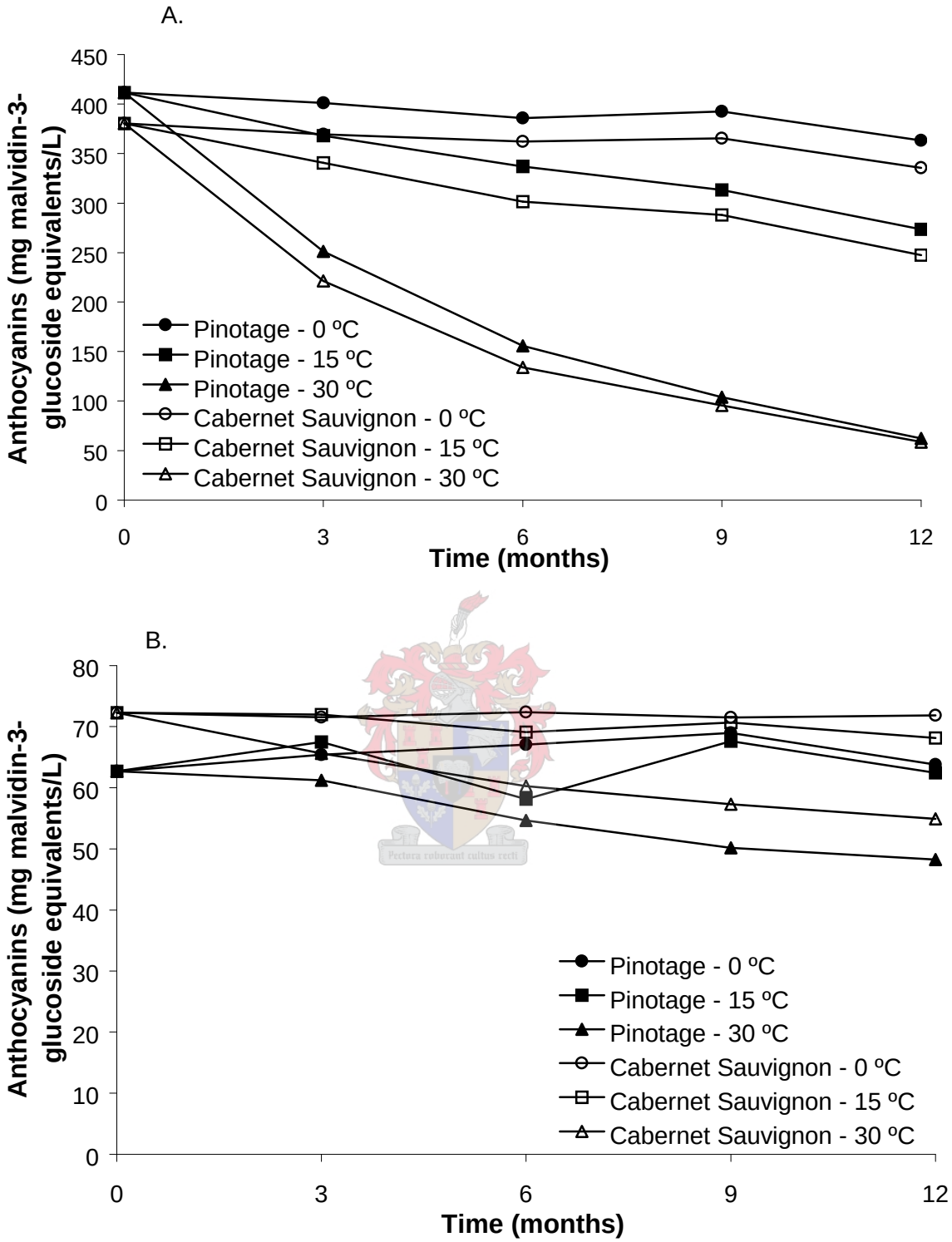

Figure 3 Change in the monomeric (A) and polymeric (B) anthocyanin content of red wines during storage at 0, 15 and 30 °C.

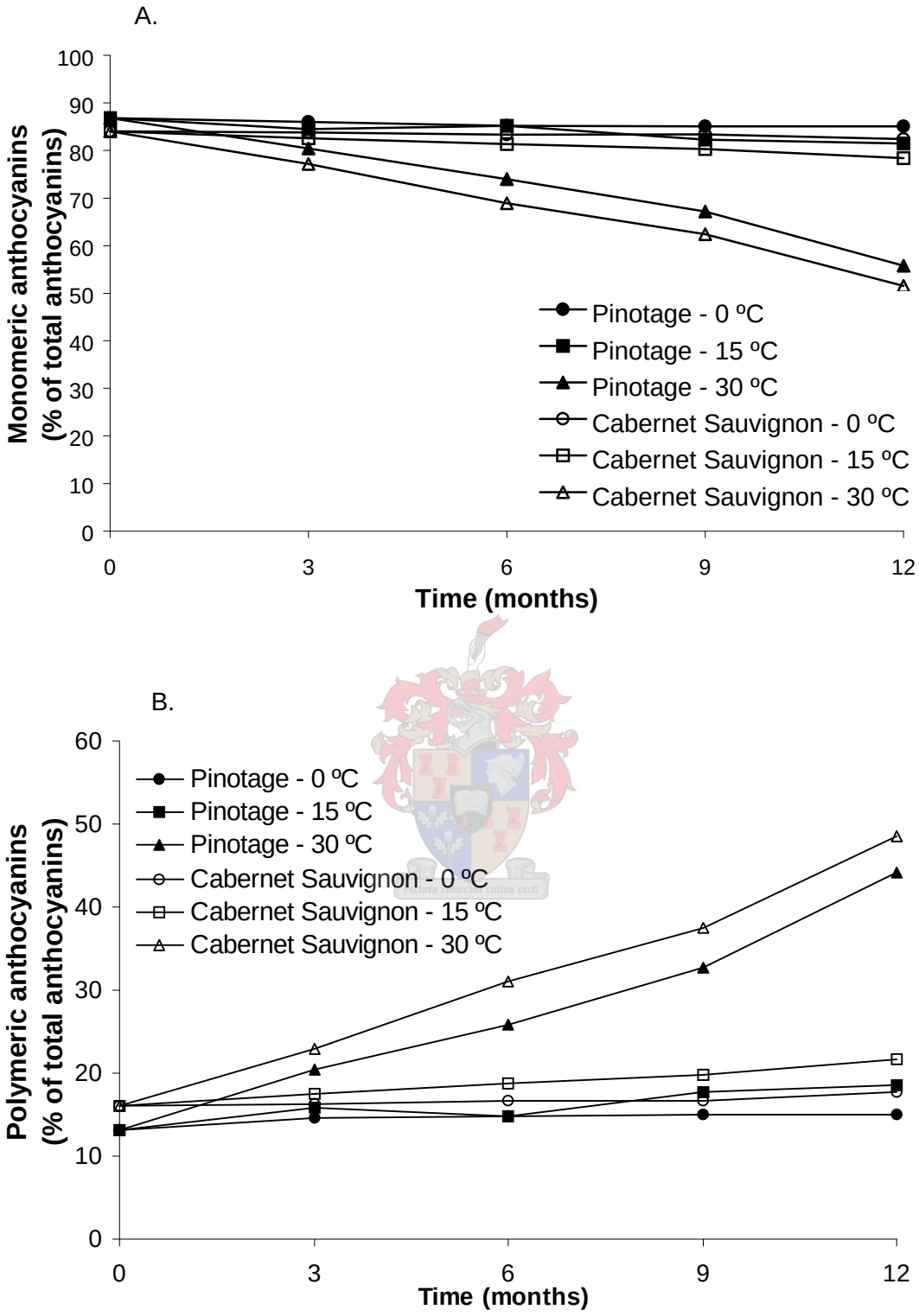

Figure 4 Change in the contribution of monomeric anthocyanins (A) and polymeric anthocyanins (B) to the total anthocyanin content of red wines during storage at 0, 15 and 30 °C.

Previously, it was shown that individual anthocyanins, and especially monomeric anthocyanins, decreased faster during storage at a higher temperature (Somers & Pocock, 1990). The present study confirms this, although for total and monomeric anthocyanins as groups. The presence of oxygen also increases anthocyanin degradation (Ribéreau-Gayon *et al*., 2000). Malvidin, the major anthocyanidin present in wines, is reported to be more sensitive to thermal degradation than cyanidin (Ribéreau-Gayon *et al*., 2000). Wines stored at 20 – 22 ºC in containers flushed with nitrogen, showed a marked decrease in anthocyanin content over a period of 7 months after fermentation (Nagel & Wulf, 1979). Similar results were also obtained by Gómez-Plaza *et al*. (2000) during in-bottle storage of a red wine at ambient temperature (not controlled) and in a cellar (temperature between 15 and 20 ºC) for up to one year. The decrease was demonstrated for individual anthocyanins using high-performance liquid chromatography (HPLC) (Nagel & Wulf, 1979; Gómez-Plaza *et al*., 2000). The decrease of total and monomeric anthocyanins in these studies, as well as the present study, could be attributed to thermal degradation, oxidative degradation and condensation reactions with other phenolic compounds such as flavanols and tartaric acid esters (Timberlake & Bridle, 1979; Somers & Pocock, 1990; Ribéreau-Gayon *et al*., 2000). The condensation reactions lead to the stabilisation of red wine colour due to the formation of polymeric pigments (Somers & Pocock, 1990).

5.3.1.3 Flavanol content

Changes in the flavanol content of the red wines during storage are shown in Figure 5A. The flavanol content of both red wines stored at 30 ºC were already significantly ($P < 0.05$) higher than the initial values after 3 months. This increase continued up to 9 months with a subsequent significant ($P < 0.05$) decrease to 12 months for Pinotage (15 and 30 ºC) and Cabernet Sauvignon (30 ºC). After 9 months the flavanol content of red wines at 0, 15 and 30 ºC was approximately 7.5, 12.7 and 30.9% higher than the initial values. At 12 months, the final flavanol content of all cultivar/temperature combinations, except for Cabernet

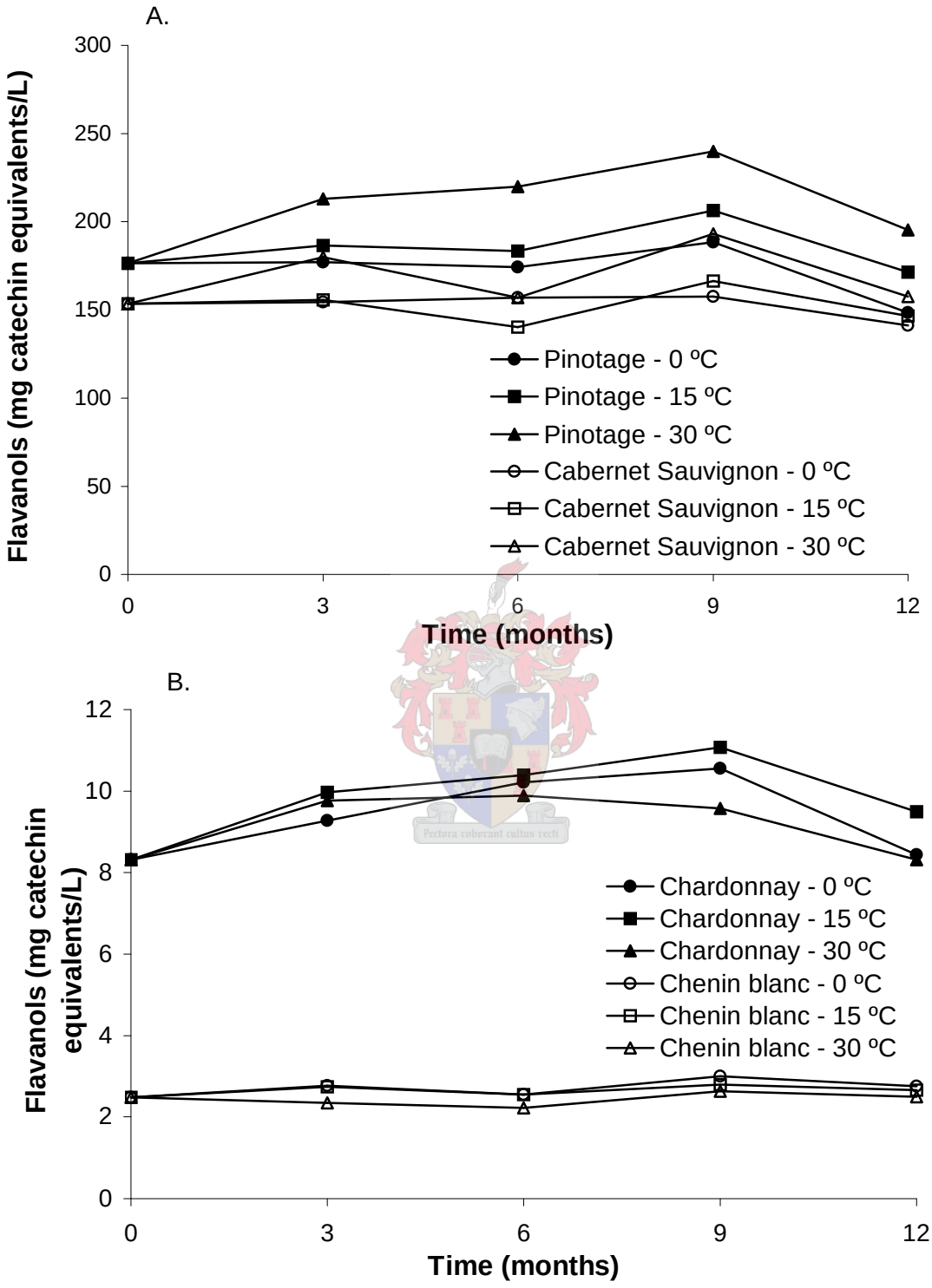

Figure 5 Change in the flavanol content of red (A) and white (B) wines during storage at 0, 15 and 30 °C.

Sauvignon wines stored at 0 ºC, did not differ significantly ($P \geq 0.05$) from the initial values (Table 1). The final flavanol contents of Pinotage and Cabernet Sauvignon wines stored at 30 ºC were significantly ($P < 0.05$) higher than for the same wines stored at 0 ºC (Table 1). A possible explanation for this result, is that condensation of flavanols and anthocyanins involving acetaldehyde, is favoured by low temperatures (Mazza, 1995). Cabernet Sauvignon also exhibited a significantly ($P < 0.05$) higher final flavanol content for storage at 30 ºC compared to 15 ºC. No significant ($P \geq 0.05$) difference was observed between 0 and 15 ºC or 15 and 30 ºC for Pinotage wines and between 0 and 15 ºC for Cabernet Sauvignon wines.

Changes in flavanol content during in-bottle ageing of Chardonnay wines at 0, 15 and 30 ºC followed a similar trend to that of red wines, with the flavanol content of Chenin blanc wines exhibiting no change during the same period (Figure 5B). After 3 months, the flavanol content of Chardonnay wines were already significantly ($P < 0.05$) higher than the initial values for all storage temperatures. After 9 months the increase in flavanol content of the Chardonnay wines was ca. 27.0, 33.2 and 15.2% for 0, 15 and 30 ºC respectively. No significant ($P \geq 0.05$) change in the flavanol content of Chenin blanc wines were observed over the first 9 months of storage, as well as after 12 months, irrespective of temperature (Table 1). No significant ($P \geq 0.05$) differences between the initial and final flavanol content were observed for Chardonnay and Chenin blanc wines at the different storage temperatures, nor did the temperature affect the final flavanol content. The low initial content of flavanols and possibly proanthocyanidins could explain the difference in evolution of flavanols between Chardonnay and Chenin blanc wines. Chardonnay wines contained four times more flavanols than Chenin blanc wines (Table 1).

Nagel & Wulf (1979) and Gómez-Plaza *et al*. (2000), using HPLC methods observed a decrease in the flavanols, (+)-catechin and (-)-epicatechin, in red wines during storage both in the presence of air and after bottling. No significant change in proanthocyanidin B2, B4 and B5 contents were observed during 12 months of storage (Gómez-Plaza *et al*., 2000). A study on white wines stored in

the presence of air showed the decrease of proanthocyanidins during a 30 day period (Mayén *et al*., 1996). Bottled white wines subjected to an accelerated browning test (50 °C) also showed a decrease in proanthocyanidin content after 12 weeks with a simultaneous increase in flavanol content (Mayén *et al*., 1997), a trend also observed in the present study for the first 9 months of storage. It is well known that disproportionation of dimeric proanthocyanidins can occur with the resultant formation of a flavanol monomer and a carbocation (Timberlake & Bridle, 1976; Haslam, 1980; Ribéreau-Gayon *et al*., 2000). The carbocation formed in this manner can react with another flavanol (resulting in dimeric proanthocyanidin), a dimeric proanthocyanidin (resulting in trimeric proanthocyanidin) or other nucleophilic compounds in wine. This could account for the initial increase in apparent flavanol content (Figures 5A and B). Flavanols, however, also participate in condensation reactions with anthocyanins in red wines (Jurd, 1967; Timberlake & Bridle, 1976), which could explain the decrease in flavanol content of Pinotage and Cabernet Sauvignon wines after the initial increase (Figure 5A). The higher final flavanol content observed for red wines stored at 30 ºC could be due to slower reaction of flavanols with anthocyanins at higher temperatures (Mazza, 1995).

Furthermore, the method of determining flavanol content that was used in the present study depends on the reaction of flavanols and proanthocyanidins with 4-dimethylaminocinnamaldehyde (DAC) (McMurrough & McDowell, 1978). When reacting with DAC, (+)-catechin and (-)-epicatechin exhibits a higher colour yield than dimeric or trimeric proanthocyanidins (McMurrough & McDowell, 1978). The apparent increase in flavanol content observed in the present study could, therefore, also in part be attributed to the differences in reactivity of the flavanols and oligomeric proanthocyanidins in the assay and is indicative of changes in phenolic composition.

5.3.1.4 Flavonol content

The flavonol content of wines decreased significantly ($P < 0.05$) over the storage period for most cultivar/temperature combinations, except for Pinotage at 15 °C,

Cabernet Sauvignon at 0 °C and Chardonnay at 15 °C (Table 1). A significant ($P < 0.05$) decrease in flavonol content was observed after 9 months for Pinotage and Cabernet Sauvignon wines stored at 30 °C, as well as after 6 months, for Cabernet Sauvignon wines stored at 15 °C. Chardonnay and Chenin blanc wines contained significantly ($P < 0.05$) less flavonols after 9 and 12 months respectively. Storage temperature had no significant ($P \geq 0.05$) effect on the final flavonol content of the red or white wines. Decreases in flavonol content after 12 months observed for Pinotage, Cabernet Sauvignon and Chardonnay wines at 30 °C were in the order of 30.5, 19.6 and 11.2% respectively. Previously, a decrease in flavonol content of white wines during post-fermentation storage was demonstrated using HPLC techniques (Mayén *et al*., 1996). These white wines were stored in contact with air for 30 days at 20 °C. Decreases in flavonol content of both red and white wines are presumably a result of oxidative degradation (Singleton, 1987). In the case of red wines, copigmentation of flavonols with anthocyanins could also contribute to the decrease in the measured flavonol content (Ribéreau-Gayon *et al*., 2000).

5.3.1.5 Tartaric acid ester content

The tartaric acid ester contents of some wines increased during the storage period, while others remained stable. Storage did not affect the tartaric acid ester content of Pinotage and Chardonnay wines significantly ($P \geq 0.05$). Cabernet Sauvignon wines contained significantly ($P < 0.05$) more tartaric acid esters already after 3 months of storage at 30 °C, but no change was observed for wines stored at 0 and 15 °C, even after 12 months. Chenin blanc followed a similar trend to Cabernet Sauvignon, except that it took 9 months for its tartaric acid ester content of the wine to increase significantly ($P < 0.05$). After 12 months, the tartaric acid ester content of Cabernet Sauvignon and Chenin blanc wines at 30 °C increased by 8.3 and 9.0% respectively.

Gómez-Plaza *et al*. (2000) and Nagel & Wulf (1979) reported a decrease in caffeoyl- and *p*-coumaroyltartaric acids in red wine during a 12 and 7 month

storage period respectively. The higher sensitivity of the HPLC methods used by Gómez-Plaza *et al*. (2000) and Nagel & Wulf (1979) compared with the spectrophotometric estimations used in the present study could explain the different trends. In another study by Mayén *et al*. (1997), the tartaric acid ester content decreased during an accelerated browning test (50 °C) of bottled white wines over a period of 12 weeks. Tartaric acid esters could participate in copigmentation and condensation reactions with anthocyanins in red wines (Ribéreau-Gayon *et al*., 2000), possibly changing their absorbance characteristics. The increase in tartaric acid esters of some wines after storage at 30 °C can be attributed to a release of these compounds from copigmentation stacks due at the high temperature as formation of these complexes are favoured by low temperatures (Mazza, 1995).

5.3.2 Antioxidant activity measured using the ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ scavenging assays

Total antioxidant activity (TAA_{ABTS}) of the red wines decreased with time at 0, 15 and 30 °C (Figure 6A). The TAA_{ABTS} values of the red wines were already significantly ($P < 0.05$) lower after 3 months, irrespective of storage temperature, while both TAA_{ABTS} and TAA_{DPPH} values of the red wines were significantly ($P < 0.05$) lower after 12 months (Table 2). Temperature did not affect the final TAA_{ABTS} and TAA_{DPPH} values of Pinotage wines significantly ($P \geq 0.05$). Only TAA_{ABTS} for Cabernet Sauvignon wines was significantly ($P < 0.05$) lower for storage at 30 °C compared to 0 °C. The TAA_{ABTS} and TAA_{DPPH} values for red wines decreased on average by 18.6 and 34.5% respectively.

Changes in TAA_{ABTS} of white wines are shown in Figure 6B. Storage of 3 months significantly ($P < 0.05$) reduced the TAA_{ABTS} of white wines. After 12 months both the TAA_{ABTS} and TAA_{DPPH} values of Chardonnay and Chenin blanc were significantly ($P < 0.05$) reduced (Table 2). Storage temperature had no significant ($P \geq 0.05$) effect on the final TAA_{ABTS} of Chenin blanc wines or TAA_{DPPH} values of Chardonnay and Chenin blanc wines, but Chardonnay wines stored at 30 °C exhibited significantly ($P < 0.05$) lower final TAA_{ABTS} values than

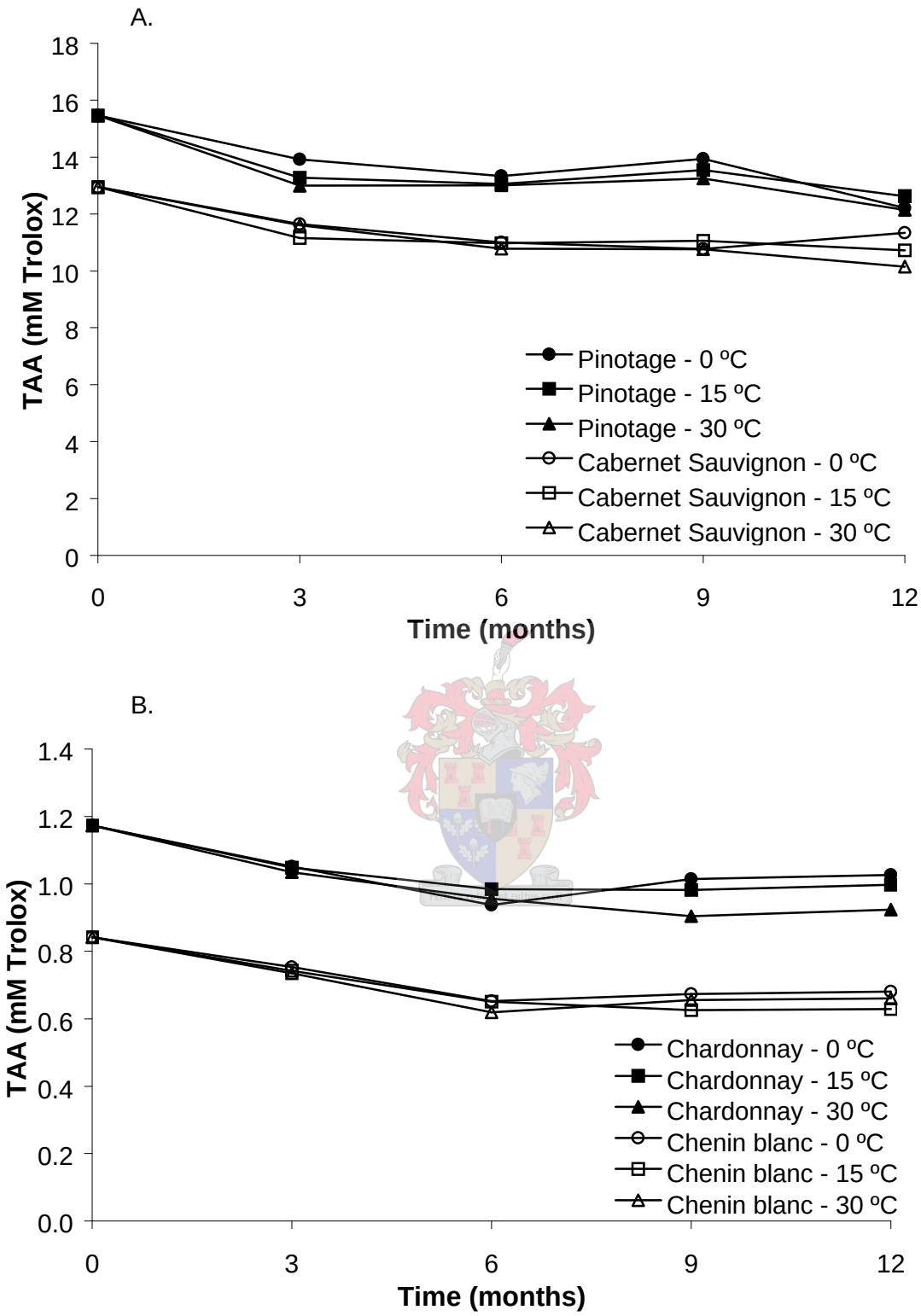

Figure 6 Change in total antioxidant activity of red (A) and white (B) wines during storage at 0, 15 and 30 °C. The ABTS radical cation scavenging assay was used to measure total antioxidant activity.

Table 2 Initial and final antioxidant activity of red and white wines stored at different temperatures[a]

Antioxidant activity	Initial[b]	Final[c] (0 ºC)	Final[c] (15 ºC)	Final[c] (30 ºC)
Pinotage				
TAA_{ABTS}[d]	15.46 a[e] (± 1.37)[f]	12.22 b (± 0.97)	12.63 b (± 0.94)	12.14 b (± 1.04)
TAA_{DPPH}[d]	19.96 a (± 0.46)	14.21 b (± 1.56)	14.88 b (± 1.17)	14.60 b (± 2.18)
AP_{ABTS}[g]	7.12 a (± 0.07)	6.57 b (± 0.10)	6.60 b (± 0.94)	6.63 b (± 1.04)
AP_{DPPH}[g]	8.84 a (± 0.34)	7.64 b (± 0.49)	7.79 ab (± 0.60)	7.95 ab (± 0.55)
Cabernet Sauvignon				
TAA_{ABTS}[d]	12.95 a (± 0.60)	11.34 b (± 0.27)	10.72 bc (± 0.40)	10.15 c (± 0.29)
TAA_{DPPH}[d]	19.87 a (± 1.41)	11.84 b (± 0.90)	11.90 b (± 0.57)	10.91 b (± 0.60)
AP_{ABTS}[g]	6.91 a (± 0.12)	6.74 ab (± 0.24)	6.44 c (± 0.09)	6.49 bc (± 0.11)
AP_{DPPH}[g]	10.80 a (± 0.34)	7.03 b (± 0.40)	7.15 b (± 0.37)	6.97 b (± 0.34)

Table 2 (continued…) Initial and final antioxidant activity of red and white wines stored at different temperatures[a]

Antioxidant activity	Initial[a]	Final[b] (0 ºC)	Final[b] (15 ºC)	Final[b] (30 ºC)
Chardonnay				
TAA_{ABTS}[d]	1.17 a (± 0.08)	1.03 b (± 0.03)	1.00 bc (± 0.03)	0.92 c (± 0.02)
TAA_{DPPH}[d]	1.93 a (± 0.15)	1.17 b (± 0.04)	1.12 b (± 0.08)	1.06 b (± 0.06)
AP_{ABTS}[g]	4.58 a (± 0.10)	4.38 b (± 0.06)	4.25 b (± 0.03)	4.34 b (± 0.03)
AP_{DPPH}[g]	7.54 a (± 0.39)	4.88 b (± 0.11)	4.76 b (± 0.14)	4.99 b (± 0.21)
Chenin blanc				
TAA_{ABTS}[d]	0.84 a (± 0.06)	0.68 b (± 0.02)	0.63 b (± 0.03)	0.66 b (± 0.01)
TAA_{DPPH}[d]	1.75 a (± 0.02)	0.76 b (± 0.06)	0.71 b (± 0.07)	0.70 b (± 0.04)
AP_{ABTS}[g]	4.18 a (± 0.13)	3.59 b (± 0.14)	3.59 b (± 0.14)	3.97 a (± 0.18)
AP_{DPPH}[g]	8.70 a (± 0.55)	3.99 b (± 0.18)	4.05 b (± 0.14)	4.20 b (± 0.31)

[a] Detailed results for each wine are reported in Addendum E.
[b] Values of parameters at 0 months of storage.
[c] Values of parameters at 12 months of storage.
[d] Total antioxidant activity as mM Trolox equivalents measured using the ABTS radical cation or DPPH radical scavenging assay.
[e] Averages in a row followed by different letters, differ significantly ($P < 0.05$). Data for each parameter for red and white wines were analysed separately.
[f] Standard deviation.
[g] Antioxidant potency (AP) = TAA (mM Trolox) X 1000 / total phenols (mg gallic acid equivalents/L.

those stored at 0 °C. The $DPPH^{\bullet}$ scavenging assay was, therefore, less sensitive in discriminating between cultivar/temperature treatments compared with the $ABTS^{\bullet+}$ scavenging assay. The TAA_{ABTS} and TAA_{DPPH} values for the white wines decreased on average by 18.9 and 50.2% respectively. Previously a decrease in free radical scavenging activity, expressed as the rate of reaction with $DPPH^{\bullet}$, was observed by Manzocco *et al*. (1998) for 3 red wines (1995, 1996 and 1973 vintages) presumably stored at ambient temperature.

The decrease in antioxidant activity could be ascribed to a decrease in the total phenol content and complex changes in the phenolic composition. The decrease in total phenol, monomeric anthocyanin and polymeric anthocyanin content, as wel as an increase in flavanol and tartaric acid ester content for certain wines especially at 30 °C, resulted in the significant ($P < 0.05$) decrease in TAA_{ABTS}, although moderate at this stage. The initial rapid decrease in anthocyanin content of red wines at 15 and 30 °C could be linked to a decrease in total antioxidant activity, but the initial increase in flavanol content would, however, have the opposite effect. This would explain the moderate decrease in total antioxidant activity that was observed. When considering Chardonnay, the increase in flavanol content would be counteracted by a simultaneous decrease in flavonol content. Flavonol compounds are generally more potent antioxidants than the flavanols (Rice-Evans *et al*., 1996).

In order to take the decrease in total phenol content into account, the antioxidant potency of wine phenolic fractions was calculated. Pinotage wines stored at 0, 15 and 30 °C and Cabernet Sauvignon wines stored at 15 and 30 °C already exhibited a significantly ($P < 0.05$) lower AP_{ABTS} after 3 months. The AP_{ABTS} of Cabernet Sauvignon wines stored at 0 °C was significantly ($P < 0.05$) lower than the initial value after 3, 6 and 9 months. All temperatures significantly ($P < 0.05$) reduced the AP_{ABTS} for Pinotage and Cabernet Sauvignon wines, except for Cabernet Sauvignon at 0 °C (Table 2). In the case of the $DPPH^{\bullet}$ scavenging assay, the AP values for Cabernet Sauvignon stored at 0, 15 and 30 °C were significantly ($P < 0.05$) reduced, but for Pinotage this was only true for storage at 0 °C.

After 3 months, Chardonnay wines stored at 0, 15 and 30 °C and Chenin blanc wines stored at 0 and 15 °C exhibited a significant ($P < 0.05$) decrease in AP_{ABTS}. Storage at 30 °C resulted in a significant ($P < 0.05$) decrease in AP_{ABTS} for Chenin blanc after 6 months, but 9 and 12 months storage had no significant ($P \geq 0.05$) effect. The lower temperatures, 0 and 15 °C, gave a significant ($P < 0.05$) reduction in AP_{ABTS} after 12 months. AP_{DPPH} of Chenin blanc wines was significantly ($P < 0.05$) lower after 12 months for storage at 0, 15 and 30 °C (Table 2). The final AP_{ABTS} and AP_{DPPH} for Chardonnay were significantly ($P < 0.05$) lower than the initial values at 0, 15 and 30 °C. This indicates that the changes in potency of phenolic compounds are not the same between cultivars. These differences are presumably due to differences in original phenolic composition between Pinotage and Cabernet Sauvignon wines, as well as between Chardonnay and Chenin blanc wines. Larrauri *et al*. (1999) reported an increase in antiradical efficiency (a parameter combining the concentration of sample required to obtain 50% scavenging and the time to reach a steady state) and a decrease in EC_{50} value with increasing ageing time of red wines. This change was attributed to an increase in tannic acid content as measured by HPLC. The fact that older wines were also subjected to longer wood treatments would account for the increase in tannic acid content. The effects of wood maturation and in-bottle ageing were, therefore, not separated which makes interpretation of these results difficult. In the present study, however, it was shown that the efficiency of wines to scavenge free radicals decreased during storage as evidenced by the decrease in antioxidant potency.

The two free radical scavenging assays used, namely the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays, exhibited similar results for the evolution of antioxidant activity of wines during in-bottle ageing. The TAA_{ABTS} and TAA_{DPPH} of the red and white wines both decreased during storage at all temperatures over a period of 12 months. Differentiation between the temperatures was possible using the TAA_{ABTS} for Cabernet Sauvignon and Chardonnay wines, but the TAA_{DPPH} could not discriminate between different temperature treatments. The antioxidant potency of wine phenolic fractions calculated from TAA_{ABTS} and

TAA_{DPPH} did not give the same trends. In the case of Pinotage, the AP_{ABTS} discriminated better between the initial and final values than the AP_{DPPH}, while for Chenin blanc the opposite was true. Both these parameters did not differentiate between temperature treatments of the red and white wines, except for AP_{ABTS} in the case of Cabernet Sauvignon. It would appear that the $ABTS^{\bullet+}$ scavenging assay is the more effective method to differentiate between wines subjected to different temperature treatments with subtle differences in their phenolic composition.

5.3.3 Correlation analysis

Correlation coefficients of total phenols and phenolic groups with TAA_{ABTS} for wines at all temperature/time combinations are reported in Table 3. The TAA_{ABTS} correlated ($P < 0.001$) with the total phenol content for red ($r = 0.934$) and white ($r = 0.907$) wines. Of the phenolic groups flavonols ($r = 0.84$, $P < 0.001$) and tartaric acid esters ($r = 0.71$, $P < 0.001$) in red wines and flavanols ($r = 0.808$, $P < 0.001$), flavonols ($r = 0.87$, $P < 0.001$) and tartaric acid esters ($r = 0.78$, $P < 0.001$) in white wines exhibited a good correlation with TAA_{ABTS}. The flavanol ($r = 0.490$, $P < 0.001$) and monomeric anthocyanins ($r = 0.500$, $P > 0.001$) content of red wine weakly correlated with TAA_{ABTS}. The high correlation coefficients for total phenol content with TAA_{ABTS} shows that the decrease in total antioxidant activity could be best explained by the decrease in total phenol content during in-bottle ageing of red and white wines. Flavanols and tartaric acid esters in white wines and flavonols in red wines seem to be the major determinants of antioxidant activity in terms of phenolic group contents.

When considering the mechanisms of the assays determining total phenols and antioxidant activity, the reason for the high correlation of total phenols with antioxidant activity can be found. The Folin-Ciocalteau reagent used to estimate total phenol content reacts with free hydroxyl groups of phenolic compounds to produce a blue colour (Singleton *et al*., 1999). These hydroxyl groups are also the main determinants of antioxidant activity although other constituents and the position of the hydroxyl groups can modify this activity (Rice-Evans *et al*., 1996).

Table 3 Correlation of the total antioxidant activity of red and white wines with the contents of phenolic groups

	TAA_{ABTS}[a]	
	r	**P**
Red wine		
Total phenols	0.93	< 0.001
Monomeric anthocyanins	0.50	< 0.001
Polymeric anthocyanins	0.11	0.30
Flavanols	0.49	< 0.001
Flavonols	0.84	< 0.001
Tartaric acid esters	0.71	< 0.001
White wine		
Total phenols	0.91	< 0.001
Flavanols	0.81	< 0.001
Flavonols	0.87	< 0.001
Tartaric acid esters	0.78	< 0.001

[a] Total antioxidant activity as mM Trolox equivalents determined according to the ABTS radical cation scavenging assay.

The weak correlation obtained for monomeric anthocyanins with antioxidant activity could suggest that compounds formed after condensation with other phenolic groups retains some of the antioxidant activity associated with the original compounds, while at the same time a decrease in concentration is observed. In the Folin-Ciocalteau assay, they would still react to some extent, which gives a further explanation for the correlation of total phenols with antioxidant activity.

The phenolic groups determined in the present study only gave a broad picture of the effect of changes in phenolic composition on the antioxidant activity of wines during ageing. Quantitative and qualitative changes in individual phenolic compounds will play a role in this regard. The reaction or degradation rate of individual compounds would be important. An example is malvidin, with a lower antioxidant potency than cyanidin (Re *et al*., 1999), and higher susceptibility to thermal degradation (Ribéreau-Gayon *et al*., 2000).

5.4 Conclusions

The phenolic composition of red and white wines changed considerably during storage after bottling, resulting in a decrease of antioxidant activity. In red wines, the rapid decrease in anthocyanins with concurrent changes in flavanol and total phenol content served as indication of the complex reactions taking place in the wine during in-bottle ageing.

The distinct changes in phenolic groups affected not only the total antioxidant activity, but also the antioxidant potency of wine phenolic fractions when compared on an equal total phenol basis. Quantitative changes in individual phenolic compounds or changes in oxidative status would contribute to this phenomenon. The use of empirical methods estimating phenolic groups gave only a partial explanation of the evolution of antioxidant activity of wines during in-bottle ageing. In future, the change in individual phenolic compounds during ageing should receive attention to fully explain their contribution to total antioxidant activity of wines.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Wine has been important as an integral part of the human diet throughout history, but it is only recently that emphasis has been placed on its possible health promoting properties. Evidence from epidemiological studies (St Leger *et al*., 1979; Renaud & De Lorgeril, 1992), clinical trials (Marchioli, 1999; Estruch, 2000) and *ex vivo* studies (Fuhrman *et al*., 1995; Carbonneau *et al*., 1998) strengthens this view. The antioxidant properties of wine phenolic compounds are believed to be the major contributors to its disease-preventing properties (Kinsella *et al*., 1995). Ethanol also plays a role by increasing the high-density lipoprotein content of blood, which confers a protective effect against atherosclerosis (Gorinstein *et al*., 1998; Rakic *et al*., 1998). Due to the high content and accessibility of phenolic compounds in wine compared to that in fruit and vegetables, the moderate consumption of wine would contribute significantly to increase antioxidant intake. It has, therefore, been proposed to consider red wine in nutritional recommendations (Ursini *et al*., 1999). However, more information is still needed on the factors determining antioxidant activity of wines and the role of wine phenolic compounds as antioxidants.

Given this background, and the fact that consumers are increasingly becoming aware of the health benefits of antioxidants, it is not surprising that wineries are offered the opportunity of exploiting the new niche market developing for foods and beverages with health-promoting properties. To fully realise the market advantage of “antioxidant-rich” wines, typical total antioxidant activity data for a large selection of wines are needed as a basis for comparison. Investigations on the impact of terroir, as well as vineyard and vinification practices, on wine antioxidant activity and properties will also be imperative in this regard. In the present study, a large selection of red (46) and white (40) commercial cultivar wines from the 1998 and 1999 seasons respectively, was obtained from commercial wineries situated in the cold, moderate and warm climatic wine-producing regions of the Western Cape

(South Africa). The red wines included Cabernet Sauvignon, Merlot, Shiraz, Ruby Cabernet, as well as Pinotage, a unique South African cultivar wine. The white wine selection comprised Chenin blanc, Chardonnay, Sauvignon blanc and Colombar. Due to variations in terroir, as well as vineyard and vinification practices during the production of these wines, considerable variability in phenolic composition and antioxidant activity was expected. The antioxidant behaviour of these wines was evaluated in terms of their ability to scavenge free radicals and inhibit lipid peroxidation in relation to the contribution of different phenolic groups to the antioxidant activity of wines. The hydroxyl radical scavenging, metal chelating and pro-oxidant activity of two red (Pinotage and Cabernet Sauvignon) and two white (Chardonnay and Chenin blanc) experimental wines were also investigated using the deoxyribose assay. Experimental Pinotage, Cabernet Sauvignon, Chardonnay and Chenin blanc wines, prepared using standard vinification processes, were used to evaluate the effect of in-bottle ageing on the antioxidant activity of red and white cultivar wines.

The antioxidant activity of the commercial cultivar wines was evaluated using the 2,2'-azino-di-(3-ethylbenzthialozinesulphonate) radical cation ($ABTS^{\bullet+}$) and the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging assays, a microsomal lipid peroxidation (MLP) assay and the deoxyribose assay. The $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays measure the hydrogen-donating ability of wines and give an indication of the relative total antioxidant activity of wines, but do not allow evaluation of the behaviour of antioxidants in a biological system (Brand-Williams *et al*., 1995; Re *et al*., 1999). The MLP assay, on the other hand, measures the ability of wines to inhibit lipid peroxidation in an *in vitro* biological membrane system (Yen & Hsieh, 1998). In the MLP assay, the free radical species scavenged include hydroxyl and peroxyl radicals, which are expected to occur *in vivo*, while the the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ species are synthetic and therefore not physiologically relevant. Peroxidation of lipids in membranes is associated with ageing and age-related diseases such as cancer (O'Brien, 1987; Cutler, 1991; Wiseman, 1996; Rice-Evans & Packer, 1998). Lastly, the deoxyribose assay (Aruoma, 1994) can be used to measure hydroxyl radical scavenging activity, metal

chelating ability and pro-oxidant activity depending on assay conditions. The latter is relevant to evaluate the ability of antioxidants to act as pro-oxidants by stimulating hydroxyl radical generation in an environment containing iron (Aruoma, 1994). Endogenous iron is usually bound and unavailable for reaction, but can be mobilised during disease conditions, creating the ideal environment for pro-oxidant action by powerful antioxidants (Halliwell & Gutteridge, 1990). These assays were selected to investigate some of the important antioxidant properties of the red and white wines selected.

The total antioxidant activity of the commercial red wines in the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays was almost fifteen times higher than that of the commercial white wines, but differences between cultivars for both red and white wines were not very pronounced. Ruby Cabernet and Merlot wines exhibited the lowest and highest total antioxidant activity ($P < 0.05$) of the red cultivar wines with the unique South African cultivar wine, Pinotage, exhibiting a total antioxidant activity comparable to that of all cultivars analysed. Amongst the white wines, Chardonnay and Chenin blanc wines represented the highest and lowest total antioxidant activity ($P < 0.05$), while that of Sauvignon blanc and Colombar wines was not significantly ($P \geq 0.05$) different to the other white cultivar wines. The total antioxidant activity measured for wines in this study was in the same range as those reported for wines produced elsewhere (Campos & Lissi, 1996; Verhagen *et al*., 1996; Simonetti *et al*., 1997; Soleas *et al*., 1997; Fogliano *et al*., 1999; Pellegrini *et al*., 2000; Landrault *et al*., 2001).

When considering the commercial wines at the same total phenol content, the antioxidant potency (AP) of red wine phenolic fractions was twice as high in the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays and three times as high in the MLP assay as that of white wines. On the other hand, the white wines (Chardonnay and Chenin blanc) were more effective hydroxyl radical scavengers and metal chelators than the red wines (Pinotage and Cabernet Sauvignon) at the same total phenol content. In addition, differences in assays must also be considered when evaluating the antioxidant potency. The free radical scavenging assays utilise synthetic free radical species in a single phase, while the MLP assay consists of a lipid (hydrophobic) and an

aqueous (hydrophilic) phase. Partitioning of antioxidant molecules between the two phases due to their hydrophobic/hydrophilic nature is, therefore, an important factor determining antioxidant activity in addition to their ability to scavenge biologically relevant free radical species such as hydroxyl and peroxyl radicals. The increased difference in the antioxidant activity between red and white wines when using the MLP assay compared to the free radical scavenging assays, implies that the partitioning of phenolic compounds may play a greater role in determining the antioxidant activity of red wines than white wines. Hydroxyl radical scavenging and metal chelating ability were measured in an aqueous phase using the sugar moiety of DNA, namely deoxyribose, as an oxidation substrate. The fact that two experimental white wines exhibited a greater hydroxyl radical scavenging activity compared to that of two experimental red wines, while the opposite was observed when evaluating the $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ scavenging activity of commercial red and white wines, is possibly due to differences in the size and reactivity of the radicals involved. Hydroxyl radicals are very small and highly reactive (Halliwell & Gutteridge, 1990), while the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ are large, synthetic molecules with lower reactivity (Yoshida *et al*., 1989). It is, therefore, clear that no single assay is able to give a full picture of the antioxidant activity, but different types of assays are needed to determine different aspects of antioxidant activity.

Pro-oxidant activity, i.e. ability to promote hydroxyl radical generation in the presence of iron, was more pronounced for the two experimental red wines (Pinotage and Cabernet Sauvignon wine) than for the two experimental white wines (Chardonnay and Chenin blanc wine). The maximum pro-oxidant activity for red wines occurred at a higher total phenol content than was the case for white wines. The red wines, which contained more potent antioxidants than white wines, thus also exhibited greater pro-oxidant activity. Previously, different tea fractions exhibiting the highest antioxidant activity were also the most effective pro-oxidants (Winterton, 1999). This result, therefore, supports the movement cautioning against the use of high doses of potent phenolic compounds as antioxidants (Skibola & Smith, 2000), as high

antioxidant activity in one set of conditions in many cases also corresponds to high pro-oxidant activity under different conditions.

The antioxidant activity of the wines in these assays was dependent on the phenolic composition, as has been observed by other investigators (Fogliano *et al*., 1999; Frankel *et al*., 1995; Ghiselli *et al*., 1998; Landrault *et al*., 2001; Meyer *et al*., 1997; Simonetti *et al*., 1997). Antioxidant activity of wines in the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays, as well as the MLP assay, correlated with their total phenol content. This largely explains the greater antioxidant efficacy of red wines compared to white wines, as red wines have a higher total phenol content than white wines. In addition, when comparing the wines on the basis of an equal total phenol content, red wine phenolic fractions also exhibited higher antioxidant potency than that of white wines in all assays used, except for the deoxyribose assay. This indicates that red wine phenolic fractions are more effective as antioxidants than that of white wine, whether due to a higher concentration or the efficacy of individual phenolic compounds.

The phenolic groups exhibiting a good correlation ($r \geq 0.7$, $P < 0.001$) with antioxidant activity in the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays were flavanols for red wines, and tartaric acid esters for white wines. Tartaric acid esters were the major phenolic group found in white wines, while flavanols together with anthocyanins were the most common phenolic groups in red wines. Weaker correlations ($r < 0.7$, $P < 0.001$) with free radical scavenging activity were obtained for the polymeric anthocyanins and tartaric acid esters in red wines and flavonols in white wines. These groups were present in lower concentrations than those exhibiting stronger correlations with antioxidant activity. In the MLP assay, however, the flavanol content of both red and white wines and tartaric acid ester content of white wines correlated well ($r \geq 0.7$, $P < 0.001$) with antioxidant activity. The fact that flavanol content of white wines correlated strongly with antioxidant activity in the MLP assay, but not in the free radical scavenging assays, suggests that flavanols are more able to closely interact with the microsomal membrane by being positioned at the interface of the lipid and aqueous phase (Terao, 2001) or by being incorporated into the membrane (Saija *et al*., 1995; Van Dijk *et al*.,

2000) due to its planar structure. This would not be true for other phenolic groups, which correlated to the same extent with the antioxidant activity measured using both types of assays.

In all the antioxidant assays, no correlation ($P > 0.1$) was observed between antioxidant activity and anthocyanin content of red cultivar wines. This result was unexpected, as they are one of the major phenolic groups in red wines. The correlation of the anthocyanin content of red wines with inhibitory activity in the low-density lipoprotein peroxidation assay has been reported (Frankel *et al*., 1995; Ghiselli *et al*., 1998), although Landrault *et al*. (2001) found no correlation ($P = 0.1$) of free radical scavenging activity with the concentration of a number of individual anthocyanins. A possible explanation is that the glycoside group of the anthocyanins in red wines diminishes its free radical scavenging capacity (Rice-Evans *et al*., 1996), and hinders incorporation of the molecules into the microsomal membrane (Saija *et al*., 1995). In the MLP assay, carried out at pH 7.4 and 37 ºC for one hour, the anthocyanins would be in the quinoidal base form. This form is less active as an antioxidant than the flavylium ion, the major form at the pH of wine (pH < 3.5) (Lapidot *et al*., 1999). Degradation of the anthocyanins during the incubation period could also be involved, as the stability of anthocyanins at pH 7.4 is less than would normally be the case at the lower pH of wine (Cabrita *et al*., 2000).

Ascorbic acid present in some of the commercial white wines also modified their antioxidant activity. Ascorbic acid is an effective free radical scavenger, but in systems containing iron (Fe^{2+}/Fe^{3+}), it exhibits pro-oxidant activity (Sevanian & Ursini, 2000). Using a free radical scavenging assay such as the $ABTS^{\bullet+}$ and the $DPPH^{\bullet}$ scavenging assays, ascorbic acid will contribute to the measured antioxidant activity of wines. Its contribution can presumably be calculated and subtracted to obtain the contribution of phenolic compounds alone (Fogliano *et al*., 1999; Saucier & Waterhouse, 1999). In the present study, only two wines contained large amounts of ascorbic acid, which have contributed significantly to the total antioxidant activity. The data for these two wines were removed from the data set, as correction of the EC_{50} values and the initial scavenging rate of $DPPH^{\bullet}$ to account for the contribution

of ascorbic acid was not possible. The possibility that the ascorbic acid contribution is not additive due to the complex mixture of components in wines, in comparison with the (+)-catechin solution used by Saucier & Waterhouse (1999), supported the exclusion of wines containing large amounts of ascorbic acid from the data set. Removal of the data obtained from wines containing ascorbic acid improved the correlation of antioxidant activity with total phenol content. In the MLP assay, initiation of lipid peroxidation with iron(II) and hydrogen peroxide in the presence of ascorbic acid is enhanced, due to recycling of Fe^{3+} to Fe^{2+} by ascorbic acid (Sevanian & Ursini, 2000). Fe^{2+} is a more effective initiator of lipid peroxidation than Fe^{3+} (Halliwell & Gutteridge, 1990). Even small quantities of ascorbic acid, e.g. 10 mg/L, had a noticeable effect on the % MLP inhibition of white wines.

Ascorbic acid is added to white wines by some winemakers to prevent oxidative browning, due to its antioxidant action (Rice-Evans *et al*., 1996). In theory, ascorbic acid will be oxidised preferentially before the *ortho*-dihydrophenolic compounds in white wine due to its greater oxidisability. Oxidation of these compounds to *ortho*-quinones leads to the formation of yellow and brown pigments (Singleton, 1987; Guyot *et al*., 1996), which is detrimental to the sensory quality of white wines. Bradshaw *et al*. (2001) have, however, reported that ascorbic acid added to a (+)-catechin solution promotes non-enzymatic browning. The practice of adding ascorbic acid to white wines could, therefore, not only be detrimental to the sensory quality of these wines, but also affect the antioxidant properties of white wines.

During the in-bottle ageing study, the storage of the wines at 0, 15 and 30 ºC for 12 months resulted in decreasing free radical scavenging activity for all cultivar/temperature combinations, except for Cabernet Sauvignon wines stored at 0 ºC, which did not change. Decreases ($P < 0.05$) in total antioxidant activity were already observed after three months of storage. By taking the decrease in total phenol content during ageing into account, it could be shown that the antioxidant potency of the red and white wine phenolic fractions decreased during storage. This result was attributed to changes in phenolic group content and composition. As wines are not normally consumed directly after production for a number of reasons and a reduction of between 15 and 60% in total antioxidant activity can take place even under

favourable storage conditions (15 ºC), the possible use of total antioxidant activity values to market wines should be approached with caution. More information of the behaviour of wine phenolic compounds and their effect on antioxidant activity is needed before this should be attempted.

Apart from reduction in the total phenol content (6 – 17%), the monomeric anthocyanin (12 – 85%) and flavonol (11 – 30%) contents also decreased during in-bottle ageing, while the tartaric acid ester content was stable for most cultivar/temperature combinations. The decrease in monomeric anthocyanin content was strongly affected by temperature. Storage at 30 ºC cause a significant ($P < 0.05$) reduction in the monomeric anthocyanin content of Pinotage and Cabernet Sauvignon wines after only three months. Storage at 15 ºC also reduced the monomeric anthocyanin content of Pinotage and Cabernet Sauvignon wines significantly ($P < 0.05$) after three and six months respectively, while storage at 0 ºC only caused a significant ($P < 0.05$) decrease in monomeric anthocyanin content after twelve months for both cultivar wines. The tartaric acid ester content of Cabernet Sauvignon wines and Chenin blanc wines increased (8 – 9%) during storage at 30 ºC, presumably due to changes in copigmentation with anthocyanins. Previously, anthocyanin and tartaric acid ester contents, measured using HPLC methods, were shown to decrease during in-bottle ageing of red wines (Nagel & Wulf, 1979; Gómez-Plaza *et al*., 2000), while a decrease in flavonol (Mayén *et al*., 1996) and tartaric acid ester (Mayén *et al*., 1997) contents was reported for white wines stored in the presence of air. The increase in tartaric acid ester content obtained in this study for wines stored at 30 ºC could be related to less copigmentation of anthocyanins with tartaric acid esters due to the higher storage temperature, as wines in the study of Mayén *et al*. (1997) were stored at 50 ºC, resulting in a decrease of tartaric acid ester content.

The flavanol content of red wines and Chardonnay wines increased for the first nine months, with a subsequent decrease to twelve months. This phenomenon was not observed in Chenin blanc wines, which contained a quarter of the flavanol content of the Chardonnay wines. A significant ($P < 0.05$) increase was manifested already after 3 months for wines stored at 30 ºC. The increase of flavanols in both red and white wines could be attributed to self-dissociation of proanthocyanidins (Haslam, 1980), while

decreases in the flavanol content of red wines during the later stages of ageing are presumably due to condensation reactions involving anthocyanins (Timberlake & Bridle, 1976). In white wines, subjected to an accelerated browning test, a decrease in proanthocyanidins with a simultaneous increase in flavanol content was observed during a twelve week period (Mayén *et al*., 1997). A decrease in flavanol content (Nagel & Wulf, 1979) and no significant ($P \geq 0.05$) change in proanthocyanidin content (Gómez-Plaza *et al*., 2000) have, however, also been reported. The effect of these changes in phenolic composition on other antioxidant properties such as inhibition of lipid peroxidation and metal chelating ability, as well as pro-oxidant activity, needs to be investigated in future. A better understanding of the changes in individual compounds and their specific contribution to total antioxidant activity is needed to explain the effects of ageing on the total antioxidant activity of wines and the antioxidant potency of wine phenolic fractions.

Data from the in-bottle ageing experiment were also analysed to obtain correlation coefficients of phenolic group contents with total antioxidant activity, measured using the $ABTS^{\bullet+}$ scavenging assay. Similar to the study of the large selection of cultivar wines, the total phenol content of the red and white wines used for the in-bottle ageing experiment, correlated well ($r \geq 0.7$, $P < 0.001$) with antioxidant activity. The decrease in antioxidant activity of wines during ageing could, therefore, be attributed to a decrease in total phenol content. The flavonol and tartaric acid ester contents of red wines and flavanol, flavonol and tartaric acid ester contents of white wines also correlated well ($r \geq 0.7$, $P < 0.001$) with antioxidant activity. A very weak correlation ($r \leq 0.5$, $P < 0.001$) with antioxidant activity was observed for the flavanol content in red wines, although a good correlation ($r \geq 0.7$, $P < 0.001$) was obtained when different cultivar wines were evaluated in the free radical scavenging and MLP assays. This would be explained by the fact that in the latter case, single point measurements were made, while the flavanol content increased and then decreased during ageing. The very weak correlation ($r \leq 0.05$, $P < 0.001$) of the monomeric anthocyanin content of these wines with antioxidant activity is contrary to what was found for the cultivar wines in the free radical scavenging and MLP assays. As both the monomeric

anthocyanin content and the antioxidant activity of wines decreased over time, some correlation between these parameters would be expected. However, due to the differences in trends observed for the decrease of monomeric anthocyanin content compared to antioxidant activity, this correlation was very weak.

A plethora of different assays exists to evaluate the antioxidant activity of phenolic compounds, foods and beverages. These assays are based on a wide range of different principles. Some assays measure only one parameter of antioxidant activity, such as free radical scavenging activity or metal chelating ability, while in others several factors, such as inhibition of lipid peroxidation, metal chelating ability and partitioning between lipid and aqueous phases also play a role. Even when deciding on one type of assay, many specific assays exist. The need for comparison of samples from different production years and wine-producing regions necessitates standardisation of antioxidant activity testing of foods and beverages. Differences in protocols used in different laboratories complicate comparisons. As an example, the two free radical scavenging assays used in the present study did not give the same results for the same samples. The $DPPH^{\bullet}$ scavenging assay was more effective in discriminating between different cultivar wines, whereas the $ABTS^{\bullet+}$ scavenging assay differentiated more effectively between temperature treatments for the in-bottle storage of wines. A standard method should be easy, fast and reproducible when applied in different laboratories. Automation will be needed to allow for greater precision and fast screening of many samples for quality control. The tea industry has realised the need for such standardisation (Wiseman *et al*., 2001), and interest in adopting a standard method for the measurement of total antioxidant activity of the indigenous South African herbal teas, has been expressed (E. Joubert, ARC Infruitec-Nietvoorbij, South Africa, personal communication). However, no guidelines for the implementation of such a standardised methodology have been forthcoming.

During the present study, the importance of the phenolic groups of red and white wines in determining antioxidant activity was explored. Distinctions between the antioxidant behaviour of red and white cultivar wines in different

assays were identified and broad correlations of phenolic groups with antioxidant activity in different types of assays were observed. Data obtained from the present study could in future be used as basis for comparison with South African and international wines.

Due to the greater health awareness of consumers in recent years, antioxidant content or antioxidant potential could become a major factor in determining the acceptability and marketability of wine and other foodstuffs, especially in niche markets. This development necessitates evaluation of factors such as vineyard and vinification practices on the antioxidant activity of red and white wines. In this regard, it will also be necessary to explore the relationship between the sensory quality of wines and their antioxidant activity, as a very high total phenol content might confer exceptional antioxidant properties, but would not necessarily be acceptable in terms of flavour and colour. Investigations into the contribution of individual phenolic compounds or combinations thereof, and how they interact with test substrates, are needed to improve our understanding of the antioxidant capacity of wines. Information concerning these factors could be helpful when considering the optimisation of the antioxidant activity of a wine while retaining sensory quality. Another area of research that is still largely unexplored is the mechanism of uptake and distribution of phenolic compounds in the body. Metabolisation of these compounds will play a role in determining their eventual *in vivo* activity. The benefits of this proposed research to the South African wine industry should not be underestimated, but to realise this, a concerted effort of researchers and winemakers involved in various aspects of producing good wine, is needed.

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Addendum A

The effect of sulphur dioxide content on activity of wines in the total phenol, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays

Materials and Methods

Two experimental wines, Pinotage and Chardonnay, containing 85 and 40 mg SO_2/L respectively, were produced at the experimental winery of Nietvoorbij and used to test the possible synergistic effect of sulphur dioxide on the total phenol, $ABTS^{\bullet+}$ scavenging and $DPPH^{\bullet}$ scavenging assays. Additional sulphur dioxide in the form of sodium metabisulphite dissolved in purified water was added to give a series of wine mixtures. Synergism in the case of these assays was calculated by subtracting the calculated activity of the mixture from the measured activity:

$$\% \text{ Synergism} = \frac{\text{Activity}_{\text{wine}+SO_2} - (\text{Activity}_{\text{wine}} + \text{Activity}_{SO_2})}{\text{Activity}_{\text{wine}+SO_2}} \times 100$$

where $\text{Activity}_{\text{wine}+SO_2}$ = activity of wine with additional SO_2

$\text{Activity}_{\text{wine}}$ = TAA of wine without additional SO_2

Activity_{SO2} = TAA of SO_2

Results and Discussion

A possible source of interference with the measurement of total phenol content and free radical scavenging is sulphur dioxide added to wine as a preservative (Saucier & Waterhouse, 1999). Typical levels found in South African wines are 40 – 80 mg/L total sulphur dioxide. The activity of sulphur dioxide and wine with and without additional sulphur dioxide in the total phenol, ABTS radical cation scavenging and DPPH radical scavenging assays are reported in Table 1. Sulphur dioxide alone showed no activity in the total phenol, ABTS radical cation scavenging and DPPH radical scavenging assay (results not shown) at amounts similar to that used in other studies (Fogliano *et al*., 1999; Saucier & Waterhouse, 1999). Synergism of 18.99% and 49.94%

was observed in the total phenol assay for a white wine at additional levels of 50 and 150 mg SO_2/L respectively, to the SO_2 already present in the wine. At these levels of sulphur dioxide addition to red wine, the amount of sulphur dioxide in the reaction volume had no significant effect due to higher dilution of red wine samples. In the ABTS radical cation and DPPH radical scavenging assays some positive (ca. 20%) synergism were observed respectively at all levels of sulphur dioxide for the white wine only. No dose-response relationship could be established between the level of sulphur dioxide addition and the magnitude of the synergism as found by Saucier & Waterhouse (1999). Saucier & Waterhouse (1999) obtained a synergistic effect of approximately 50% at 50, 100, 150 and 200 mg SO_2/L additions to a catechin solution. Differences in results obtained in this study and that of Saucier & Waterhouse (1999) is that in our study a wine comprising of a complex mixture of phenolic and other compounds were used to test the synergistic effect of sulphur dioxide, while Saucier & Waterhouse (1999) evaluated the effect on a single compound, namely (+)-catechin. Another possible is differences in experimental procedure followed, although the same assay ($ABTS^{\bullet+}$ scavenging assay). In the present study, $ABTS^{\bullet+}$ were generated before addition of samples to the reaction mixture, while Saucier & Waterhouse (1999) used enzymatic radical generation in the presence of the sample under investigation (Saucier & Waterhouse, 1999).

Table 1 Synergism of sulphur dioxide in the total phenol, ABTS radical cation scavenging and DPPH radical scavenging assays as determined for a red and white wine

Sample[a]	Total phenols[b]	% Synergism[c]	TAA_{ABTS}[d]	% Synergism[c]	TAA_{DPPH}[d]	% Synergism[c]
Pinotage + 0 mg SO_2/L	1873.95 a[e] (± 222.6)[f]	0.00 a (± 0.00)	11.71 a (± 1.24)	0.00 a (± 0.00)	13.56 a (± 1.68)	0.00 a (± 0.00)
Pinotage + 50 mg SO_2/L	1899.72 a (± 195.69)	1.73 a (± 1.69)	11.62 a (± 1.36)	0.11 a (± 0.94)	13.79 a (± 1.68)	1.38 a (± 0.17)
Pinotage + 100 mg SO_2/L	1924.36 a (± 203.61)	2.08 a (± 1.41)	11.66 a (± 1.13)	1.04 a (± 1.06)	13.93 a (± 1.72)	2.44 a (± 0.00)
Pinotage + 150 mg SO_2/L	1905.32 a (± 330.38)	3.30 a (± 5.54)	10.98 a (± 2.71)	-8.02 a (± 13.34)	13.57 a (± 2.60)	-0.61 a (± 0.86)
Chardonnay + 0 mg SO_2/L	228.20 a (± 5.81)	0.00 a (± 0.00)	0.96 a (± 0.07)	0.00 a (± 0.00)	1.11 a (± 0.28)	0.00 a (± 0.00)
Chardonnay + 50 mg SO_2/L	269.28 b (± 5.81)	18.99 b (± 0.48)	1.15 ab (± 0.00)	15.72 ab (± 7.90)	1.08 a (± 0.04)	23.69 a (± 46.28)
Chardonnay + 100 mg SO_2/L	311.20 c (± 13.34)	34.26 c (± 2.43)	1.27 ab (± 0.17)	22.28 b (± 9.55)	1.13 a (± 0.02)	21.84 a (± 43.67)
Chardonnay + 150 mg SO_2/L	354.53 d (± 12.81)	49.94 d (± 1.80)	1.32 b (± 0.12)	20.00 ab (± 5.02)	1.14 a (± 0.05)	13.20 a (± 43.50)

[a] The SO_2 values represent additional amounts added to wine.
[b] Total phenols expressed as mg gallic acid equivalents/L.
[c] % Synergism = [$Activity_{wine+SO2}$ – ($Activity_{wine}$ + $Activity_{SO2}$) / $Activity_{wine+SO2}$] X 100
[d] Total antioxidant activity as mM Trolox equivalents determined according to the ABTS radical cation or DPPH radical scavenging assay.
[e] Averages in a column followed by different letters, differ significantly ($P < 0.05$). Data for red and white wines were analysed separately.
[f] Standard deviation.

Addendum B

The actual values for the content of total phenols and phenolic groups, as well as the relative importance of each group for individual cultivar wines.

Wine[a]	Total phenols[b]	Total antho[c]	% Total antho[d]	Free antho[c]	Polymeric antho[d]	Flava[e]	% Flava[d]	Flavo[f]	% Flavo[d]	TAE[g]	% TAE[d]
CS1	1971.9	122.98	6.24	68.73	54.25	194.82	9.88	3.44	0.17	137.23	6.96
CS2	2451.5	178.78	7.29	90.70	88.08	226.69	9.25	54.56	2.23	186.52	7.61
CS3	2502.6	237.90	9.51	155.61	82.29	293.66	11.73	63.44	2.54	205.67	8.22
CS4	2805.3	147.28	5.25	81.65	65.62	369.66	13.18	91.22	3.25	213.48	7.61
CS5	2480.7	179.20	7.22	96.91	82.29	276.82	11.16	33.44	1.35	163.83	6.60
CS6	2055.3	218.76	10.64	146.07	72.68	231.38	11.26	22.33	1.09	138.30	6.73
CS7	2293.6	241.72	10.54	137.32	104.40	220.18	9.60	47.89	2.09	200.71	8.75
CS8	2088.9	197.64	9.46	100.23	97.41	205.86	9.86	36.78	1.76	184.40	8.83
CS9	2568.4	212.47	8.27	121.56	90.91	261.48	10.18	89.00	3.47	236.17	9.20
CS10	2226.3	242.63	10.90	154.06	88.58	195.70	8.79	65.67	2.95	215.60	9.68
RC1	1827.4	221.12	12.10	139.41	81.71	177.69	9.72	81.67	4.47	205.22	11.23
RC2	2062.5	276.54	13.41	183.34	93.20	198.21	9.61	89.44	4.34	210.29	10.20
RC3	2273.8	210.31	9.25	132.92	77.39	235.71	10.37	88.33	3.88	211.01	9.28
RC4	1778.4	213.49	12.01	141.46	72.04	155.66	8.75	41.61	2.34	191.70	10.78
RC5	1763.7	298.78	16.94	243.25	55.53	151.55	8.59	28.71	1.63	186.03	10.55
RC6	1702.3	264.73	15.55	189.49	75.24	160.08	9.40	25.48	1.50	180.36	10.59
RC7	2703.8	288.72	10.68	288.72	147.63	245.08	9.06	110.43	4.08	318.65	11.79
P1	1753.0	213.95	12.21	147.37	66.57	149.01	8.50	69.44	3.96	213.19	12.16
P2	1517.9	111.30	7.33	51.44	59.86	115.69	7.62	49.44	3.26	177.68	11.71
P3	2235.1	226.13	10.12	141.80	84.33	227.20	10.17	118.33	5.29	278.41	12.46
P4	2420.2	180.83	7.47	90.13	90.70	249.13	10.29	50.22	2.07	223.62	9.24
P5	2573.7	195.63	7.60	115.28	80.34	260.71	10.13	37.31	1.45	244.18	9.49

Addendum B (continued …)

Wine[a]	Total phenols[b]	Total antho[c]	% Total antho[d]	Free antho[c]	Polymeric antho[d]	Flava[e]	% Flava[d]	Flavo[f]	% Flavo[d]	TAE[g]	% TAE[d]
P6	2320.8	235.91	10.17	111.19	124.73	234.83	10.12	58.82	2.53	256.24	11.04
P7	2608.8	190.73	7.31	118.35	72.38	294.89	11.30	93.23	3.57	272.36	10.44
P8	2831.0	268.80	9.50	186.07	82.73	313.90	11.09	74.95	2.65	268.89	9.50
P9	2583.9	250.02	9.68	162.05	87.97	269.53	10.43	63.12	2.44	256.39	9.92
P10	2579.5	243.76	9.45	157.50	86.26	275.84	10.69	51.29	1.99	215.42	8.35
Sh1	1462.8	155.57	10.64	96.96	58.61	138.94	9.50	58.33	3.99	190.00	12.99
Sh2	2117.6	228.17	10.78	139.52	88.65	205.08	9.69	152.78	7.22	304.49	14.38
Sh3	2854.2	229.88	8.05	135.54	94.34	319.40	11.19	145.00	5.08	300.87	10.54
Sh4	2810.5	271.94	9.68	171.12	100.82	323.89	11.52	129.79	4.62	347.73	12.37
Sh5	1890.9	174.23	9.21	115.62	58.61	205.60	10.87	41.61	2.20	212.98	11.26
Sh6	2559.1	212.81	8.32	128.14	84.67	241.11	9.42	119.03	4.65	305.18	11.93
Sh7	2778.4	277.31	9.98	169.94	107.37	327.46	11.79	99.68	3.59	291.81	10.50
Sh8	2538.6	228.17	8.99	142.25	85.92	240.60	9.48	64.19	2.53	245.28	9.66
Sh9	2559.1	224.19	8.76	136.79	87.40	257.28	10.05	88.93	3.48	239.03	9.34
Sh10	2553.2	200.97	7.87	117.22	83.76	272.27	10.66	96.45	3.78	280.69	10.99
M1	3165.2	255.71	8.08	112.78	142.94	401.84	12.70	120.56	3.81	260.29	8.22
M2	2699.4	199.15	7.38	124.39	74.77	243.63	9.03	100.56	3.73	257.39	9.54
M3	2317.0	225.21	9.72	134.74	90.47	253.91	10.96	108.33	4.68	232.75	10.05
M4	2728.7	223.74	8.20	137.93	85.81	333.96	12.24	81.40	2.98	245.60	9.00
M5	1937.7	193.58	9.99	112.21	81.37	179.58	9.27	69.57	3.59	234.97	12.13
M6	2409.9	252.53	10.48	153.29	99.24	245.27	10.18	119.03	4.94	317.94	13.19
M7	2113.2	204.28	9.67	143.62	60.66	221.25	10.47	52.37	2.48	177.22	8.39
M8	2946.5	246.38	8.36	141.46	104.93	308.53	10.47	83.55	2.84	327.22	11.11
M9	2171.6	187.20	8.62	115.19	72.04	204.38	9.41	62.04	2.86	220.28	10.14

Addendum B (continued …)

Wine[a]	Total phenols[b]	Flava[e]	% Flava[d]	Flavo[f]	% Flavo[d]	TAE[g]	% TAE[d]
SB1	268.8	6.40	2.38	5.73	2.13	47.55	17.69
SB2	233.2	6.98	2.99	4.79	2.05	35.82	15.36
SB3	320.1	7.87	2.46	5.57	1.74	41.99	13.12
SB4	272.5	12.17	4.46	6.07	2.23	53.72	19.71
SB5	290.5	5.81	2.00	10.46	3.60	52.48	18.07
SB6	214.5	6.35	2.96	2.23	1.04	34.04	15.87
SB7	253.3	6.07	2.40	7.40	2.92	46.67	18.42
SB8	349.6	2.06	0.59	3.46	0.99	39.86	11.40
SB9	263.3	9.24	3.51	6.34	2.41	44.61	16.94
SB10	284.2	1.95	0.69	11.90	4.19	58.09	20.44
Chen1	199.4	3.84	1.93	4.17	2.09	22.70	11.38
Chen2	338.7	7.40	2.18	11.28	3.33	46.68	13.78
Chen3	200.9	1.67	0.83	7.17	3.57	25.38	12.63
Chen4	227.5	4.30	1.89	3.73	1.64	31.01	13.63
Chen5	212.9	3.94	1.85	2.98	1.40	33.43	15.70
Chen6	231.5	3.26	1.41	4.81	2.08	38.60	16.68
Chen7	243.0	5.00	2.06	8.03	3.31	42.38	17.44
Chen8	196.8	1.35	0.69	3.19	1.62	28.14	14.30
Chen9	235.0	2.72	1.16	8.46	3.60	39.25	16.70
Chen10	334.7	2.46	0.73	9.54	2.85	45.15	13.49
Char1	282.1	3.51	1.24	12.50	4.43	39.14	13.88
Char2	223.6	7.98	3.57	15.28	6.83	42.62	19.06
Char3	302.5	9.33	3.08	17.00	5.62	49.94	16.51
Char4	326.5	12.61	3.86	14.05	4.30	47.11	14.43
Char5	291.4	2.00	0.68	12.12	4.16	48.53	16.65

Addendum B (continued …)

Wine[a]	Total phenols[b]	Flava[e]	% Flava[d]	Flavo[f]	% Flavo[d]	TAE[g]	% TAE[d]
Char6	301.0	14.11	4.69	14.27	4.74	50.66	16.83
Char7	305.6	14.12	4.62	8.57	2.80	49.11	16.07
Char8	278.5	6.76	2.43	8.68	3.12	37.51	13.47
Char9	321.5	11.75	3.65	12.12	3.77	52.17	16.23
Char10	294.6	13.08	4.44	6.53	2.22	46.75	15.87
Col1	266.6	3.87	1.45	9.28	3.48	38.86	14.58
Col2	374.8	8.97	2.39	17.72	4.73	62.33	16.63
Col3	315.1	3.54	1.12	8.50	2.70	39.22	12.45
Col4	275.8	8.72	3.16	16.53	5.99	60.87	22.07
Col5	189.3	2.77	1.47	1.80	0.95	21.37	11.29
Col6	267.4	3.99	1.49	5.24	1.96	36.40	13.61
Col7	219.4	4.53	2.06	1.90	0.87	26.75	12.19
Col8	439.7	3.56	0.81	9.54	2.17	46.26	10.52
Col9	237.6	6.95	2.93	4.27	1.80	32.48	13.67
Col10	269.5	6.23	2.31	5.45	2.02	35.29	13.09

a Sample codes for wines is as described in Table 1 of Chapter 3.
b mg gallic acid equivalents/L.
c Anthocyanin content expressed as mg malvidin-3-glucoside equivalents/L.
d % Phenolic group = g phenolic group per 100 g total phenols.
fe Flavanol content expressed as mg catechin equivalents/L.
f Flavonol content expressed as mg quercetin equivalents/L.
g Tataric acid ester content expressed as mg caffeic acid equivalents/L.

Addendum C

The actual values for free radical scavenging activity parameters for the of individual cultivar wines.

Wine[a]	TAA_{ABTS}[b]	AP_{ABTS}[c]	TAA_{DPPH}[b]	AP_{DPPH}[c]	EC_{50}[d]	Initial scavenging rate[e]	RSE[f]
CS1	12.600	6.39	8.049	4.08	86.09	0.0356	0.414
CS2	15.845	6.46	13.271	5.41	57.50	0.0337	0.587
CS3	16.860	6.74	16.686	6.67	51.29	0.0290	0.565
CS4	17.344	6.18	19.006	6.78	45.53	0.0292	0.641
CS5	15.394	6.21	12.659	5.10	63.67	0.0345	0.543
CS6	14.909	7.25	10.600	5.16	77.69	0.0409	0.527
CS7	14.673	6.40	10.551	4.60	77.33	0.0415	0.536
CS8	14.068	6.73	11.297	5.41	42.15	0.0232	0.550
CS9	15.013	5.85	10.410	4.05	68.45	0.0344	0.502
CS10	14.033	6.30	11.372	5.11	65.94	0.0197	0.299
P1	11.337	6.47	10.593	6.04	60.56	0.0322	0.531
P2	10.071	6.64	7.002	4.61	69.33	0.0296	0.427
P3	13.602	6.09	10.279	4.60	94.33	0.0345	0.365
P4	15.865	6.56	16.078	6.64	47.33	0.0261	0.551
P5	16.225	6.30	12.193	4.74	80.73	0.0405	0.502
P6	14.523	6.26	12.143	5.23	74.13	0.0464	0.625
P7	16.006	6.14	12.086	4.63	63.32	0.0303	0.478
P8	19.501	6.89	12.454	4.40	66.08	0.0337	0.510
P9	17.899	6.93	13.663	5.29	59.24	0.0319	0.539
P10	17.844	6.92	12.642	4.90	63.74	0.0346	0.543

Addendum C (continued …)

Wine[a]	TAA_{ABTS}[b]	AP_{ABTS}[c]	TAA_{DPPH}[b]	AP_{DPPH}[c]	EC_{50}[d]	Initial scavenging rate[e]	RSE[f]
M1	19.166	6.06	17.361	5.49	46.72	0.0259	0.555
M2	17.692	6.55	12.082	4.48	72.58	0.0314	0.432
M3	14.068	6.07	12.625	5.45	57.81	0.0302	0.522
M4	18.155	6.65	12.756	4.67	72.95	0.0350	0.480
M5	12.334	6.37	8.945	4.62	77.29	0.0332	0.429
M6	15.984	6.63	11.345	4.71	80.93	0.0390	0.482
M7	13.876	6.57	10.223	4.84	73.23	0.0344	0.469
M8	17.095	5.80	13.662	4.64	77.74	0.0398	0.512
M9	13.433	6.19	10.196	4.69	75.95	0.0334	0.439
Sh1	9.200	6.29	6.134	4.19	96.86	0.0283	0.292
Sh2	13.192	6.23	10.624	5.02	68.71	0.0305	0.443
Sh3	17.236	6.04	12.523	4.39	75.95	0.0344	0.452
Sh4	16.568	5.89	11.954	4.25	70.94	0.0357	0.504
Sh5	12.084	6.39	8.924	4.72	86.01	0.0332	0.386
Sh6	15.969	6.24	10.359	4.05	91.40	0.0364	0.398
Sh7	15.999	5.76	14.534	5.23	83.59	0.0400	0.479
Sh8	14.928	5.88	13.011	5.13	71.62	0.0474	0.661
Sh9	17.514	6.84	12.685	4.96	72.88	0.0345	0.473
Sh10	15.817	6.20	14.421	5.65	62.91	0.0292	0.464
RC1	11.252	6.16	10.334	5.66	66.85	0.0350	0.524
RC2	12.635	6.13	8.382	4.06	78.27	0.0332	0.424
RC3	15.309	6.73	10.236	4.50	81.99	0.0387	0.472
RC4	13.071	7.35	7.172	4.03	74.01	0.0332	0.448
RC5	10.004	5.67	7.517	4.26	89.16	0.0354	0.397
RC6	11.834	6.95	9.752	5.73	61.61	0.0284	0.460
RC7	18.143	6.71	13.177	4.87	65.10	0.0350	0.538

Addendum C (continued …)

Wine[a]	TAA_{ABTS}[b]	AP_{ABTS}[c]	TAA_{DPPH}[b]	AP_{DPPH}[c]	EC_{50}[d]	Initial scavenging rate[e]	RSE[f]
SB1	0.782	2.91	0.503	1.87	154.06	0.0216	0.140
SB2	0.830	3.56	0.687	2.95	136.66	0.0256	0.188
SB3	1.072	3.35	0.717	2.24	135.38	0.0200	0.148
SB4	0.993	3.64	0.712	2.61	120.15	0.0263	0.219
SB5	1.187	4.09	0.895	3.08	107.65	0.0211	0.196
SB6	0.713	3.32	0.444	2.07	139.69	0.0245	0.175
SB7	0.682	2.69	0.555	2.19	138.60	0.0204	0.147
SB8	1.225	3.50	0.920	2.63	111.41	0.0163	0.146
SB9	0.955	3.63	0.789	3.00	122.30	0.0241	0.197
SB10	1.056	3.72	0.377	1.32	147.19	0.0241	0.164
Chen1	0.531	2.66	0.337	1.69	218.90	0.0223	0.102
Chen2	1.240	3.66	1.002	2.96	128.37	0.0154	0.120
Chen3	0.554	2.76	0.321	1.60	169.33	0.0231	0.136
Chen4	0.841	3.70	0.446	1.96	167.21	0.0257	0.154
Chen5	0.620	2.91	0.561	2.64	131.35	0.0245	0.186
Chen6	0.836	3.61	0.685	2.96	122.63	0.0205	0.167
Chen7	0.760	3.13	0.423	1.74	169.74	0.0275	0.162
Chen8	0.647	3.29	0.540	2.74	169.64	0.0288	0.170
Chen9	0.747	3.18	0.482	2.05	148.83	0.0246	0.166
Chen10	1.222	3.65	0.638	1.91	149.42	0.0200	0.134

Addendum C (continued …)

Wine[a]	TAA_{ABTS}[b]	AP_{ABTS}[c]	TAA_{DPPH}[b]	AP_{DPPH}[c]	EC_{50}[d]	Initial scavenging rate[e]	RSE[f]
Char1	0.937	3.32	0.640	2.27	136.28	0.0191	0.140
Char2	0.841	3.76	0.536	2.40	131.61	0.0230	0.175
Char3	1.035	3.42	0.838	2.77	118.28	0.0258	0.218
Char4	1.144	3.50	0.775	2.37	135.93	0.0267	0.197
Char5	0.960	3.29	0.656	2.25	129.81	0.0288	0.222
Char6	0.997	3.31	0.690	2.29	123.63	0.0227	0.184
Char7	1.232	4.03	0.763	2.50	118.36	0.0245	0.207
Char8	0.996	3.58	0.803	2.88	122.85	0.0279	0.227
Char9	1.255	3.90	0.665	2.07	134.25	0.0250	0.186
Char10	1.205	4.09	0.819	2.78	120.51	0.0195	0.161
Col1	1.027	3.85	0.508	1.91	167.52	0.0191	0.114
Col2	1.401	3.74	0.955	2.55	134.69	0.0286	0.212
Col3	0.856	2.72	0.612	1.94	171.28	0.0254	0.148
Col4	0.980	3.55	0.657	2.38	136.81	0.0277	0.203
Col5	0.544	2.87	0.359	1.90	177.93	0.0277	0.156
Col6	0.999	3.73	0.514	1.92	167.14	0.0303	0.181
Col7	0.630	2.87	0.414	1.89	170.22	0.0265	0.155
Col8	1.400	3.18	1.019	2.32	146.04	0.0183	0.125
Col9	0.709	2.98	0.346	1.46	166.38	0.0241	0.145
Col10	0.915	3.39	0.426	1.58	155.34	0.0321	0.207

[a] Sample codes for wines is as described in Table 1 of Chapter 3.

[b] Total antioxidant activity expressed as mM Trolox equivalents.

[c] Antioxidant potency (AP) = TAA (mM Trolox) X 1000 / total phenols (mg gallic acid equivalents/L).

[d] EC_{50} = concentration of wine in mg gallic acid equivalents/l required to obtain 50% scavenging.

[e] The initial scavenging rates of wines is the negative of the slope of the line for [DPPH] against time for the first minute of the reaction.

[f] RSE (Radical Scavenging Efficiency) = $initial\ scavenging\ rate \times 1000 / EC_{50}$

Addendum D

The actual parameters for the inhibition of microsomal lipid peroxidation of individual cultivar wines.

Wine[a]	Total phenols[b]	% Inhibition	AP[c]
CS1	3.94	63.91	16.20
CS2	4.90	63.20	12.89
CS3	5.01	70.73	14.13
CS4	5.61	81.18	14.47
CS5	4.96	69.20	13.95
CS6	4.11	62.53	15.21
CS7	4.59	67.95	14.81
CS8	4.18	55.80	13.36
CS9	5.14	77.33	15.05
CS10	4.45	61.88	13.90
P1	3.51	38.32	10.93
P2	3.04	42.90	14.13
P3	4.47	55.42	12.40
P4	4.84	68.50	14.15
P5	5.15	67.64	13.14
P6	4.64	67.74	14.59
P7	5.22	70.48	13.51
P8	5.66	72.92	12.88
P9	5.17	69.00	13.35
P10	5.16	64.84	12.57
M1	6.33	90.03	14.22
M2	5.40	70.55	13.07
M3	4.63	74.83	16.15
M4	5.46	83.91	15.38
M5	3.88	60.98	15.74
M6	4.82	81.21	16.85
M7	4.23	60.89	14.41
M8	5.89	82.02	13.92
M9	4.34	68.91	15.87
Sh1	2.93	35.79	12.24
Sh2	4.24	61.39	14.49
Sh3	5.71	83.23	14.58
Sh4	5.62	83.96	14.94
Sh5	3.78	57.98	15.33
Sh6	5.12	77.86	15.21
Sh7	5.56	87.38	15.72
Sh8	5.08	71.89	14.16
Sh9	5.12	63.05	12.32
Sh10	5.11	71.21	13.94
RC1	3.65	50.16	13.72
RC2	4.13	64.23	15.57
RC3	4.55	63.74	14.02
RC4	3.56	49.48	13.91
RC5	3.53	50.99	14.45
RC6	3.40	53.18	15.62
RC7	5.41	75.34	13.93

Addendum D (continued …)

Wine[a]	Total phenols[b]	% Inhibition	AP[c]
SB1	10.75	44.72	4.16
SB2	9.33	38.66	4.14
SB3	12.81	61.61	4.81
SB4	10.90	76.00	6.97
SB5	11.62	57.32	4.93
SB6	8.58	42.61	4.97
SB7	10.13	49.12	4.85
SB8	13.98	25.89	1.85
SB9	10.53	62.66	5.95
SB10	11.37	31.75	2.79
Chen1	7.98	23.85	2.99
Chen2	13.55	77.84	5.75
Chen3	8.04	25.32	3.15
Chen4	9.10	34.92	3.84
Chen5	8.52	38.25	4.49
Chen6	9.26	36.36	3.93
Chen7	9.72	46.45	4.78
Chen8	7.87	23.90	3.04
Chen9	9.40	27.33	2.91
Chen10	13.39	40.98	3.06
Char1	11.28	38.04	3.37
Char2	8.95	45.31	5.07
Char3	12.10	63.59	5.25
Char4	13.06	71.54	5.48
Char5	11.66	51.85	4.45
Char6	12.04	62.64	5.20
Char7	12.23	61.56	5.04
Char8	11.14	30.31	2.72
Char9	12.86	65.10	5.06
Char10	11.78	85.46	7.25
Col1	10.66	35.55	3.33
Col2	14.99	84.46	5.63
Col3	12.60	31.84	2.53
Col4	11.03	56.73	5.14
Col5	7.57	25.32	3.34
Col6	10.70	34.51	3.23
Col7	8.78	19.87	2.26
Col8	17.59	44.79	2.55
Col9	9.50	33.17	3.49
Col10	10.78	42.73	3.96

[a] Sample codes for wines is as described in Table 1 of Chapter 3.

[b] Total phenols expressed as mg gallic acid equivalents/L in the reaction mixture.

[c] Antioxidant potency = % inhibition / total phenol content in the reaction mixture (mg gallic acid equivalents/L).

Addendum E

Table 1 Total phenol content[a] of individual wines during in-bottle ageing

Sample		0°C				15°C				30°C			
	Control	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]
Pinotage Batch 1	1993.8	1879.0	1800.5	1809.4	1752.0	1947.7	1810.3	1765.3	1765.8	1845.7	1728.0	1715.0	1679.3
Pinotage Batch 2	2359.0	2294.9	2186.0	2127.6	2063.1	2235.5	2189.0	2113.9	2021.9	2150.8	2153.5	2087.4	1975.6
Pinotage Batch 3	2163.2	2042.8	2000.5	1908.6	1769.3	2147.1	2010.9	1954.1	1952.7	2005.1	1934.9	1900.3	1837.6
Pinotage Average	**2172.0**	**2072.2**	**1995.6**	**1948.5**	**1861.5**	**2110.1**	**2003.4**	**1944.4**	**1913.5**	**2000.5**	**1938.8**	**1900.9**	**1830.9**
Cabernet Sauvignon Batch 1	1889.6	1850.5	1782.5	1705.1	1693.7	1752.9	1735.5	1685.5	1725.4	1830.0	1739.6	1657.9	1631.4
Cabernet Sauvignon Batch 2	1946.5	1792.0	1700.5	1749.3	1715.1	1866.1	1489.2	1707.5	1684.9	1771.7	1709.7	1683.2	1518.9
Cabernet Sauvignon Batch 3	1786.8	1772.4	1710.6	1433.7	1640.1	1697.8	1637.2	1595.9	1586.1	1706.3	1613.2	1524.6	1546.1
Cabernet Sauvignon Average	**1874.3**	**1805.0**	**1731.2**	**1629.4**	**1683.0**	**1772.3**	**1620.6**	**1663.0**	**1665.5**	**1769.4**	**1687.5**	**1621.9**	**1565.5**
Chardonnay **Batch** 1	265.0	252.3	247.8	235.7	238.2	252.9	246.6	239.5	237.5	245.3	235.5	223.1	215.8
Chardonnay Batch 2	239.4	258.4	246.5	236.4	237.7	258.0	230.9	229.8	225.7	249.1	229.6	212.4	212.1
Chardonnay Batch 3	264.6	250.3	250.5	236.7	243.9	252.4	244.3	238.0	241.1	239.9	225.7	212.9	210.7
Chardonnay Average	**256.3**	**253.7**	**248.2**	**236.3**	**239.9**	**254.4**	**240.6**	**235.8**	**234.8**	**244.8**	**230.3**	**216.1**	**212.9**
Chenin blanc Batch 1	210.8	212.7	207.6	*193.5*	202.7	252.9	200.5	181.0	183.1	200.3	189.6	177.0	178.6
Chenin blanc Batch 2	202.7	193.5	196.4	180.8	181.2	182.3	184.8	174.8	183.9	178.5	172.6	164.9	163.2
Chenin blanc **Batch** 3	190.8	188.7	181.7	169.1	186.1	185.3	164.3	158.0	159.2	177.1	172.3	151.8	158.6
Chenin blanc Average	**201.4**	**198.3**	**195.3**	**181.2**	**190.0**	**206.8**	**183.2**	**171.3**	**175.4**	**185.3**	**178.2**	**164.6**	**166.8**

[a] mg gallic acid equivalents/L.
[b] Months of storage.

Addendum E (continued …)

Table 2 Anthocyanin content[a] of individual wines during in-bottle ageing

Sample		0°C				15°C				30°C			
	Control	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]
Monomeric anthocyanins													
Pinotage Batch 1	342.55	339.56	319.38	328.92	303.75	309.07	272.93	255.42	229.03	208.41	125.49	79.818	44.79
Pinotage Batch 2	449.20	424.15	417.27	420.15	399.31	401.31	377.81	338.34	291.12	265.06	161.63	108.53	64.63
Pinotage Batch 3	442.99	439.44	420.49	428.36	386.12	393.33	360.07	346.32	300.21	279.81	179.70	123.28	76.94
Pinotage Average	**411.58**	**401.05**	**385.71**	**392.48**	**363.06**	**367.90**	**336.94**	**313.36**	**273.45**	**251.10**	**155.61**	**103.87**	**62.12**
Cabernet Sauvignon Batch 1	372.71	359.85	351.64	359.4	329.25	329.47	294.44	275.48	234.36	210.41	127.04	89.796	52.10
Cabernet Sauvignon Batch 2	405.97	394.88	390.00	394.66	358.52	364.28	313.73	313.18	269.39	242.34	147.66	105.54	66.29
Cabernet Sauvignon Batch 3	362.51	353.20	344.33	342.11	319.05	327.48	296.21	275.82	238.57	211.52	126.82	90.904	57.42
Cabernet Sauvignon Average	**380.39**	**369.31**	**361.99**	**365.39**	**335.61**	**340.41**	**301.46**	**288.16**	**247.44**	**221.42**	**133.84**	**95.41**	**58.61**
Polymeric anthocyanins													
Pinotage Batch 1	52.55	54.32	55.87	56.76	54.54	56.98	54.10	55.429	51.66	51.22	43.68	41.905	41.24
Pinotage Batch 2	68.07	72.50	73.39	77.601	70.28	72.95	48.56	72.28	67.18	64.96	60.31	54.543	51.88
Pinotage Batch 3	67.40	69.40	71.84	72.502	66.52	72.50	71.84	75.162	68.51	67.40	60.09	54.099	51.66
Pinotage Average	**62.67**	**65.41**	**67.03**	**68.95**	**63.78**	**67.48**	**58.16**	**67.62**	**62.45**	**61.19**	**54.69**	**50.18**	**48.26**
Cabernet Sauvignon Batch 1	74.72	72.95	74.94	76.049	72.50	73.39	72.72	71.837	70.28	66.96	60.31	59.199	57.42
Cabernet Sauvignon Batch 2	75.16	75.38	74.94	77.823	76.71	76.27	68.29	74.719	71.61	69.40	62.75	59.864	55.43
Cabernet Sauvignon Batch 3	66.96	66.29	67.18	65.185	66.29	66.29	66.29	65.407	62.52	60.53	57.65	52.991	51.88
Cabernet Sauvignon Average	**72.28**	**71.54**	**72.35**	**71.50**	**71.84**	**71.98**	**69.10**	**70.65**	**68.14**	**65.63**	**60.23**	**57.35**	**54.91**

[a] Anthocyanin content expressed as mg malvidin-3-glucoside equivalents/L.

[b] Months of storage.

Addendum E (continued …)

Table 3 Flavanol content[a] of individual wines during in-bottle ageing

Sample		0°C				15°C				30°C			
	Control	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]
Pinotage Batch 1	165.52	157.47	158.11	170.40	133.96	173.05	161.32	186.96	149.54	202.08	200.26	210.00	167.97
Pinotage Batch 2	193.95	199.93	193.28	216.00	170.83	195.23	212.01	220.28	185.77	231.23	240.72	278.84	217.36
Pinotage Batch 3	169.58	173.76	171.14	178.67	140.27	191.09	176.80	211.51	179.17	205.14	218.39	230.80	200.63
Pinotage Average	**176.35**	**177.05**	**174.18**	**188.36**	**148.36**	**186.46**	**183.38**	**206.25**	**171.49**	**212.82**	**219.79**	**239.88**	**195.32**
Cabernet Sauvignon Batch 1	158.35	157.63	150.34	173.76	145.60	153.94	155.30	166.14	146.80	180.57	176.05	199.89	160.19
Cabernet Sauvignon Batch 2	155.01	157.47	178.29	157.62	139.14	165.04	123.37	179.83	153.01	182.96	135.63	191.95	153.78
Cabernet Sauvignon Batch 3	146.88	148.55	141.85	141.28	138.66	147.56	142.53	153.11	140.27	176.03	158.83	186.96	158.80
Cabernet Sauvignon Average	**153.41**	**154.55**	**156.82**	**157.55**	**141.13**	**155.51**	**140.40**	**166.36**	**146.69**	**179.86**	**156.84**	**192.94**	**157.59**
Chardonnay **Batch** 1	9.28	9.17	10.69	10.69	6.61	9.92	10.72	11.91	9.27	10.41	10.80	10.14	8.46
Chardonnay Batch 2	7.04	9.96	9.99	11.31	9.31	10.20	10.26	10.11	9.51	9.36	9.94	10.36	8.72
Chardonnay Batch 3	8.62	8.68	9.94	9.66	9.39	9.80	10.18	11.20	9.69	9.52	8.91	8.22	7.78
Chardonnay Average	**8.31**	**9.27**	**10.21**	**10.56**	**8.44**	**9.97**	**10.39**	**11.08**	**9.49**	**9.76**	**9.88**	**9.57**	**8.32**
Chenin blanc Batch 1	2.51	2.78	2.70	3.30	2.85	2.90	2.67	2.66	2.71	2.20	2.39	2.83	2.53
Chenin blanc Batch 2	2.53	2.90	2.49	2.64	2.77	2.61	2.77	3.31	2.86	2.37	2.30	2.49	2.68
Chenin blanc **Batch** 3	2.38	2.60	2.47	3.07	2.62	2.73	2.21	2.41	2.40	2.45	2.00	2.57	2.28
Chenin blanc Average	**2.48**	**2.76**	**2.55**	**3.00**	**2.75**	**2.75**	**2.55**	**2.79**	**2.66**	**2.34**	**2.23**	**2.63**	**2.50**

[a] Flavanol content expressed as mg catechin equivalents/L.
[b] Months of storage.

Addendum E (continued …)

Table 4 Flavonol content[a] of individual wines during in-bottle ageing

Sample		0°C				15°C				30°C			
	Control	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]
Pinotage Batch 1	125.44	118.78	116.56	105.44	96.56	122.11	114.33	105.44	109.89	115.44	106.56	98.78	83.22
Pinotage Batch 2	155.44	153.22	142.11	135.44	136.56	154.33	145.44	128.78	116.56	135.44	142.11	126.56	129.89
Pinotage Batch 3	153.22	142.11	145.44	132.11	108.78	147.67	142.11	134.33	137.67	149.89	134.33	128.78	108.78
Pinotage Average	**144.70**	**138.04**	**134.70**	**124.33**	**113.96**	**141.37**	**133.96**	**122.85**	**121.37**	**133.59**	**127.67**	**118.04**	**107.30**
Cabernet Sauvignon Batch 1	127.67	111.00	111.00	106.56	114.33	122.11	109.89	109.89	94.33	117.67	113.22	113.22	108.78
Cabernet Sauvignon Batch 2	127.67	115.44	105.44	104.33	97.67	107.67	93.22	95.44	104.33	117.67	113.22	85.44	91.00
Cabernet Sauvignon Batch 3	115.44	96.56	104.33	63.22	97.67	105.44	101.00	67.67	87.67	99.89	99.89	67.67	98.78
Cabernet Sauvignon Average	**123.59**	**107.67**	**106.93**	**91.37**	**103.22**	**111.74**	**101.37**	**91.00**	**95.44**	**111.74**	**108.78**	**88.78**	**99.52**
Chardonnay **Batch** 1	20.99	18.88	19.10	15.54	17.77	22.99	19.43	15.43	18.77	19.54	19.10	15.66	17.77
Chardonnay Batch 2	17.66	19.21	18.77	14.66	17.88	18.77	18.66	14.88	18.10	19.77	19.77	14.88	17.43
Chardonnay Batch 3	20.43	19.32	18.54	18.54	18.21	19.66	19.66	19.21	18.66	19.54	19.10	18.77	17.88
Chardonnay Average	**19.69**	**19.14**	**18.80**	**16.25**	**17.95**	**20.47**	**19.25**	**16.51**	**18.51**	**19.62**	**19.32**	**16.43**	**17.69**
Chenin blanc Batch 1	13.88	13.54	12.66	12.77	11.54	12.54	12.43	13.66	12.43	14.43	13.66	13.54	12.54
Chenin blanc Batch 2	14.32	12.93	12.99	12.99	12.32	13.66	13.77	13.43	12.43	13.10	13.88	13.77	12.77
Chenin blanc **Batch** 3	13.10	13.32	12.32	12.10	10.43	12.66	12.21	12.43	11.54	13.54	12.54	13.66	11.43
Chenin blanc Average	**13.77**	**13.27**	**12.66**	**12.62**	**11.43**	**12.95**	**12.80**	**13.17**	**12.14**	**13.69**	**13.36**	**13.66**	**12.25**

[a] Flavonol content expressed as mg quercetin equivalents/L.

[b] Months of storage.

Addendum E (continued …)

Table 5 Tartaric acid ester content[a] of individual wines during in-bottle ageing

Sample		0°C				15°C				30°C			
	Control	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]
Pinotage Batch 1	273.04	276.67	270.87	278.84	279.57	288.99	272.32	281.01	286.81	291.16	273.77	278.84	275.94
Pinotage Batch 2	318.70	335.36	318.70	327.39	326.67	343.33	326.67	325.22	323.04	330.29	330.29	323.77	328.84
Pinotage Batch 3	310.72	319.42	312.90	310.72	296.23	327.39	314.35	317.97	323.77	333.19	321.59	315.80	312.90
Pinotage Average	**300.82**	**310.48**	**300.82**	**305.65**	**300.82**	**319.90**	**304.44**	**308.07**	**311.21**	**318.21**	**308.55**	**306.14**	**305.89**
Cabernet Sauvignon Batch 1	205.65	206.38	204.93	199.13	210.00	206.38	204.93	207.10	209.28	225.94	223.04	220.87	223.04
Cabernet Sauvignon Batch 2	209.28	215.80	204.20	203.48	208.55	215.07	197.68	211.45	212.90	230.29	228.12	229.57	223.04
Cabernet Sauvignon Batch 3	194.78	196.23	195.51	187.54	196.23	200.58	196.96	195.51	197.68	214.35	209.28	210.00	214.35
Cabernet Sauvignon Average	**203.24**	**206.14**	**201.55**	**196.71**	**204.93**	**207.34**	**199.86**	**204.69**	**206.62**	**223.53**	**220.14**	**220.14**	**220.14**
Chardonnay **Batch** 1	56.72	57.45	56.36	58.17	56.43	58.61	57.01	58.83	56.94	59.77	57.01	59.26	57.01
Chardonnay Batch 2	47.09	56.00	54.70	55.78	55.20	56.22	54.84	56.22	55.49	58.03	57.16	57.09	55.71
Chardonnay Batch 3	53.75	55.13	53.83	54.84	54.04	56.00	55.06	55.86	54.91	55.86	56.29	56.51	54.77
Chardonnay Average	**52.52**	**56.19**	**54.96**	**56.27**	**55.23**	**56.94**	**55.64**	**56.97**	**55.78**	**57.88**	**56.82**	**57.62**	**55.83**
Chenin blanc Batch 1	39.62	39.48	39.12	39.77	38.61	40.13	39.55	41.29	40.42	42.74	43.61	43.90	44.04
Chenin blanc Batch 2	41.87	42.41	41.80	41.94	41.94	41.72	42.38	42.88	42.23	42.30	45.57	45.13	44.48
Chenin blanc **Batch** 3	37.45	38.39	37.81	37.59	36.43	38.39	38.17	38.10	38.68	40.93	41.22	41.43	41.07
Chenin blanc Average	**39.65**	**40.09**	**39.57**	**39.77**	**39.00**	**40.08**	**40.03**	**40.76**	**40.44**	**41.99**	**43.46**	**43.49**	**43.20**

[a] Tataric acid ester content expressed as mg caffeic acid equivalents/L.

[b] Months of storage.

Addendum E (continued …)

Table 6 Total antioxidant activity[a] of individual wines during in-bottle ageing

Sample		0°C				15°C				30°C			
	Control	**3** [b]	**6** [b]	**9** [b]	**12** [b]	**3** [b]	**6** [b]	**9** [b]	**12** [b]	**3** [b]	**6** [b]	**9** [b]	**12** [b]
Pinotage Batch 1	14.23	13.73	12.37	12.87	11.65	12.80	11.71	12.63	11.63	11.85	12.02	12.27	11.12
Pinotage Batch 2	16.94	14.86	13.59	14.79	13.33	13.35	14.50	14.89	13.49	14.30	13.60	14.57	13.19
Pinotage Batch 3	15.22	13.18	14.05	14.18	11.67	13.68	12.96	13.12	12.78	12.85	13.40	12.91	12.13
Pinotage Average	**15.46**	**13.92**	**13.34**	**13.94**	**12.22**	**13.27**	**13.06**	**13.54**	**12.63**	**13.00**	**13.01**	**13.25**	**12.14**
Cabernet Sauvignon Batch 1	13.31	12.10	10.92	11.17	11.63	10.94	11.87	11.54	10.93	12.01	10.40	10.82	10.40
Cabernet Sauvignon Batch 2	13.28	11.69	11.49	11.64	11.09	12.29	9.53	11.01	10.96	11.56	11.59	11.08	9.84
Cabernet Sauvignon Batch 3	12.25	11.16	10.59	9.49	11.30	10.23	11.50	10.63	10.26	11.24	10.34	10.38	10.21
Cabernet Sauvignon Average	**12.95**	**11.65**	**11.00**	**10.76**	**11.34**	**11.15**	**10.97**	**11.06**	**10.72**	**11.60**	**10.78**	**10.76**	**10.15**
Chardonnay **Batch** 1	1.19	1.04	1.10	1.03	1.02	1.11	1.03	1.00	1.00	0.99	1.00	0.93	0.94
Chardonnay Batch 2	1.09	1.05	0.67	1.00	1.00	1.03	1.00	0.96	0.96	1.02	0.90	0.87	0.92
Chardonnay Batch 3	1.24	1.07	1.05	1.01	1.06	1.01	0.93	0.99	1.03	1.09	0.96	0.91	0.91
Chardonnay Average	**1.17**	**1.05**	**0.94**	**1.01**	**1.03**	**1.05**	**0.98**	**0.98**	**1.00**	**1.03**	**0.96**	**0.90**	**0.92**
Chenin blanc Batch 1	0.88	0.74	0.66	0.72	0.70	0.75	0.74	0.65	0.64	0.77	0.61	0.68	0.67
Chenin blanc Batch 2	0.87	0.82	0.74	0.68	0.68	0.73	0.57	0.64	0.66	0.75	0.68	0.65	0.65
Chenin blanc **Batch** 3	0.77	0.70	0.55	0.62	0.67	0.75	0.64	0.58	0.60	0.69	0.57	0.63	0.66
Chenin blanc Average	**0.84**	**0.75**	**0.65**	**0.67**	**0.68**	**0.74**	**0.65**	**0.63**	**0.63**	**0.74**	**0.62**	**0.66**	**0.66**

[a] Total antioxidant activity measured using the ABTS radical cation scavenging assay expressed as mM Trolox equivalents.
[b] Months of storage.

Addendum E (continued …)

Table 7 AP[a] of individual wines during in-bottle ageing

Sample		0°C				15°C				30°C			
	Control	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]	3[b]	6[b]	9[b]	12[b]
Pinotage Batch 1	7.57	7.31	6.87	7.11	6.65	6.57	6.47	7.15	6.59	6.42	6.96	7.16	6.62
Pinotage Batch 2	7.72	6.47	6.22	6.95	6.46	5.97	6.63	7.04	6.67	6.65	6.31	6.98	6.67
Pinotage Batch 3	7.30	6.45	7.03	7.43	6.59	6.37	6.45	6.71	6.54	6.41	6.93	6.79	6.60
Pinotage Average	**7.53**	**6.74**	**6.70**	**7.16**	**6.57**	**6.30**	**6.51**	**6.97**	**6.60**	**6.49**	**6.73**	**6.98**	**6.63**
Cabernet Sauvignon Batch 1	7.40	6.54	6.13	6.55	6.74	6.24	6.84	6.85	6.33	6.56	5.98	6.53	6.07
Cabernet Sauvignon Batch 2	7.41	6.52	6.76	6.65	6.46	6.59	6.40	6.45	7.22	6.52	6.78	6.58	6.48
Cabernet Sauvignon Batch 3	7.11	6.30	6.19	6.62	6.89	6.02	7.03	6.66	6.47	6.59	6.41	6.81	6.60
Cabernet Sauvignon Average	**7.31**	**6.45**	**6.36**	**6.61**	**6.70**	**6.28**	**6.76**	**6.65**	**6.67**	**6.56**	**6.39**	**6.64**	**6.38**
Chardonnay **Batch** 1	4.32	4.11	4.43	4.38	4.28	4.40	4.16	4.19	4.22	4.05	4.25	4.18	4.36
Chardonnay Batch 2	4.48	4.05	2.71	4.24	4.22	3.98	4.31	4.17	4.26	4.09	3.94	4.10	4.36
Chardonnay Batch 3	4.64	4.28	4.18	4.26	4.34	3.99	3.81	4.15	4.27	4.56	4.26	4.27	4.31
Chardonnay Average	**4.48**	**4.15**	**3.78**	**4.30**	**4.28**	**4.12**	**4.10**	**4.17**	**4.25**	**4.23**	**4.15**	**4.18**	**4.34**
Chenin blanc Batch 1	4.02	3.46	3.18	3.72	3.45	3.88	3.67	3.61	3.47	3.83	3.22	3.84	3.78
Chenin blanc Batch 2	4.25	4.23	3.79	3.77	3.74	3.99	3.10	3.68	3.57	4.18	3.94	3.96	3.98
Chenin blanc **Batch** 3	4.01	3.73	3.05	3.66	3.58	4.03	3.91	3.66	3.74	3.91	3.29	4.18	4.14
Chenin blanc Average	**4.09**	**3.81**	**3.34**	**3.72**	**3.59**	**3.96**	**3.56**	**3.65**	**3.59**	**3.97**	**3.48**	**3.99**	**3.97**

[a] Antioxidant potency (AP) = Total antioxidant activity (mM Trolox measured using the ABTS radical cation scavenging assay) X 1000 / total phenols (mg gallic acid equivalents/L).

[b] Months of storage.