

ANTIOXIDANT STATUS OF SOUTH AFRICAN BEVERAGES AND ITS
ROLE ON THE CHEMICAL PARAMETERS IN HUMAN BLOOD

SAMUEL MEURU WANJIKU

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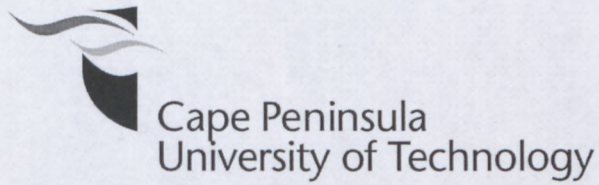
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ROLE ON THE CHEMICAL PARAMETERS IN HUMAN BLOOD

By

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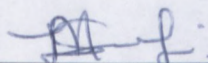
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DECLARATION

I, SAMUEL MBURU WANJIKU, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



Signed

28/08/09.

Date

ABSTRACT

In recent years antioxidants present in various beverages have attracted a great deal of research interest due to their potential benefits to human health. Epidemiological evidence shows an indirect relationship between the intake of beverages rich in antioxidants and the reduced incidences of some chronic diseases, certain cancers and coronary heart disease mortality.

The study was divided into two phases. The aim of the first phase was to compile a database of antioxidant capacities (Oxygen radical absorbance capacity, Ferric reducing antioxidant potential and Trolox equivalent antioxidant capacity) and contents (polyphenols, flavonols, flavanols, anthocyanins and vitamin C) of selected South African beverages. The second phase of the project included an intervention study to investigate the short *term in vivo* effects of consumption of an antioxidant-rich beverage, rooibos, on the antioxidant and oxidative stress status of human participants. The parameters that were investigated in human plasma included the total antioxidant capacity (ORAC and FRAP) and content (total polyphenols) as well as the reduced-oxidised glutathione ratios (GSH/GSSG) in erythrocytes.

Results from this study were used to compile a database for antioxidant capacities and contents of selected South African beverages. The results showed that, in the case of fruit juices their antioxidant capacity and content covered a wide range. This could be attributed to the juice's differences in fruit composition, e.g. either as a single fruit or as a combination of two or more fruits. In the case of wines, red wines had the highest antioxidant capacity for all the beverages tested and had 22 times higher polyphenols compared to that of white wines. Results also indicated that black tea and coffee had a higher antioxidant capacity and polyphenol content than the local herbal tea, rooibos. The duration of extraction (brewing) of the teas also influenced the antioxidant capacity. Correlations between the three antioxidant capacity assays were very high in the case of wines and teas, but lower in the case of fruit juices. This antioxidant database for South African beverages is useful not only to consumers, but also nutritionist, researchers, dieticians and functional food formulators.

Results of the *in vivo* study showed an increase of just more than 6% in the plasma antioxidant capacity (FRAP) 45 minutes after the volunteers consumed 500 mL of

rooibos. The polyphenol content of the plasma remained unchanged, which meant that the increase in the antioxidant capacity could not be directly linked to the polyphenol content of the plasma. The GSH/GSSG erythrocyte ratio was increased at 90 minutes and 180 minutes after rooibos was consumed. Although none of the increases or decreases for any of the parameters tested showed statistical significance, the results were comparable to similar studies which focused on other antioxidant rich beverages such as red wine, green tea and coffee.

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To you all my heartfelt appreciation for making it happen and may God bless you.

DEDICATION

To my Grandmother, with love and gratitude, for the sacrifice, motivation and inspiration.

GLOSSARY

Clarification of terms and abbreviations

°C: Degrees Celsius

¹O₂: Singlet oxygen

A: Absorbance

AAO: Ascorbic acid oxidase

AAPH: 2,2'-Azobis (2-amidinopropane) hydrochloride

ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) diammonium salt

AIDS: Acquired immunodeficiency syndrome

ANOVA: Analysis of variance

Antioxidant activity: Corresponds to the rate constant of a single antioxidant against a given free radical

Antioxidant capacity: Measure of the moles of a given free radical scavenged by a test solution, independently from the activity of any one antioxidant present in the mixture

ATP: Adenosine triphosphate

AUC: Area under the curve

BHT: Butylated hydroxytoluene

CE: Catechin equivalents

-CH₂-: Methylene group

CPUT: Cape Peninsula University of Technology

CT: Cycle time

Cu: Copper

Cytochrome P-450: A group of hemoproteins which forms multi-component electron transfer chains, useful in metabolism of endogenous and exogenous molecules e.g. drugs and toxins in humans

DMACA: 4-dimethylaminocinnamaldehyde

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DTNB: 5-5'-dithiobis [2-nitrobenzoic acid]

EC: Epicatechin

ECG: Epicatechin gallate

EDTA: Ethylenediaminetetraacetic acid

EGC: Epigallocatechin

EGCG: Epigallocatechin gallate

EtOH: Ethanol

Fe: Iron

Fenton reaction: The oxidation of ferrous Iron (II) to ferric iron (III), a hydroxyl radical and a hydroxyl anion by hydrogen peroxide

FL: Fluorescein

FRAP: Ferric reducing antioxidant power/potential

Free radical: A molecule having an unpaired electron in its outer orbit hence unstable and thus very reactive.

GAE: Gallic acid equivalents

GC: Gallocatechin
GCG: Gallocatechin gallate
GPx: Glutathione peroxidase
GR: Glutathione reductase
GSH: Reduced Glutathione
GSSG: Oxidised Glutathione
H₂O: Water
H₂O₂: Hydrogen peroxide
HCl: Hydrochloric acid
HO[•]: Hydroxyl radical
***In vitro*:** In an experimental situation outside the organism. Biological or chemical work done in the test tube (*in vitro* is Latin for "in glass") rather than in living systems
***In vivo*:** In a living cell or organism
K: Kappa
Kw: Weighted Kappa
LDL: Low density lipoproteins
M2VP: 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate
MBH: Membrane bound haemoglobin
MDA: Malondialdehyde
MeOH: Methanol
MPA: Metaphosphoric acid
MTT: [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]
Na₂CO₃: Sodium carbonate
NaCl: Sodium chloride
NADPH: Reduced nicotinamide dinucleotide phosphate
NaOH: Sodium hydroxide
O₂^{•-}: Superoxide radical
OCl: Hypochlorite
ONOO⁻: Peroxynitrate
ORAC: Oxygen radical absorbance capacity
Oxidation: The loss of electrons by a substance.
PE: Phycoerythrin
Peroxidation: Oxidation initiated by free radicals with the end products being hydroperoxides
Phenols: Groups of antioxidants occurring in wines and beverages having at least one phenol ring
PMS: 5-methylphenazinium methosulfate
Pro-oxidant: A free radical of pathological importance or a toxic substance that can cause damage to biological molecules
PUFA'S: Polyunsaturated fatty acids
QE: Quercetin equivalents
r: Pearson correlation coefficient
RBC: Red blood cells

RDA: Recommended daily allowance

Reactive oxygen species: Free radicals containing oxygen

Reducing agent: a substance that donates electrons to another thereby causing that substance to be reduced

Reduction: The gain of electrons by a substance

ROS: Reactive oxygen species

RPM: Revolutions per minute

SOD: Superoxide dismutase

TAC: Total antioxidant capacity

TBA: Thiobarbituric acid

TBARS: Thiobarbituric acid reactive substances

TE: Trolox equivalents

TEAC: Trolox equivalent antioxidant capacity

TNB: 5-thionitrobenzoic acid

TPTZ: 2,4,6-Tri [2-pyridyl]-s-triazine

TRAP: Total radical trapping antioxidant parameter

Trolox: 6-Hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid

μM: Micromolar

US: United States

ΔA: Absorbance difference

CHAPTER ONE INTRODUCTION

Fruits juices, wines and other beverages have been known from time immemorial to have health promoting effects. Scientists have exploited this fact and the specific properties in these beverages that combat harmful oxidation in the body have remained a fertile ground for research. It is known that moderate consumption of red wine and fruit juices can reduce the risk for cardiovascular disease (Bub *et al.*, 2003: 90; Modun *et al.*, 2008: 250). The protective effects of wine have been attributed to polyphenols that are efficient scavengers of free radicals and breakers of lipid peroxidative chain reactions (Lotito & Fraga, 1999: 125).

Data gathered from epidemiological studies has also shown an indirect correlation between the intake of food rich in phenolic compounds and the reduced incidences of some chronic diseases, certain cancers and coronary heart disease mortality (Steinmetz & Potter, 1996: 1027; Hertog *et al.*, 1995: 381). The use of wine and its association with health has been suspected from the earliest of civilizations. Antioxidants present in various wines and beverages have attracted a great deal of research interest of late due to the potential benefits for human health (López-Vélez *et al.*, 2003).

The importance of eating at least five servings of fruits and vegetables every day is common knowledge to most people, although this is rarely achieved, if at all. Since the normal diet does not provide enough antioxidants due to the low fruit and vegetable intake, ways to improve them become important. To supplement the diet, it is necessary to determine the antioxidant capacities of the components of the foods (Wu *et al.*, 2004: 408). Several assays have been developed to determine the antioxidant capacities of various dietary components (Wu *et al.*, 2004: 408; Yeum *et al.*, 2004: 98). However for the assays to be relevant the following should be considered: 1) the heterogeneity of the antioxidants i.e. the hydrophilic (water soluble) antioxidants, the lipophilic (lipid soluble) antioxidants and the insoluble antioxidants. This is important because the choice of the methods of extraction and the solvents to extract the antioxidants depends on this to a large extent and 2) the cooperative synergistic

interactions as some antioxidants show a higher antioxidant capacity in the presence of others than on their own (Yeum *et al.*, 2004: 98).

Even under normal conditions, aerobic metabolism in biological systems creates reactive oxygen species that are free radicals (Cheeseman & Slater, 1993: 481). The human body, which is in constant metabolism to provide energy to drive other vital processes, produces toxic substances in the form of free radicals. Prevention of free radical formation and the removal of those formed are the role of endogenous micronutrients produced by our body. In order to combat harmful oxidation in our bodies, we must supplement our natural defences, with micronutrients from other sources, hence the importance of drinking reasonable amounts of healthy beverages. The measurement of the antioxidant capacity of the various sources of these antioxidants therefore becomes important (Prior & Cao, 1999: 1173).

Defence against free radical damage of biological molecules, such as deoxyribonucleic acid or lipids, requires both intra- and extracellular antioxidant activity. Oxidative stress or injury occurs if free radicals are in excess. Studies have shown that intracellular antioxidants are predominantly enzymes, the most common being superoxide dismutase, GPx, GR and catalase. Extracellular antioxidants in plasma are of much lower molecular mass and include vitamin C, vitamin E, carotenoids, and glutathione (Packer & Colman, 1999: 14).

In the past two decades, the possible role of flavonoids acting as antioxidants has been investigated. The major extracellular antioxidants are of dietary origin or are influenced by the diet. One study showed that red wine protected low-density lipoproteins from peroxidation (Frankel *et al.*, 1993). Another study showed that five commonly occurring flavonoids in various common foodstuffs significantly reduced the risk of coronary artery disease in elderly men who consumed the largest amount of these foods (Packer & Colman, 1999: 117). Several studies have shown that more than 80% of the daily flavonoid intake in humans comes from beverages, such as tea, coffee, fruit juices and wine (Coimbra *et al.*, 2006: 791; Lotito & Frei, 2006: 1728).

The consumption of saturated fat in France is greater than that in other developed countries; but mortality from coronary artery disease in France is only about one-third

of that of other the countries. It has been suggested that the French benefit from the consumption of red wine as it has a high flavonoid (subgroup of polyphenols) content (Renaud & DeLorgeril, 1992: 1523). Besides antioxidant activity, polyphenols also have anti-inflammatory effects and may protect low-density lipoproteins (LDL) against oxidative modification (Frankel *et al.*, 1993: 454).

The contemporary consumer is becoming progressively more aware of the connection between antioxidants and health and requires this data to be available on the products label. The manufacturers of food products and beverages have recognised this fact and have started including detailed information on their packaging. Therefore a reliable compositional data base will not only help the consumers and manufacturers but also the nutritionists and medical researchers in studies of dietary factors and aetiology of diseases as well as in epidemiological research which is based on composition tables (Wu *et al.*, 2004: 418).

Currently there is no database available for antioxidant capacity of South African beverages. Existing research work on beverages in South Africa has bypassed this area and hence the health benefits of these beverages remain underutilised and underexploited. An easy to understand and an up to date record which correlates with the required daily allowance (RDA) for antioxidants is required for the consumers, medical researchers, nutritionists and manufacturers.

This study was divided into two parts. The aim of the first part of the study was to develop a database of antioxidant status of South African beverages. Since herbal teas and fruit juices are also major sources of polyphenols they were included in this study and compared to red wine. The second part of the project was a pilot intervention study to investigate the short *term in vivo* effects of the consumption of one of the beverages on antioxidant status and oxidative stress of human participants. These effects were monitored during specific intervals (0, 45, 90, 180 minutes) over a 3-hour period after consumption of a standard amount (500 mL) of one of the beverages (in this case Rooibos herbal tea). Rooibos was selected in preference of the others as it is an exclusively indigenous South African herbal tea. The parameters that were investigated in the human plasma and erythrocytes are: antioxidant capacity (ORAC assay) and content (total polyphenols), reduced-oxidised glutathione ratio and

uric acid (FRAP assay).

The research involved both *in vitro* and *in vivo* measurement of antioxidant status. Since beverages used in South Africa are vast, this study focused only on selected beverages from leading supermarkets in the Western Cape region as a departure point. The aim of this research was to compile an up-to-date compositional database of antioxidants in beverages and to investigate the short term effects of consumption of one of the beverages on plasma antioxidant status.

The *in vitro* study had the following objectives:

- To determine the antioxidant capacity of selected South African beverages.
- To determine the antioxidant quantity of selected South African beverages.
- To compare the antioxidant capacity of the different beverages.

The *in vivo* study had the following objectives:

- To determine the modulatory effects of rooibos on blood polyphenol levels and blood antioxidant capacity.
- To determine the effects of rooibos on the oxidative stress status in the blood.

CHAPTER TWO

LITERATURE STUDY

2. Free radicals, antioxidants, oxidative stress and human disease

In order to appreciate the role of antioxidants in oxidative stress and to comprehend their vital connection to human disease; the knowledge of the following basic terminologies is vital:

- 1) Free radicals,
- 2) Reactive oxygen species (ROS),
- 3) Reducing agent (reductant),
- 4) Oxidising agents (oxidant), and
- 5) Pro-oxidant.

A free radical can be defined as a highly reactive atom which is capable of independent existence and has an unpaired electron in its outer orbit (Young & Woodside, 2001: 176; Willcox *et al.*, 2004: 275). The unpaired electron is always in search of a partner to make it stable, thus increasing its reactivity. The removal of an electron from a co-reactant avails a partner for the unpaired electron but results in the reduction of the radical and thus forming a new radical by oxidation of the co-reactant (Benzie, 2003: 115). Examples of free radicals include: superoxide anion ($O_2^{\cdot-}$), alkoxy, hydroxyl radical (HO^{\cdot}), and alkoxy singlet oxygen (1O_2) (Langseth, 1993: 5; Evans & Halliwell, 2001: 568).

Oxygen, a prevalent and generously available component of the air humans' breathe, is vital for aerobic respiration. However, this oxygen is also a source of oxidative damage to important biological systems and sites, endangering their structure and function (Benzie, 2003: 114). The oxygen is reduced within the cells to produce free radicals containing oxygen otherwise known as ROS (Morrissey & O'Brien, 1998: 463).

Reactive oxygen species are the most important category of free radicals in living organisms (Miller *et al.*, 1990: 95). They are generated in substantial amounts by internal metabolic processes in aerobic organisms during mitochondrial respiration when oxygen is reduced to water (Halliwell & Gutteridge, 1999: 5; Benzie, 2003: 115). These reactive oxygen species include free radicals which can briefly exist independently and contain single or additional unpaired electrons in their outer orbits, thus are unstable, have very short half-life and are very reactive (Fang *et al.*, 2002: 872). Free radicals may be derived from single atoms such as hydrogen or chlorine or may involve two or more atoms e.g. the hydroxyl radical and the nitric oxide radical.

The superoxide radical is an important oxidant produced continuously and inevitably in the mitochondria from electron leakage during passage along the respiratory chain (Valko *et al.*, 2004: 38; Kovacic *et al.*, 2005: 2601). The generation of the superoxide radical, which serves as a source of numerous other ROS including hydrogen peroxide and the very reactive peroxyxynitrite and hydroxyl radical, is greatly enhanced during exercise, post ischaemic reperfusion and during inflammation (Halliwell & Gutteridge, 1999: 6; Benzie, 2000: 54; McCord, 2000: 653). The splitting of oxygen by high-energy ionizing radiation, produces the highly reactive hydroxyl radical, and can also absorb electro-magnetic radiation, to form the energized singlet oxygen molecule (Benzie, 2003: 116). Biological systems such as phagocyte activation, lipid oxidation, the arachidonate pathway, auto-oxidation of catecholamines, reduced flavins and hemoproteins and iron-mediated reactions are also capable of producing reactive oxygen species (Halliwell, 1994: 721).

In the human body, the mitochondria, the skin, areas of inflammation or post-ischaemic reperfusion face strong challenges of ROS (Benzie, 2003: 116). Although superoxide is usually accepted to be an oxidant, it also acts as a reducing agent by donating its extra electron to an oxidised transition metal ion, e.g. Fe^{3+} or Cu^{2+} . The enzyme superoxide dismutase (SOD), which occurs in mitochondrial and cytosol, catalyses the conversion of superoxide into hydrogen peroxide (Halliwell, 1994: 721). The hydrogen peroxide (H_2O_2) produced may react in turn with reduced metal ions in the Haber-Weiss reaction (Fenton reaction when the metal ion is iron), forming the highly reactive hydroxyl radical, which causes random damage to biological molecules (McCord, 2000: 653).

Free radicals and reactive oxygen species are formed either deliberately to serve vital biological functions or inadvertently, for example, activated phagocytes may produce ROS for killing some strains of bacteria and fungi (Bredt, 1999: 578; Jabs, 1999: 231; Willcox *et al.*, 2004: 275). The human body produces reactive oxygen species constantly through the normal physiological and metabolic processes e.g. aerobic respiration. Kehrer & Smith (1994) have identified the four endogenous sources of ROS as:

- i. By products of normal aerobic respiration whereby molecular oxygen is reduced in the cells to generate adenosine triphosphate (ATP) consequently producing O_2^- , H_2O_2 and OH^\cdot .
- ii. Peroximes, major sites of oxygen consumption in cells, which produces H_2O_2 as a by product of their metabolic functions and is then degraded by catalase. Some of the H_2O_2 escapes the degradation and may leak when peroximes are damaged into other compartments and thereby increasing oxidative damage.

- iii. Cytochrome P-450 mixed function oxidase system which constitutes a primary defence against xenobiotics and endogenous substances also enhance production of free radicals.
- iv. Some phagocytic cells in an oxidative burst of O_2 , H_2O_2 , hypochlorite ($-OCl$) and nitric oxide produces O_2^- intentionally to destroy cells infected with bacteria or virus infected cells.

Other exogenous sources, which may also increase the endogenous free radical load, include high intakes of iron and copper, cigarette smoke, ionizing radiation, air pollutants, chemicals such as ethyl alcohol, ozone, halogenated hydrogen and lipid oxidation products in foods (Fang *et al.*, 2002: 874).

Free radicals react by either joining their unpaired electrons with other radicals and producing more damaging products e.g. the highly reactive peroxynitrite produced by combination of superoxide and nitric oxide as shown in Figure 2.1 (an important vasodilator) (Halliwell, 1994: 724):

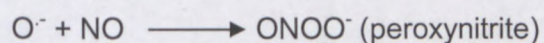


Figure 2.1

They may also react with non radicals, generating a chain reaction of free radicals thus adding more to the radical pool. These propagate some processes, e.g. in lipid peroxidation where reactive radicals remove atoms of hydrogen from polyunsaturated fatty acid's side chain, leaving unpaired electrons on the carbon. If the hydroxyl radical production is near deoxyribonucleic acid (DNA), the free radicals attack the nitrogen bases of the molecule causing mutations, e.g. guanine is converted to 8-hydroxyguanine, a good biomarker of DNA damage (Halliwell, 1994: 721).

Heme proteins and transition metals, resulting from tissue damage or injury, causes formation of free radicals (Halliwell, 1989: 317). They may also activate or increase the enzymes that catalyses free radical producing reactions (Valko *et al.*, 2005: 1161). Tissue damage, as a consequence of chronic diseases for instance heart diseases, cancer, arthritis, cataracts and others, could as well be linked with increased production of mediators such as prostaglandins, leucotrienes, interferons and tumour necrosis factors leading to inflammation and consequently production of more free radicals (Willcox *et al.*, 2004: 277). Macrophages, which have been activated by tissue injury, are important sources of free radicals (Morrissey & O'Brien, 1998: 464).

Disease conditions causing tissue damage and haemolysis, such as

haemoglobinopathies, iron overload, sickle cell anaemia, malaria and cerebrospinal haemorrhage, releases iron and other transition metals which catalyses free radical reactions consequently producing peroxy radicals (Papap, 1999: 1001). Premature babies and even normal term babies have iron, which is capable of catalysing free radical reactions. If exposed to high oxygen concentrations, premature babies develop retinopathy due to the oxidative damage caused by free radicals. Living organisms consequently, have developed intricate antioxidant systems to manage production and diminish damage from free radicals species and ROS (Morrissey & O'Brien, 1998: 463).

However, free radicals are not always harmful. Sometimes they play an important biological role, e.g. oxygen radicals are involved in significant roles such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity in cells (Fang *et al.*, 2002: 873; Valko *et al.*, 2007: 53). Other free radicals, such as nitric oxide, are essential for regulating the relaxation and proliferation of vascular smooth muscle, leukocyte adhesion, platelet aggregation angiogenesis, thrombosis, vascular tone and hemodynamics. Nitric oxide produced by neurons serves as a neurotransmitter. Nitric oxide produced by activated macrophages is an important mediator of the immune response.

Free radicals, therefore serve not only as signalling and regulatory molecules but also as extremely damaging and cytotoxic oxidants (Fridovich, 1999: 13). Living organisms as a result, have evolved mechanisms of maintaining equilibrium between harmful and beneficial effects of free radicals. This is referred to as redox regulation, which maintains a type of redox homeostasis in the organisms (Valko *et al.*, 2007: 47).

Reduction of a substance can be defined as an increase of electrons whereas oxidation refers to a loss of electrons (Prior & Cao, 1999: 1173). It follows that, a reducing agent is a substance that is capable of donating electrons and as a consequence causes the reduction of another reactant. An oxidizing agent on the other hand is a substance that accepts electrons and therefore causing another reactant to be oxidized (Prior & Cao, 1999: 1173). Oxidation and reduction reactions occur hand in hand in biological systems, i.e. an oxidation reaction at one point causes reduction elsewhere in the system. Redox reactions form the basis of aerobic respiration whereby atmospheric oxygen is utilized in a chain of chemical reactions to metabolize food chemicals which in turn provide chemical energy for living organisms (Willcox *et al.*, 2004: 275). A pro-oxidant can be defined as a toxic substance that can cause oxidative damage to biomolecules such as proteins, lipids and nucleic acids, resulting

in various pathological conditions and or diseases. In chemical terms a pro-oxidant can be said to be an oxidant of pathological importance (Prior & Cao, 1999: 1173).

2.1 Oxidative stress

Papas (1996: 77) defined antioxidant status as the balance between antioxidants and pro-oxidants in living organisms. This equilibrium, although dynamic in the human body, slightly favours the pro-oxidants. This is evident from the ongoing damage to biomolecules such as DNA, lipids, carbohydrates, proteins, uric acids etc. As a result the body has developed a repair mechanism which includes enzymes such as ligases, nucleases, polymerases, proteinases and phospholipases to deal with this slight imbalance (Morrissey & O'Brien, 1998: 465; Young & Woodside, 2001: 178). The body also uses a type of negative feedback regulation mechanism in a process referred to as redox homeostasis which produces endogenous antioxidants to correct this imbalance (Valko *et al.*, 2007: 47).

Oxidative stress which has been defined by Willcox *et al.* (2004: 279) as a serious imbalance between free radicals and antioxidant defence system is said to result from excessive production of reactive oxygen species and free radicals or from the failure of the antioxidant defence system. Consequently the ensuing injury is referred to as oxidative damage and is known to be damaging to the purine and pyrimidine bases together with the deoxyribose backbone of the DNA molecule. The resulting oxidative damage of the DNA molecule is implicated in mutagenesis, carcinogenesis and ageing (Valko *et al.*, 2007: 47).

Lipid peroxidation reaction has been the most convenient marker of oxidative stress in living systems. The lipid peroxidation progression is started by a free radical assault on a polyunsaturated fatty acid side-chain in a membrane or lipoprotein. As a result, a lipid radical that reacts with oxygen is formed which leads to the formation of a peroxy radical that may further react with other lipids and produce a new lipid radical (Halliwell, 1994: 722). The reaction will lead to a cascade chain reaction which is sustained until a termination process by a chain breaking antioxidant occurs. The estimation of the lipid peroxidation process in general is not easy as lipid peroxidation is an intricate process that occurs in several stages (Nalsen, 2006: 134). Oxidative stress in living systems can be estimated by measuring the degree of lipid peroxidation in the systems (Laguette, *et al.*, 2007: 273).

2.1.1 Lipid peroxidation

There are three different mechanisms that are known to be capable of inducing lipid peroxidation:

- Auto-oxidation by free radicals
- Photo-oxidation
- Enzyme action

Auto-oxidation is a radical chain reaction linking three processes: Initiation, propagation and termination (Laguerre *et al.*, 2007: 250).

2.1.1.a Initiation

Initiation is whereby a lipid system, e.g. a polyunsaturated fatty acid (PUFA), is attacked by a sufficiently active ROS (e.g. hydroxyl or singlet oxygen) which abstracts a hydrogen atom from its methylene group (-CH₂-). The attack generates a carbon centred free radical for the polyunsaturated fatty acids (Fang *et al.*, 2002: 874). The occurrence of a double bond in the polyunsaturated fatty acid makes the C-H bonds on the carbon atom next to the double bond weaker and thus the removal of H becomes easier. This reaction forms conjugated dienes which in the presence of oxygen combines with the oxygen to form a peroxy radical (Halliwell, 1994: 724).

Figure 2.2 depicts the formation of a peroxy radical by peroxidation of polyunsaturated fatty acid.

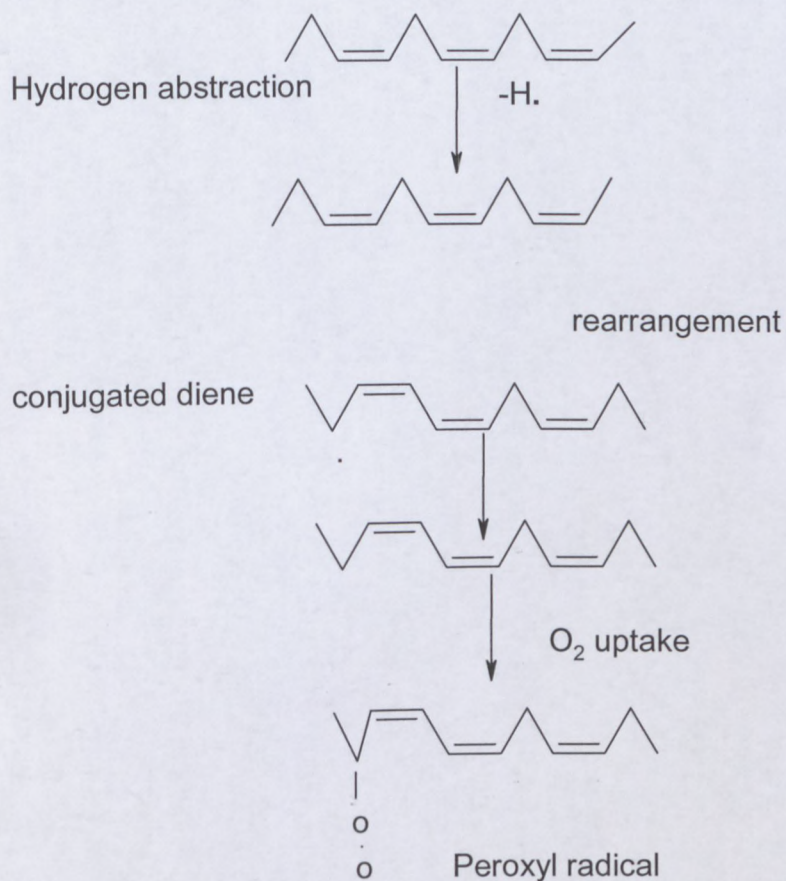
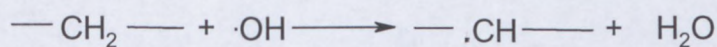


Figure 2.2: Lipid peroxidation mechanism (Adapted from Halliwell, 1994: 722).

2.1.1.b Propagation

In the presence of metals, e.g. copper or iron, the peroxy radical formed during the initiation process is capable of removing hydrogen ions from adjacent PUFA's, thus causing the propagation or continuation of the reaction. The peroxy radical unites with the hydrogen to produce peroxide in what is referred to as propagation stage as indicated in Figure 2.3 (Halliwell, 1994: 722).

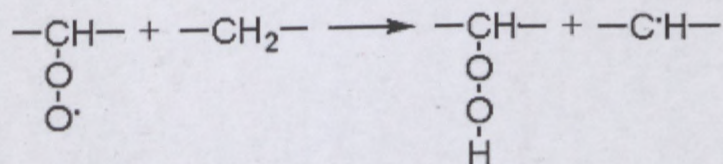


Figure 2.3: Propagation of lipid peroxidation (Adapted from Halliwell, 1994: 722).

2.1.1.c Termination

Termination occurs when the peroxy radical reacts with a chain breaking antioxidant, e.g. α -tocopherol, to produce a hydroperoxide in the cell membrane (Willcox *et al.*, 2004: 278).

2.1.2 Photo-oxidation

This occurs when the singlet oxygen radical reacts with double bonds of the unsaturated fatty acids in the presence of sensitizers like porphyrins, myoglobin, riboflavin, bilirubin, or erythrosine etc. Oxygen is added to the carbon at both ends of a double bond which assumes a trans configuration, producing hydroperoxides (Laguerre *et al.*, 2007: 250).

2.1.3 Enzymatic peroxidation

Lipoxygenases are capable of catalyzing reactions between singlet oxygen and PUFA's e.g. arachidonic acid to produce hydroperoxides. Cyclooxygenases are also capable of catalysing the addition of molecular oxygen to various PUFA's converting them into endoperoxides, which are biological active molecule intermediates in the transformation of fatty acids to prostaglandins (Laguerre *et al.*, 2007: 250).

2.2. Oxidative stress and disease

Cells are capable of dealing with mild oxidative stress via their antioxidant defence mechanisms, however, severe oxidative stress causes damage to biomolecules, cells and tissues. Prevention of the oxidative stress is therefore paramount for good health and prevention of disease (Morrissey & O'Brien, 1998: 469). Several studies indicate that oxidative stress may be implicated in the progression of numerous disorders which includes atherosclerosis, inflammatory conditions, certain cancers and the aging process. This is a result of evidence of increased amounts of oxidative damage products, e.g. lipid peroxidation in body fluids (Young & Woodside, 2001: 182). However, oxidative stress may be a secondary phenomenon and not the primary cause of the disease (Gutteridge, 1993: 142). The following summarizes some of the data supporting a contributory role of free radicals in some diseases.

2.2.1 Cancer

Some clinical and epidemiological data from experimental models support a role or involvement of free radicals in the pathogenesis of cancer (Valko et al., 2006: 12). Extremely insidious or metastatic cancer cells need a particular level of oxidative stress to sustain equilibrium between propagation and apoptosis. These cells produce great quantities of hydrogen peroxide, an important signal molecule in the continued existence of cancer cells. Antioxidants have been known to repress these hydrogen peroxide signal molecules hampering cancer cell production. Oxidative damage to lipids and proteins, e.g. DNA repair enzymes may also cause DNA mutations (Willcox et al., 2004: 281).

Dietary and non dietary antioxidants and free radical inhibitors have been shown to protect animal models against free radicals and may be chemoprotective in humans (Guyton & Kensler, 1993: 524). Substantial evidence that antioxidants such as β -carotene, vitamin E, vitamin A and selenium are associated with reduced cancer risks exist (Omen et al., 1996: 1152). Goldstein & Witz (1990: 4) listed evidence that suggest that free radicals are implicated in tumour promotion and progression;

- i. Free radical producing compounds such as organic peroxides act as tumour promoters and progressors,
- ii. Chemical promoters (e.g. phorbol esters, benzoyl peroxide) stimulate endogenous production of ROS,
- iii. ROS producing systems copy the actions of tumour promoters in the cell culture,
- iv. Tumour promoters aggravate fast and continued changes in cellular antioxidant enzyme activities,
- v. Antioxidants inhibit tumour promotion and progression.

2.2.2 Diabetes

Investigational and clinical reports indicate that oxidative stress is a key player in the progression of both type 1 and type 2 diabetes. Oxidative stress increases diabetic complications due to DNA damage and also the risk of atherosclerosis in diabetic patients (Willcox et al., 2004: 287).

2.2.3 Coronary heart disease

Oxidation of low density lipoproteins (LDL), a key cholesterol transporting protein in

human blood plasma has been implicated as the initiator of atherosclerosis, which is a primary cause of cardiovascular diseases. LDL are rich in cholesterol, linoleic, arachidonic and docosahexaenoic acid which are highly susceptible to peroxidation by oxidative attack through oxygen radicals (Langseth, 1993: 13).

2.2.4 Eye disease

Age related eye diseases, e.g. maculopathy and cataracts, are a major health predicament the world over (Langseth, 1993: 18). In less developed countries, cataracts are a major cause of blindness. Oxidative processes have been implicated in the causation of both cataract and age-related maculopathy and recent reports have indicated that elevated dietary intakes of antioxidants may help delay or prevent these disorders (Packer & Fuchs, 1997). The eye has defence systems which protect the lens from oxidative damage. Antioxidants and antioxidant enzymes inactivate free radicals while proteases remove damaged proteins from the lens. However, when oxidized proteins accumulate (due to age or inefficient defence systems) damage to the lens proteins becomes irreversible developing into cataracts. High intake of the major dietary antioxidants (vitamins C, E and carotenoids) decreases cataract risk (Willcox *et al.*, 2004: 289). Scientific evidence shows an inverse correlation between age-related maculopathy and consumption of fruits and vegetables rich in vitamin A (Langseth, 1993: 21).

2.2.5 Other diseases

Biochemical studies (Coyle & Puttfarcken, 1993: 689) have shown that oxidation plays a role in causing several disorders of the brain and nervous system, aging and age related disorders and that antioxidants, e.g. vitamin E may help in reducing symptoms or slowing progression of some neurological disorders (Muller *et al.*, 1983: 225). Oxidative stress is thought to significantly add to the reduction of CD4⁺ lymphocyte count in acquired immunodeficiency syndrome (AIDS) patients, aiding in the progression to AIDS. Lymphocytes of AIDS patients are deficient of glutathione, making them susceptible to oxidative stress (Willcox *et al.*, 2004: 289). Cigarette smoke has also been associated with reduced sperm count and poor sperm quality and supplementation with vitamin C has been reported to enhance sperm quality in heavy smokers (Langseth, 1993: 21). Free radicals and oxidative stress play a role in inflammatory disease, e.g. rheumatoid arthritis. Products of free radicals have been detected in blood and joints of rheumatoid arthritis patients. Free radicals are important in conferring cell mediated immunity to the body and play a role in antigen presentation

and cell production. Nonetheless, at high quantities they cause lipid peroxidation of PUFA's in membranes, thereby reducing their immune functions (Willcox *et al.*, 2004: 288).

2.3 Antioxidants defence system

The human biological system constantly produces free radicals and other oxygen derived species and as a result, cells have evolved complex antioxidant defence systems to combat this. The antioxidant defence system consists of primary or preventive antioxidants which limit the initial formation of oxygen centred radicals of organic compounds. Secondary scavenging or chain breaking antioxidants are present to trap intermediate ROS and thus breaking the chain reaction. The third line of defence consists of repair systems for damaged nucleic acids, proteins and lipids (Halliwell, 1994: 722). The first primary defence mechanism consists of antioxidant enzymes such as:

- i. Catalase, found in peroxisomes in most tissues which catalyses the two stage change of hydrogen peroxide to water and oxygen.
- ii. Glutathione peroxidase and glutathione reductase which catalyses oxidation of glutathione at the cost of hydrogen peroxide in the cytosol and mitochondria (Figure 2.4).



Figure 2.4

- iii. Superoxide dismutase which catalyses dismutation of superoxide to H_2O_2 which can then be removed as shown in Figure 2.5 below.

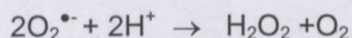


Figure 2.5

The above antioxidant defences, however, are not entirely efficient and thus repair enzymes also exist intracellular to destroy proteins damaged by free radicals, remove oxidised fatty acids from membranes and repair free radicals damaged DNA (Halliwell, 1994: 722). Other extracellular antioxidant defences exist and includes; transferrin (the plasma iron transport protein) and the iron binding protein lactoferrin, caeruloplasmin (transport form of copper), hemopexin and haptoglobin (binds free heme and heme proteins to decrease their capability to catalyse free radical damage) and albumin and urate (end products of purine metabolism which scavenges several free radicals)

(Morrissey & O'Brien, 1998: 465; Halliwell, 1989: 315).

The secondary defence system includes chain breaking antioxidants, which are small molecules that prevent further interaction of free radicals with other molecules (this may generate secondary radicals). The chain breaking antioxidants do this by receiving electrons from a radical or donate an electron to a radical to form a stable by-product (De Zwart *et al.*, 1999: 206). These antioxidants are classified into aqueous phase and lipid phase antioxidants. Lipid phase antioxidants, e.g. vitamin E and carotenoids, scavenge free radicals in membranes and lipoprotein particles thus preventing lipid peroxidation. The aqueous phase antioxidants e.g. vitamin C, uric acid, phenolic compounds and bilirubin scavenges directly radicals present in the aqueous phase section of blood plasma (Evans & Halliwell, 2001: 568).

The mechanism involving metal binding proteins (e.g. ferritin, transferrin and lactoferrin) operate as an essential constituent of the antioxidant defence system by seizing iron and copper so that they are unavailable to form hydroxyl radicals (Papad, 1999: 1001). Figure 2.6 summarises the three antioxidant defence systems in biological systems, i.e. preventative mechanisms, repair mechanisms and antioxidant defences.

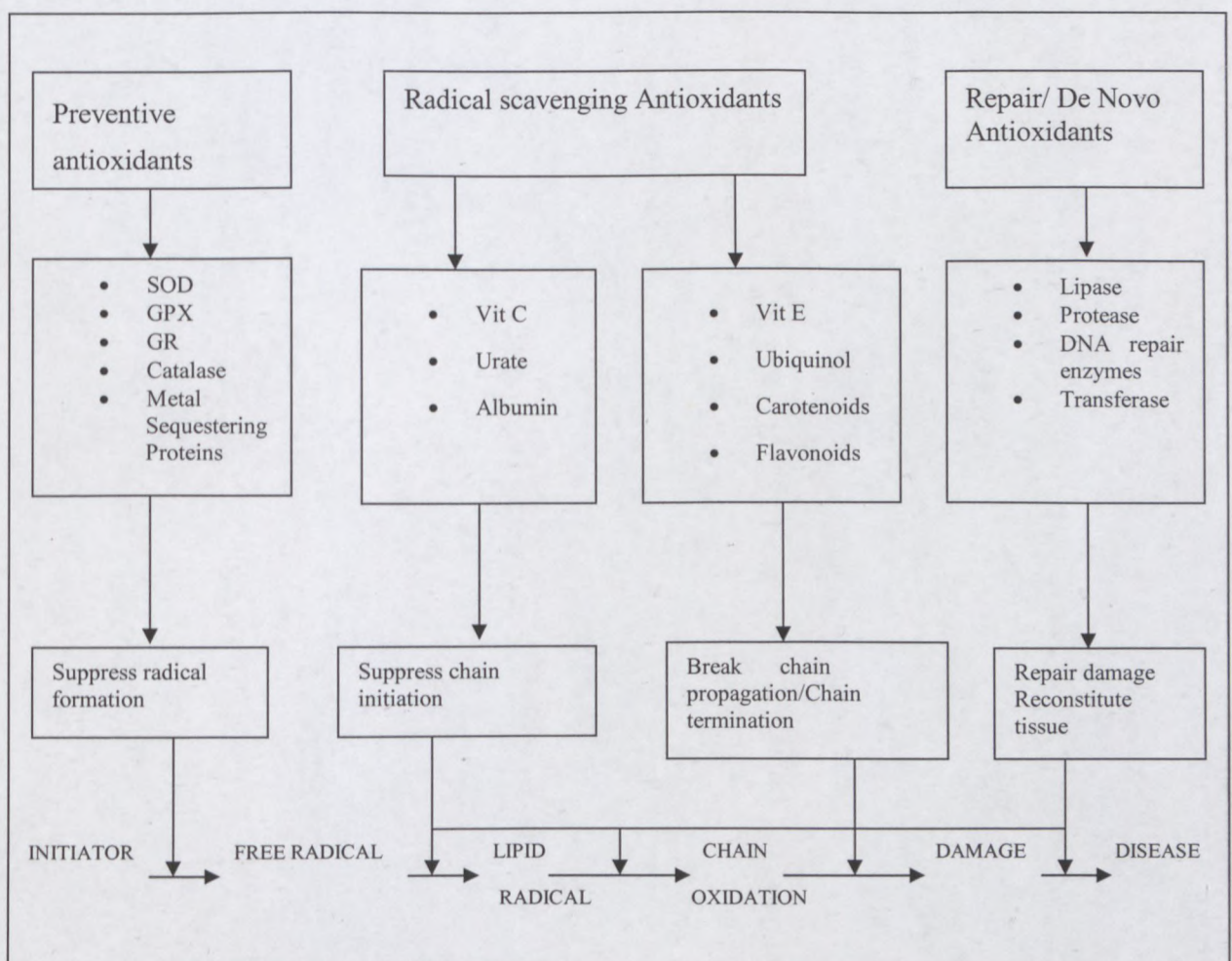


Figure 2.6: Summary of antioxidant defence system (Adapted from Willcox (2004: 279)).

2.4. Antioxidants

The protection which wines and other beverages provide against diseases has been credited to a variety of antioxidants contained in them (Serafini, *et al.*, 2000: 585). An antioxidant has been defined by Halliwell & Gutteridge (1999: 10) as a substance that, when in low concentration compared with that of an oxidisable substrate, significantly prevents or delays a pro-oxidant initiated oxidation of the substrate. An antioxidant therefore can reduce a pro-oxidant with the formed products having no or low toxicity.

Antioxidants can be generally classified as exogenous which includes natural/dietary antioxidants as well as synthetic ones (butylated hydroxyanisole, butylated hydroxy toluene, propyl gallate and tertially butylhydroquinone) and endogenous ones (Willcox *et al.*, 2004: 278). Endogenous antioxidants can be further classified as enzymatic and non-enzymatic. Enzymatic ones such as catalase, superoxide dismutase, peroxiredoxines, glutathione peroxidase, glutathione reductase, glutathione tranferase, and non-enzymatic antioxidants such as reduced glutathione, bilirubin, transferrin, cenciloplasmin, uric acid, nicotinamide adenine dinucleotide phosphate (NADPH), ubiquinol-10, manganese, copper, albumin, thiols, melatonin, vitamins (e.g. vitamin A retinol, vitamin C (ascorbic acid) and vitamin E (tocopherol), carotenoids and polyphenols (Papas, 1999: 1000). Figure 2.7 gives a general classification of antioxidants.

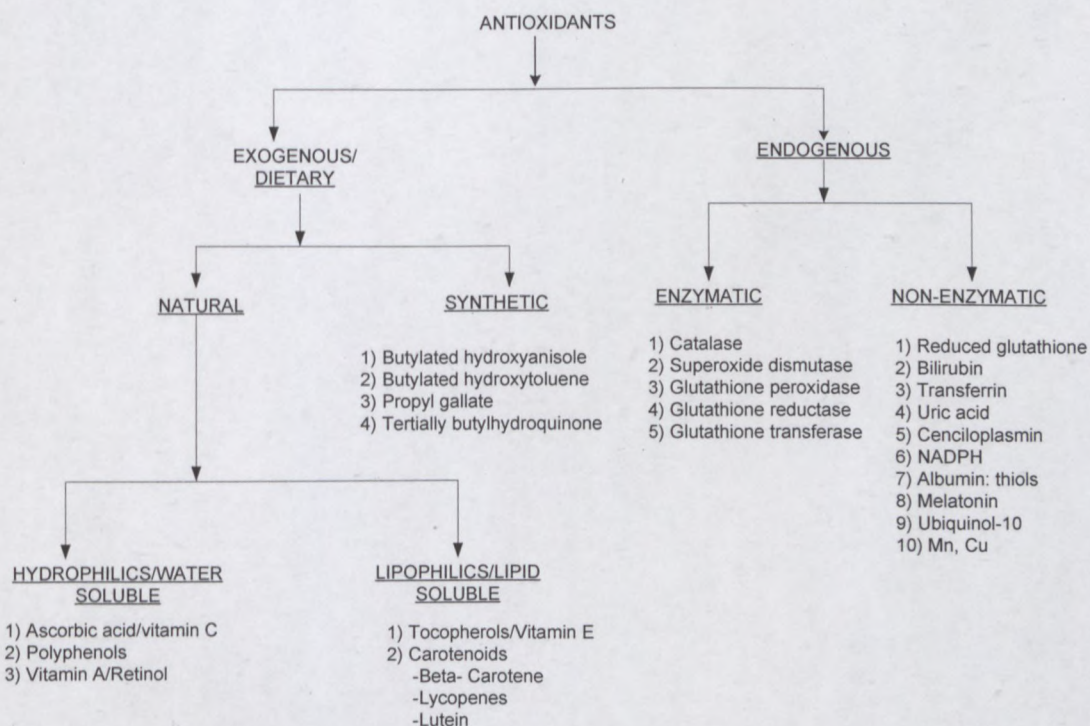


Figure 2.7: General classification of antioxidants (Compiled by author based on Willcox, *et al.*, 2004: 278).

2.4.1 Polyphenols

Polyphenols are substances that are naturally occurring in fruits and vegetables, and in beverages acquired from plants such as tea and red wine (Klimczak *et al.*, 2006: 313). The consumption of polyphenol rich diets has been associated with good health. The health effects of these compounds are dependent on the amount consumed and their bioavailability (Brannon, 2007: 8). These compounds may be classified into different groups depending on the number of phenol rings (skeleton of the chemical compound) that they have and of the structural elements that attach these rings to each other (Handique & Baruah, 2002: 163).

Polyphenols can be broadly classified into flavonoids, phenolic acids, lignans and stilbenes (Brannon, 2007: 4). The main sources of dietary polyphenols are fruits and beverages (tea and wines). Table 2.0 summarises the major categories of polyphenols, their sub-classes and their major dietary sources. Flavonoids make up the biggest collection of polyphenols. Their skeletal structure is made up of an aromatic ring compressed to a heterocyclic ring, attached to a second aromatic ring as shown in Figure 2.8 (Bors *et al.*, 1990: 343).

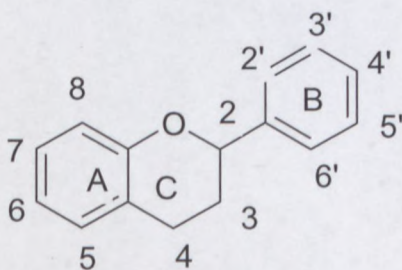


Figure 2.8: General chemical structure of flavonoids (Heim *et al.*, 2002: 573).

Flavonoids are primarily subdivided into: anthocyanins (glycosylated derivative of anthocyanidin, present in colourful flowers and fruits) and anthoxantins (colourless compounds further subdivided into several categories including flavones, flavans, flavonols, flavanols (catechins), and isoflavones (Heim *et al.*, 2002: 573). Polyphenols strongly binds metal ions, thus preventing peroxidative reactions. This binding affects the absorption of certain minerals. When consumed together, tea has been reported to reduce the assimilation of non-heme iron in a variety of food sources including lentils, chick peas, kidney beans, pinto beans, navy beans, boiled spinach, and iron fortified cereals among others (Manach *et al.*, 2004: 727).

Polyphenol contents of fruits and vegetables is affected by numerous factors which include, ripeness at the time of harvest, environmental factors such as soil type, sun exposure and rainfall, processing, methods of culinary preparation (e.g. simple peeling of fruit and vegetables), cooking and storage. The clarification and stabilization steps which removes some flavonoids responsible for loss of colour and formation of haze in the production of fruit juices, reduces the amount of flavonoids in these juices (Klimczak *et al.*, 2006: 319).

Due to the vast range of polyphenols and the numerous factors that can modify their concentration in foods and beverages, no reference food-compositional database have been prepared and only partial data for certain polyphenols such as flavones, catechins, flavonols and isoflavones have been published on the basis of direct food analysis. Thus, the need for a comprehensive compositional database for the polyphenols which should allow daily consumption to be calculated from dietary questionnaires and to correlate the polyphenol intake with incidences of certain diseases or early markers for these diseases in epidemiological studies, cannot be over emphasised (Manach *et al.*, 2004: 742). Categories, classes and food sources of polyphenols are listed in Table 2.0.

Table 2.0: Categories, classes and food sources of polyphenols (Adapted from Brannon, 2007: 3).

Category	Classes	Major Food Sources
Phenolic acids	• Ferulic acid	Dietary fibre, hemicelluloses
	• Caffeic acid - Chlorogenic acid	Many fruits and vegetables, coffee
	• Condensed tannins	Mango fruit
	• Hydrolyzable tannins - Gallotannins - Ellagitannins	Blackberries, raspberries, strawberries, wine, brandy aged in oak barrels
Flavonoids	• Flavones	Sweet red pepper, celery
	• Flavonols - Quercetin	Tea, onions, apples, many other fruits and vegetables
	• Flavanols: - Catechins: - Epicatechin (EC) - Epicatechin-3-gallate (ECG) - Epigallocatechin (EGC) - Epigallocatechin-3-gallate (EGCG)	Tea, especially green tea, chocolate, cocoa
	• Flavanones - Hesperetin	Oranges, citrus fruits
	• Isoflavones - Genistein - Daidzein	Soybeans, soy protein-containing foods
	• Anthocyanins - Cyanidin	Red fruits: cherries, plums, strawberries, raspberries, blackberries, grapes, red and black currants
	• Proanthocyanidins	Apples, pears, grapes, red wine, tea
Lignans	Enterodiol	Flaxseed, flaxseed oil
Stilbenes	Resveratrol	Red wine

2.4.2 Flavonoids

Flavonoids are a broad class of low molecular weight, secondary plant phenolics characterized by the flavan nucleus. They consist of a pyrane ring (Figure 2.8), the substitutions of which determine their classification. Thus flavonoids are benzo- γ -pyrone derivatives. The configuration of the hydroxyl, methoxy, and glycosidic side groups vary considerably in dietary flavonoids as well as the conjugation between the A- and B-rings (Heim *et al.*, 2002: 573).

Flavonoids contain a number of hydroxyl groups attached to the ring structure. The

arrangement of functional groups in the nuclear structure determines the antioxidant activity of flavonoids and their metabolites *in vitro* (Rice-Evans *et al.*, 1996: 936). The role of specific structural components in radical scavenging, chelation and oxidant activity has been strongly supported by evidence from several studies. Mainly, the flavonoids occurring in food are O-glycosides; with glucose being the most common sugar moiety (Hammerstone *et al.*, 2000: 2087). Other glucosidic units include glucorhamnose, galactose, arabinose, and rhamnose (Cook & Samman 1996: 67).

Absorption and bioavailability of flavonoids is influenced by the dosage, vehicle of administration, antecedent diet, sex difference and microbial population of the colon, as well as the type of sugars moieties, and other functional groups about the flavan nucleus. Due to their large molecular sizes, absorption of polymeric flavonoids across the intestinal epithelium requires that they are degraded to smaller, low molecular weight compounds (Manach *et al.*, 2004: 740). General chemical structures of anthocyanidins, catechins, flavonols and flavones are illustrated in Figure 2.9.

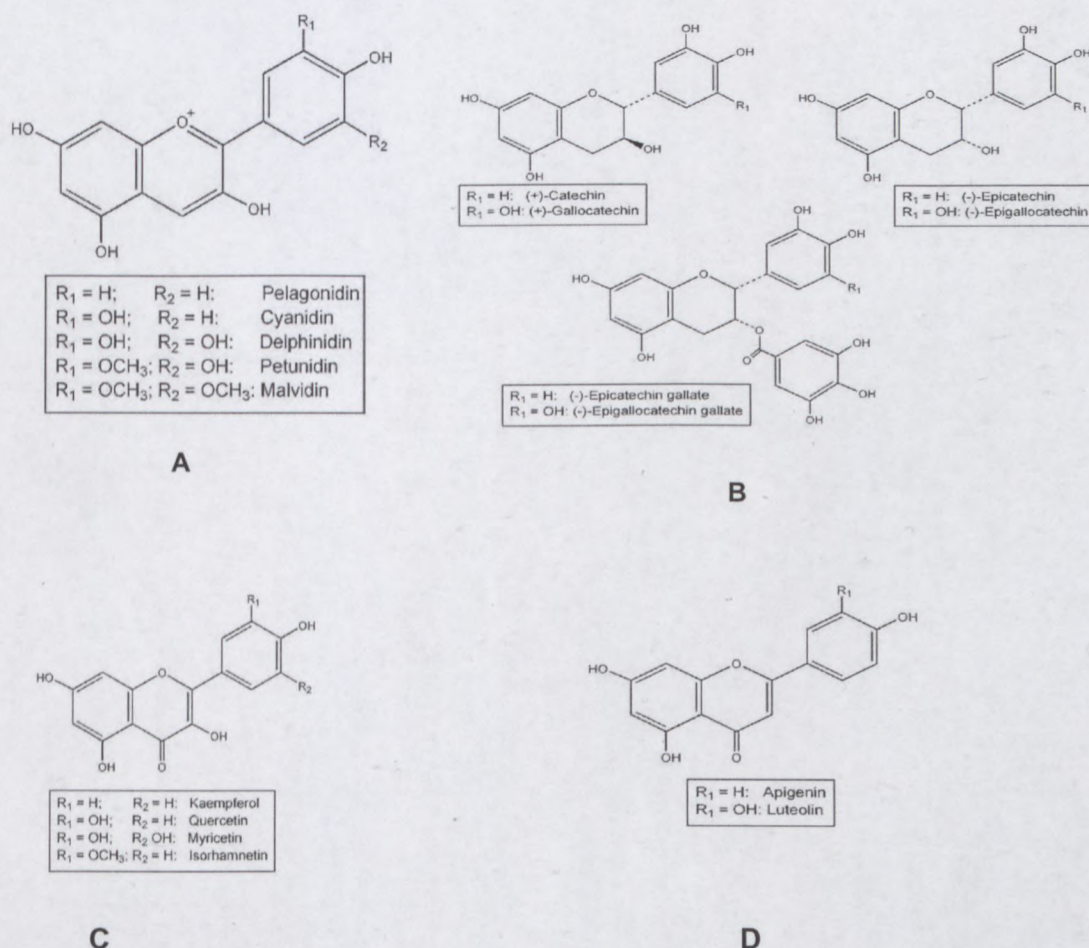


Figure 2.9 Chemical structures of anthocyanins (A) Catechins (B), Flavonols (C) and Flavones (D) (Adapted from Pannala *et al.*, 2001: 1163).

2.4.3 Phenolic acids

Phenolic acids can be broadly classified into two classes, the benzoic acid derivatives (gallic acid in tea, gallotannins in mangoes and ellagitannins in red fruits such as berries) and the cinnamic acid derivatives (Pannala *et al.*, 2001: 116; Manach *et al.*, 2004: 730). The hydroxybenzoic acids, both free and esterified, are less abundant in plants consumed by humans and consequently have not been widely studied. The hydroxycinnamic acid derivatives are the most abundant and consist of mainly caffeic-, ferulic-, p-coumaric- and sinapic acids, with caffeic acid being the most abundant of the four in most fruits (Manach *et al.*, 2004: 730).

Although hydroxycinnamic acids occur in all parts of the fruit, the highest concentration is found in the outer parts of the ripe fruit. Ferulic acid is the most abundant phenolic acids in the cereal grains (Manach *et al.*, 2004: 730). The antioxidant activities of the phenolic acids are dependent upon the number of hydroxyl groups in their nuclear structure (Rice-Evans *et al.*, 1996: 936). The general chemical structure of hydrocinnamic acids is shown in Figure 2.10.

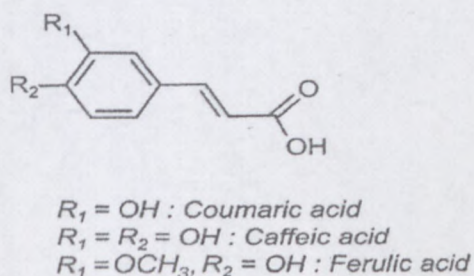


Figure 2.10: General structure of phenolic acids (Manach *et al.*, 2004: 730).

Resveratrol, a 3,4,5-trihydroxy-trans-stilbene in wines, has been linked with reduced risk of coronary heart disease, cancer, Alzheimer's disease and dementia prevention (Baxter, 2008: 2). It has also been linked with the inhibition of oxidation of low density lipoprotein (LDL), inhibition of platelet aggregation and is thought to possess preventive anti-inflammatory properties (Nikfardjam *et al.*, 2005: 74).

The curiosity in resveratrol was aroused initially by epidemiological studies indicating an indirect association between modest wine use and the possibility of coronary heart disease (also known as the French paradox) and its cancer preventive properties observed *in vitro* and *in vivo* (Goldberg *et al.*, 1995: 89). The ability of resveratrol to restrain varied cellular events which are associated with the initiation, promotion, and progression of carcinogenesis has been described by Jang and co workers (1997:

219).

2.5. Bioavailability of antioxidants

Bioavailability of antioxidants varies within the specific antioxidants. This depends on the chemical properties, deconjugation and re-conjugation for example in the intestines and liver, intestinal absorption and enzymes available in the biological system for metabolism (Manach *et al.*, 2004: 734). The bioavailability, metabolism and absorption of polyphenols are influenced by the chemical structure of the group. Most of the ingested polyphenols by humans is absorbed both in the gastro-intestinal tract and metabolised by micro flora present in the colon (Brannon, 2007: 5). The plasma half life of polyphenols is very short and repeated ingestion of polyphenols is required in order to sustain elevated plasma levels (Scalbert & Williamson, 2000: 2074). Although information on the bioavailability of antioxidants is rather scarce, antioxidants occur freely or conjugated in plasma, urine, intestines and in tissues e.g. adipose tissue liver, brain and kidneys (Scalbert & Williamson, 2000: 2074; Brannon, 2007: 5). Some groups of carotenoids also occur in breast milk. Knowledge of the bioavailability of antioxidants and their metabolism is required to evaluate their biological activity within target tissues (Heim *et al.*, 2002: 573).

2.6. Fruits and vegetables as source of antioxidants

Fruits and vegetables contain ascorbic acid, tocopherols, carotenoids and phenolic substances. The capacity of a number of the phenolic compounds to behave as potent antioxidants has been documented (Mazza & Velioglu, 1992: 115). Cao and co workers (1996: 3426) reported that vegetables such as kales, beets, pepper, broccoli, spinach, shallots, potatoes, carrots and cabbages have high antioxidant activities. Beside antioxidant nutrients such as ascorbic acid, tocopherols and carotenoids, these vegetables are also an excellent source of polyphenolic components. Food preparation may influence the antioxidant content in fruits and vegetables, particularly constituents such as tocopherols, carotenoids, ascorbic acid and polyphenols (Wu *et al.*, 2004: 408). Table 2.1 summarises the main antioxidants in some fruit sources.

Table 2.1: Main antioxidants in fruits.

FRUIT	MAIN ANTIOXIDANTS	SUBCLASS
Mango	Catechins	Flavan-3-ols
	Myricetin	Flavonols
	Luteolin	Flavones
Apples	Epicatechin	Flavan-3-ols
	Quercetin	Flavonols
	Cyanidin	Anthocyanidin
Apricots	Catechins	Flavan-3-ols
	Epicatechin	Flavan-3-ols
	Quercetin	Flavonols
Pears	Cyanidin	Anthocyanins
	Epicatechin	Flavan-3-ols
	Quercetin	Flavonols
Peach	Catechins	Flavan-3-ols
	Epicatechin	Flavan-3-ols
	Cyanidin	Anthocyanidins
Grape	Naringenin	Flavanones
	Hesperetin	Flavanones
	Quercetin	Flavonols
Orange	Hesperetin	Flavanones
	Naringenin	Flavanones
	Luteolin	Flavones
Blackberries	Cyanidin	Anthocyanins
	Catechins	Flavan-3-ols
Cranberries	Cyanidin	Anthocyanidins
	Peonidin	Anthocyanidins
	Quercetin	Flavonols
Kiwi	Luteolin	Flavones
	Epicatechin	Flavan-3-ols
	Epigallocatechin-3-gallate	Flavan-3-ols
	Luteolin	Flavones
Pineapples	Kaempferol	Flavonols
	Myricetin	Flavonols
	Cyanidin	Anthocyanidins
Raspberries	Epicatechin	Flavan-3-ols
	Pelargonidin	Anthocyanidins
	Pelargonidin	Anthocyanidins
Strawberries	Catechins	Flavan-3-ols
	Cyanidin	Anthocyanidins
	Kaempferol	Flavonols
Tomatoes	Quercetin	Flavonols
	Catechins	Flavan-3-ols
Red wine	Epicatechin	Flavan-3-ols
	Naringenin	Flavanones

Compiled by author based on the USDA flavonoid database of selected foods (USDA, 2003).

2.6.1 Vitamin C

Consumers are continually becoming knowledgeable about the nutritional importance of vitamins. The words 'with added vitamin C' are familiar on food packaging signifying an added advantage. Vitamin C, a six-carbon lactone is synthesised from glucose in the liver of the majority of mammalian species with the exception of humans, non

human primates and guinea pigs (Nishikimi & Yagi, 1996: 17). Since humans lack the enzyme, L-gulonolactone oxidase which is essential for biosynthesis of vitamin C, they must obtain ascorbate from dietary sources (Padayatty *et al.*, 2003: 19). Vitamin C is required by humans for the synthesis of collagen and bile acids and also for maintenance of skin elasticity (Packer & Colman, 1999: 78). It also participates in the vital role of iron absorption and in resistance to infections. The chemical structure of vitamin C is shown in Figure 2.12.

Vitamin C is a water-soluble, chain breaking antioxidant which occurs primarily as a monovalent anion at physiological pH. It is commonly added to foods and certain beverages to preserve the colour and aroma. The strong antioxidant effects of vitamin C, which have been confirmed *in vitro* in numerous studies, are associated with its ability to donate electrons. Ascorbate can lose one electron to form semi-dehydroascorbate, an ascorbyl radical, a comparatively stable resonance stabilised radical of little reactivity. The loss of a second electron results in the formation of dehydroascorbate (Padayatty *et al.*, 2003: 19).

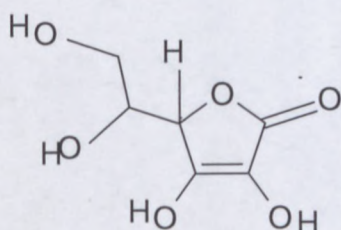


Figure 2.12: Chemical structure of vitamin C (Astley, 2003: 285).

Ascorbate participates in the essential function with the lipophilic antioxidant vitamin E of shielding the membrane from oxidative stress. Ascorbate is capable of regenerating vitamin E which is present in the membrane (Packer & Colman, 1999: 78). Ascorbate can decrease the tocopherol radical formed when vitamin E scavenges a lipid radical within the membrane. The tocopherol radical produced from the lipid radical can then be recycled back to tocopherol by ascorbate (Padayatty *et al.*, 2003: 20).

A significant feature of the antioxidant capacity of ascorbate is the capability of oxidised ascorbate to be recycled back to the reduced ascorbate. Glutathione is essential in the recycling of ascorbate by direct chemical reduction and by glutathione-dependent enzymes. Ascorbate has been known to participate in Fenton chemistry by reducing Fe (III) or Cu (II), yielding Fe (II) or Cu (I) and the ascorbyl radical. Iron (II) or Cu (I) can then catalyze the Fenton reaction with H_2O_2 , resulting in the production of hydroxyl radicals. Subsequently, the possibility that long-term mega doses of vitamin C

may cause oxidation in a living system should be taken into consideration (Carr & Frei, 1999: 1008).

Vitamin C is an essential water soluble antioxidant as it can scavenge for superoxide radical, singlet oxygen, hydrogen peroxide and hydroxyl radical directly (Packer & Colman, 1999: 78). According to available biochemical, clinical and epidemiological data, the current recommended daily allowance (RDA) for ascorbic acid is 100-120 mg/day. This RDA is enough to attain cellular saturation and optimum risk reduction of heart diseases, stroke and cancer in health individuals (Padayatty *et al.*, 2003: 22).

The vitamin C content in oranges or orange juices, ranges from 150 to 450 mg/l and one glass of orange juice can give about 30-80% of the recommended daily intake. Vitamin C has been shown to account for 65-100% of the antioxidant potential of beverages obtained from citrus but less than 5% of apple and pineapple juice (Gardner *et al.*, 1999: 472). According to Klimczak (2006: 319), the amount of vitamin C in diverse juices diminishes during storage depending on storage conditions such as temperature, oxygen and light.

2.7 Beverages as source of antioxidants

Beverages are a rich supply of flavonoids and other phenolics, which are considered important sources of dietary antioxidants (Cao *et al.*, 1996: 3426). Epidemiological studies have revealed that utilisation of phenol-rich beverages such as tea and wine, significantly reduces deaths arising from coronary heart diseases (Friedman & Kimball, 1986: 482; Cui *et al.*, 2002: 727). The protective effects of fruits and red wine consumption against coronary artery disease and certain types of cancers are to some extent accredited to the flavonoid content of these foods. *In vitro* and *in vivo* studies have confirmed that these phenolic compounds inhibit the oxidation of low density lipoproteins thereby offering important anti-atherogenic protection (Friedman & Kimball, 1986: 482; Knekt *et al.*, 1996: 479).

Phenolic compounds present in a variety of beverages have been the theme of numerous studies as a result of their possible benefits to human health (Wu *et al.*, 2004: 408). It has been established that various beverages e.g. wine, tea and fruit juices have strong antioxidant and radical scavenging activities due to their high levels of polyphenols. This adds to their ability to prevent certain diseases. Beverages, like fruit juices, are also rich in other antioxidants such as vitamin C (a strong immune booster) vitamin E and carotenoids. Although many experiments suggest strong *in*

in vitro antioxidant activity, intervention studies with vitamin C have not indicated any change in biomarkers of oxidation or clinical benefits (Langseth, 1993). Dose concentration studies of vitamin C have shown a sigmoidal association between oral dose and plasma and tissue vitamin C concentrations (Padayatty *et al.*, 2003: 29). Classification, structure and food sources of dietary flavonoids are listed in Table 2.2.

Table 2.2: Classification, structure and food sources of dietary flavonoids (Adapted from Heim *et al.*, 2002: 574).

Class	General structure	Flavonoid	Substitution Pattern	Dietary Sources	TEAC (mM)
Flavanol		(+)-catechin	3,5,7,3',4'-OH	Tea (<i>Camellia sinensis</i>) ⁶	2.4
		(-)-epicatechin	3,5,7,3',4'-OH	Tea ⁶	2.5
		Epigallocatechin gallate	3,5,7,3',4',5'-OH,3-gallate	Tea ⁶	4.75
Flavone		chrysin	5,7-OH	Fruit skins	1.43
		apigenin	5,7,4'-OH	Parsley, celery	1.45
		rutin	5,7,3',4'-OH, 3-rutinoside	Red wine ⁵ , buckwheat ⁷	2.4
		luteolin	5,7,3',4'-OH	citrus, tomato skin ⁸	2.1
		luteolin glucosides	5,7,3'-OH, 4'-glucose 5,4'-OH, 4',7-glucose	Red pepper ¹¹	1.74 0.79
Flavonol		kaempferol	3,5,7,4'-OH	Leek, broccoli, endives	1.34
		quercetin	3,5,7,3',4'-OH	grapefruit, black tea Onion, lettuce, broccoli tomato, tea, red wine berries, olive oil, appleskin	4.7
		myricetin tamarixetin	3,5,7,3',4',5'-OH 3,5,7,3'-OH,4'-OMe	Cranberry grapes, red wine	3.1
Flavanone (dihydroflavon)		naringin	5,4'-OH, 7-rhamnoglucose	Citrus, grapefruit	0.24
		naringenin	5,7,4'-OH	Citrus fruits	1.53
		taxifolin	3,5,7,3',4'-OH	Citrus fruits	1.9
		eriodictyol	5,7,3',4'-OH	Lemons ^{6,4}	1.8
		hesperidin	3,5,3'-OH,4'-OMe, 7-rutinoside	Oranges ⁹	1.08
Isoflavone		genistin	5,4'-OH, 7-glucose	Soybean ¹⁰	1.24
		genistein	5,7,4'-OH	Soybean ¹⁰	2.9
		daidzin	4'-OH, 7-glucose	Soybean ¹⁰	1.15
		daidzein	7,4'-OH	Soybean ¹⁰	1.25
Anthocyanidin		apigenidin	5,7,4'-OH	Colored fruits	2.35
		cyanidin	3,5,7,4'-OH,3,5-OMe	Cherry, raspberry, strawberry	4.42

2.7.1 Wines

The use of wine and its association with health has been reported from the earliest of civilizations. Wines have been used as a well-liked beverage and a remedy for more than 6000 years (Packer & Colman: 176). Indication of negative relationship between coronary heart disease death and wine consumption has indicated potential protective effects of wine (Hertog *et al.*, 1995: 382).

Researchers began taking a sober look at wine when numerous studies indicated that reasonable wine drinkers had a lower prevalence of heart diseases and some forms of cancers and lived longer than their teetotaler counterparts (Packer & Colman, 1999:

175). One such investigation in France, which became known as the French paradox, showed that even though the French eat a diet comparatively high in fats, and often smoke, they have one of the lowest rates of heart diseases in the world (Katalinic *et al.*, 2004: 593).

The putative good health of the French has been attributed to antioxidants compounds found in red wine and to a lesser amount in white wine and red grape juice (St. Leger *et al.*, 1979: 1018; Xia *et al.*, 1998: 384). This red wine consequence seems to be strongest amongst smokers (Packer & Colman, 1999: 176). Both red wine and red grape juices are outstanding sources of phenolic compounds such as anthocyanins, catechin, epicatechin and gallic acid.

Phenolic compounds in wines have been revealed to avert the development of blood clots, which is a major cause of both heart attacks and strokes in humans (Stanley & Mazier, 1999: 3). Phenolic compounds in wine prevent the oxidation of LDL cholesterol, which can lead to atherosclerosis and eventually to a heart attack. Wine drinkers are also less liable to develop macular degeneration, a foremost cause of blindness among people over sixty-five (Packer & Colman, 1999: 176).

The major phenolic compounds in wine comprise hydroxybenzoic acid, hydroxycinnamic acid derivatives, flavanols, flavonols and anthocyanins (Macheix *et al.*, 1990). The content of polyphenols in red wine is naturally higher than that of white wines. An elevated plasma and serum antioxidant capacity after consumption of red wine has been previously indicated (Modun *et al.*, 2008: 250).

Published studies on South African wines have been few and mainly concerned with the inhibitory activity of wine on either LDL peroxidation or microsomal lipid peroxidation. In one such study on antioxidant activity by De Beer *et al.* (2003: 902), the phenolic composition and free radical scavenging activity of 46 red and 40 white wines were evaluated using 2,2'-azinobis (3-ethylbenzothiazoline sulfonic acid) radical cation (ABTS⁺) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). Differences in phenolic contents of diverse types of grapes and vinification methods were shown to influence the phenolic composition of wines as fermentation process on grape seeds and skins. The methods of extraction and procedures for wine making and the chemical reactions that take place during the aging process also affect the phenolic contents of the wine (De Beer *et al.*, 2003: 906).

In their study on the influence of dilution and time on antioxidant activity of wines using

the ABTS⁺ method, Yu & Ong (1999: 218) found a linear association between antioxidant activity and concentration. The correspondence values increased when high concentration of phenols was not considered. The study also showed that antioxidant activity also depends on the time at which the decrease in absorbance is measured. Some researchers use a predetermined time point and others observe the reaction kinetics consequently in the study of antioxidant capacity, thus, there is lack of agreement regarding the most favourable time (Villano *et al.*, 2004: 502). This study used three different assays for measurement of total antioxidant capacity, which take into consideration these factors. Villano *et al.* (2004: 508) set up the optimum dilutions for white wines as a function of their total phenolic contents. Data regarding human plasma levels of polyphenols after drinking wine is lacking (Tedesco *et al.*, 2000: 115).

2.7.2 Tea

Tea is not only the world's most accepted beverage, it is also one of the world's healthiest (Packer & Colman, 1999: 180). There are three kinds of tea made from the tea plant (*Camellia sinensis*) i.e. green tea which is non-fermented, black tea which is fully fermented and oolong tea which is partially fermented. All these types contain antioxidants, mostly polyphenols, but are the highest in green tea. Frequent intake of tea has been linked with enhanced antioxidant status *in vivo* that could play a role in reducing the development of certain types of cancers, coronary heart disease, atherosclerosis, stroke, reduced mutagenicity, inflammation, protection against neurodegenerative diseases and increasing insulin sensitivity (Geleijnse *et al.*, 1999: 2171; Gupta *et al.*, 2002: 37).

A number of mechanisms to explain the valuable properties of tea have been proposed. These comprise binding to carcinogens, modulating the expression of antioxidant enzyme genes and inhibiting heterocyclic amine formation, reduction of protein kinase activity and modulation of inflammatory and neurological responses (Yang & Landau, 2000: 2409).

2.7.2.1 Green and black tea

Investigation into the alleged health effects of tea has confirmed the involvement of phytochemicals, predominantly phenolic acids, flavonoids, catechins and other flavanol-derivatives (Brannon, 2007: 5). The composition of tea differs with the season's age of leaf, climate and horticultural practices (Mukhtar & Ahmad, 2000: 1698). The three forms of tea differ in production processes, which influence their

chemical constituents and taste. A characteristic prepared green tea beverage was shown to contain 30-42% catechins by weight. Catechins, theaflavins and thearubigins make 3-10%, 2-6% and >20% respectively, of the water-extractable material by weight in black tea (Lambert & Yang, 2003: 202). Tea variety, weight of tea or tea bags and brewing technique affect the estimation of flavonoids. The most important type of catechins in green tea is epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC). Also present in smaller amounts are galocatechin (GC), epigallocatechin (EGC) digallate, 3-methylepicatechin gallate, catechin gallate, and galocatechin gallate (GCG) (Feng *et al.*, 2002: 214).

At present there is no compositional database of antioxidant activity of green and black teas. In their study on the effect of green tea on oxidative stress found that after drinking tea, a considerable decrease of lipid peroxidation, free radical production and oxidative stress within erythrocytes occurs, consequently protecting the individual from oxidative stress related diseases.

In general, fresh green tea leaves contains 36% polyphenols among which catechins prevail. Epigallocatechin gallate is the more dominant antioxidant of the catechins family *in vitro* and is the most abundant polyphenol in green tea (Brannon, 2007: 5; Rice-Evans, 1999: 227; Feng *et al.*, 2002: 214).

The flavonoid content of tea, in beverage form, differs according to the type of tea, blended, decaffeinated, herbal or instant, and the tea preparation itself (amount of tea used, brewing time, and water temperature). Brewed hot tea contains the highest concentration of flavonoids; instant tea contains an even lower concentration, while iced and ready-to-drink tea contains the lowest concentration. The brewing time influences the antioxidant potential of tea.

Many studies involving humans, have investigated the relationship between drinking tea and health. Various *in vitro* and *in vivo* studies have indicated that the flavonoids in green tea and black tea are efficient against free radicals or ROS. In their study, Cao *et al.* (1996: 3429) found that green tea and black tea had higher ORAC values against a peroxy radical than garlic, kale, spinach and brussels sprouts, the most potent vegetables in terms of ORAC values. Green tea was found to have a higher antioxidant potential than black tea using the FRAP assay (Langley-Evans, 2000: 187). Tea polyphenol binds strongly to proteins in more than one location. They bind strongly to proteins especially those with high contents of amino acid, proline such as milk caseins, salivary proline and gelatine. This strong binding is considered to add to the

inhibition of certain enzymes implicated in carcinogenesis. Though there have been many discrepancies, it can be safely concluded that tea drinking is linked with safety from heart disease and cancer and that it has antifungal and antibacterial effects (Brannon, 2007: 2).

2.7.2.a Black tea

Black tea is obtained, when tea leaves (*Camellia sinensis*) are fully fermented by microorganisms and enzymatic oxidation in the production processes (Brannon, 2007: 2). About 75% of catechins contained in the leaves of black tea undergo enzymatic transformation consisting of oxidation and partial polymerisation (McKay & Blumberg, 2002: 2). The primary catechins in black tea include theaflavins and thearubigins. During the process of making black tea the catechins are converted to theaflavins and thearubigins, the phytochemicals that give black tea and oolong tea their characteristic colour and flavour. The approximate black tea beverage components of solid extracts are: catechins (10-20%), theaflavins (3-6%), thearubigins (12-18%), flavonols (6-8%), phenolic acids and depsides (10-12%). Components of black tea whose antioxidative properties have been explicitly proved are catechin, EC, EGC, ECG and EGCG (Mukhtar & Ahmad, 2000: 1699).

2.7.2.b Ice teas

As consumers continue searching for healthier options, real brewed ice tea is inherently becoming an excellent option for them due to their antioxidant content. Ice teas are refreshing soft drinks made with real tea, blended with water and fruit flavours (Henning *et al.*, 2003: 226). Since they are made from real tea leaves they are a source of antioxidants especially flavonoids. Ice teas come in different flavours such as mango, apple, citrus, lemon, apple and peach. The amount of antioxidants in ice teas also varies according to the flavours (Henning *et al.*, 2003: 234).

2.7.4 Herbal teas (rooibos and honeybush)

Rooibos is a herbal infusion obtained from a native South African plant, *Aspalathus linearis* (Morton, 1983: 164). Its confirmed antioxidant properties and its caffeine free nature have made it popular internationally (Joubert *et al.*, 2008: 376). Rooibos and honeybush another indigenous herbal tea (*Cyclopia intermedia*), contain an intricate mixture of polyphenolic compounds of which aspalathin (a dihydrochalcone), is common in rooibos and mangiferin (a xanthone) in honeybush (Ferreira *et al.*, 1998:

3407; Von Gadow *et al.*, 1997: 633; Rabe *et al.*, 1994: 1560). Rooibos tea has been used as a traditional cure for infantile colic, allergies, asthma and dermatological problems since the early 1900's (Joubert *et al.*, 2008: 378).

Green Rooibos and the traditional "red bush" rooibos are produced differently thus giving them diverse flavours. In the case of red rooibos the leaves and stems are picked, bruised and left to ferment naturally to give its unique colour and aroma. With Green rooibos natural fermentation and oxidation processes are stopped after harvesting by steaming or heating, rolling and drying (Montego tea).

A number of studies on herbal teas have been done but so far very few have quantified the antioxidant capacity or compiled a database there of. In most of these studies, fermented rooibos has been used. Fermented rooibos has been shown to scavenge the physiologically relevant ROS, superoxide radical anion, hydroxyl radical and exhibit protective effects against oxidative stress caused by hydrogen peroxide radicals (Joubert *et al.*, 2004: 133; Lee & Jang, 2004: 285). In a number of human studies, rooibos has been shown to have health benefits. In one such study, rooibos was shown to decrease the incidence of disease within 2-3 days in patients with a viral infection (herpes simplex) after ingestion of a dilute infusion of this herbal tea. The infusion also decreased the itching sensation in another group of patients with atopic dermatitis with similar effects on steroid- induced dermatitis (Shindo & Kato, 1991: 385).

The effect of rooibos on the antioxidant status of blood plasma after a wash out period of 1 week has been studied by Sauter (2004). After receiving a twice daily oral dose of 250 mg extract of rooibos in tablet form for two weeks and with a restricted diet to ensure a low flavonoid intake during this time, no significant changes in blood parameters or antioxidant status of the plasma of the 20 participants as measured by ABTS^{•+} and Cu²⁺- induced low-density lipoprotein oxidation test system was demonstrated. An 8-week randomised placebo-controlled intervention study conducted on workers occupationally exposed to lead to monitor the effect of drinking rooibos on their antioxidant status indicated a decreased lipid peroxidation level and an increased GSH concentration but no effect on the lead levels (Nikolova *et al.*, 2007: 120). Subsequent studies are in progress to further evaluate the health benefits of rooibos.

2.7.4 Coffee

Coffee, a drink prepared by extraction in boiling water of ground roasted beans, is recognised for its tonifying merits, its stimulating effects on the nervous system, enhancement of perception and reduction of fatigue (Gonzalez *et al.*, 2004: 133). These actions are linked to caffeine intake. Antioxidant properties of coffee have been linked to prevention of diabetes, arteriosclerosis, neurodigestive diseases and cancers (Health, 2009). Coffee improves gastric secretion and urine production, may decrease serum uric acid concentration, lessen the danger of acquiring gallstone disease, inhibit histamines, immediate type allergic reactions and decrease D-galactosamine induced liver injury (Gonzalez *et al.*, 2004: 133). In recent times, the foremost use of coffee has been for lessening of drowsiness and fatigue and mental alertness but lately, research findings on its antioxidant activity have amplified interest in the product (Parras *et al.*, 2007: 582). These health benefits of coffee are associated with the phenolic compounds contained in coffee, predominately chlorogenic acids such as caffeic acid, ferulic acid, p-coumaric acid and caffeoylquinic acid (Clifford & Knight, 2004: 458).

2.7.5 Fruit juices

The high levels of flavonoids in apples gives credibility to the popular assertion that an 'apple a day keeps the doctor away'. Citrus fruits including grapefruit, oranges, lemons, limes tangelos and tangerines have been acknowledged as strong cancer fighters (Steinmetz, *et al.*, 1996: 1027). Well recognised for being excellent sources of vitamin C, and various phytochemicals, fruits contain flavonoids and lycopene, which may protect against prostate cancer in men and breast cancer in women (Bub *et al.*, 2003: 93). Red grape juice has been revealed to protect against heart disease, comparable to red wine (Packer & Colman 1999: 176). Encouraging health benefits of the use of red grape juice, such as the enhancement of the endothelial function, boost of the serum antioxidant capability, defence of low dietary lipoproteins against oxidation, reduction of native plasma protein oxidation and reduction of platelet aggregation, have also been reported (Chou *et al.*, 2001: 555).

Fruit juice consumption has been shown to modulate the antioxidant status in healthy volunteers, reduce the level of oxidative DNA damage and enhance immune functions (Bub *et al.*, 2003: 93). However inadequate information exists on the absorption, distribution, and excretion of polyphenols in humans and especially about the bioavailability of polyphenols from fruit juices in humans.

Little research on antioxidant activities of fruit juices in South Africa has been done, if

at all. There is no information regarding antioxidant properties of South African fruit juices. Many manufacturers place high vitamin C contents on labels of juices as it is one of the parameters used to assess their legitimacy. Nevertheless, at some point in storage and in particular elevated temperatures and occurrence of heavy metals, it breaks down and consequently regular measurement of vitamin C in fruit juices is imperative for substantiating the dietary claims.

2.8 Compositional database

A food compositional database is a list, a catalogue or collection of nutritional data information about a particular food type, which is organized so that it can easily be accessed, managed, and updated. The recognition of the involvement of diet in the development of many diseases (McGovern, 1977) has led to an expansion in the number and range of studies of the relationship between diet, health and disease. This has led to a greater focus on nutrient data. Willett (1998) has drawn attention to this and to the need for databases to be reviewed regularly.

“Knowledge of the chemical composition of the foods is the first essential step in dietary treatment of disease or in any quantitative study of human nutrition” (McCance & Widdowson, 1964). Compositional databases are used in research studies to determine the properties of diet on health, reproduction, growth and development. They can also be used for devising diets with specific nutrient composition in clinical practice, in the formulation of ration scales and in the devising of emergency food supplies. On a national scale and globally, compositional databases are used in the evaluation of nutritional value of food consumed by individuals and populations (Greenfield & Southgate, 2003: 2).

Evidence arising from recent epidemiological studies has resulted in a growth in the production of national and international guidance on choosing a healthy diet. Compositional databases provide a foundation for the development of education programmes by choosing healthy diets. As part of this guidance to consumers many governments require nutrition labelling of foods. It is a requirement in some countries that producers of food products show their analytical and compositional data of their products. However, most countries allow the use of compositional data taken from an authoritative compilation, such as a national food composition database, as a substitute for direct analysis. This development has added a quasi-regulatory role to food composition databases and strengthens the need for maintenance of data quality in terms of both the representativeness of the samples and the quality of the analytical

data (Greenfield & Southgate, 2003: 2).

2.8.1 Criteria for a comprehensive food composition database

A good food composition database should meet the following criteria: the data should be representative, of sound analytical quality, a comprehensive coverage of foods, and nutrients (Greenfield & Southgate, 2003: 4). The data should also be compatible with other databases, easy to use and understand and should have few missing data. However foods, being biological materials even the best compiled database show disparities in composition and hence it cannot accurately forecast the composition of any given single sample of a food. A database can only give an estimate of the composition (Greenfield & Southgate, 2003: 14). Initially food databases were based on analysis in the laboratories of researchers but later researchers started compiling databases from scrutinized data produced by recognised laboratories. Southgate (1974: 6) identified two methods of compiling database and described them as the direct method and the indirect method.

2.8.2 Direct method

This is based on the analysis of samples in the laboratories of researchers whereby strong control of the sampling, analysis and quality control procedures yields highly dependable data. However, the direct method is expensive and time-consuming, and adds pressure on the analytical resources available in many parts of the world (Greenfield & Southgate, 2003: 6).

2.8.3 Indirect method

The indirect method of data compilation which utilise data obtained from published literature or unpublished laboratory reports does not guarantee the quality of the data and great care must be taken when appraising the data to be included in the databases. Some values may be imputed or calculated or even taken from other tables or databases and it is impossible to refer back to the original source. These values will have a low degree of confidence. Although the indirect method requires less in terms of analytical resources than the direct method, the indirect method is generally employed when analytical resources are inadequate, or the food supply is largely drawn from food imported from other countries where compositional data are available (Greenfield & Southgate, 2003: 6).

2.8.4 Combination method

This is the most cost effective method of compiling compositional databases. It combines both direct and indirect methods and contains original analytical values together with values taken from literature and other databases as well as calculated and imputed values. A large amount of modern food databases have been compiled using this method (Greenfield & Southgate, 2003: 6).

2.8.5 Sampling

The quality of the sampling of foods to be included in the composition database and analytical data will greatly determine the quality of the database. Sampling is a very difficult phase of database preparation and requires the compilers to make spontaneous decision and concessions. The sampling method should be able to give a representative sample in time and space of the food to be used in the database. The selection of a representative sample should be based on the knowledge of the nature and population of the sample being studied (Greenfield & Southgate, 2003: 63).

The major sources of food samples for analysis for a composition database depending on their intended use include, bulk commodities, wholesale commodities, retail food, field or garden foods and foods as consumed (Greenfield & Southgate, 2003: 69). Retail foods constitute the main source of foods included in food composition databases and the major concern of the sampling protocol is to ensure that the complete range of sales outlets is represented. Since food varies in composition, the approach to sampling and the design of the sampling and analytical protocol should take into consideration other factors like: geographical samples (diversity of soil and climate), seasonal samples, physiological state and maturity, and cultivars and breeds (Greenfield & Southgate, 2003: 69).

2.8.6 Sampling methods

The main sampling methods applicable to food composition database are: stratified, random, selective and convenience sampling methods.

2.8.7 Stratified sampling method

This is whereby the population of the food samples is classified into strata based on the most important causes of variation e.g. regions, seasons or retail sale points. It is the most suitable method for use on composition database work (Greenfield & Southgate, 2003: 70).

2.8.8 Random sampling method

This is whereby sample collection ensures that every item in the population of the food being sampled has an equal chance of being collected and incorporated into the samples to be analyzed. It is a theoretically ideal method of sampling, but practically difficult to achieve (Greenfield & Southgate, 2003: 70).

2.8.9 Selective sampling method

This is whereby samples are taken according to a sampling plan that excludes material with certain characteristics used in the analysis or selects only those with defined characteristics. It is commonly used in the analysis of contaminants and only with caution for database work (Greenfield & Southgate, 2003: 70).

2.9.0 Convenience sampling method

This is whereby the samples are taken on the basis of expediency, cost or other reasons not directly related to sampling parameters. It is rarely suitable for database work. It is essential to observe that in all the above methods the compositional data obtained can only be an approximation of the composition of the food and all have limitations imposed by the variation in the composition of foods. However, some form of stratified sampling method even where there is no evidence of regional differences in composition still remains the method of choice (Greenfield & Southgate, 2003: 71).

2.9.1 Experimental procedure

After sampling, the analytical sample should be aliquoted and stored at -40°C until the day of analysis. It is desirable to store a number of identical analytical samples as a range of analytical procedures will be performed requiring a different portion of the sample over a considerable time period.

2.9.2 Existing databases

In recent years antioxidants have attracted a lot of interest from medical researchers and nutritionists because of their possibility to prevent chronic and degenerative diseases e.g. cancer and cardiovascular disease and aging (Young & Woodside, 2001: 181). There are numerous antioxidant compounds in a majority of foods and the total antioxidant capacity of a given food is the integrated action from the various compounds. It is critical to bear in mind the likely relations and synergism of the components of a given food when evaluating their total antioxidant activity and health promotion effects (Wu *et al.*, 2004: 408).

Wu *et al.* (2004: 407) has compiled an ORAC_{FL} database of 28 food samples from four regions during two different seasons representing ordinary foods in the human diets in the USA. In this study fruits, vegetables, nuts and dried fruits were sampled from retail outlets in 12 cities around the USA in two different seasons. Other large scale analyses of food to evaluate their antioxidant capacities using different methods and radical sources have been done (Pellergrini *et al.*, 2003: 2813). An example of a database of the TAC of 28 foods from two different seasons is shown in Table 2.3.

Table 2.3: An example of database of TAC of 28 foods from two different seasons (Wu *et al.*, 2004:413).

Table 3
Lipophilic (L-ORAC_{FL}), hydrophilic (H-ORAC_{FL}), and TAC of 28 foods from two different seasons^a

Sample name	Moisture (%)	L-ORAC _{FL} (μmol TE/g)		Mean (μmol TE/g)	H-ORAC _{FL} (μmol TE/g)		Mean (μmol TE/g)	TAC (μmol TE/g)	L-ORAC _{FL} /TAC (%)
		Pass 1 ^b	Pass 2 ^b		Pass 1	Pass 2			
<i>Fruits</i>									
Honeydew	90.0	0.09±0.03	0.12±0.07	0.11±0.05	3.09±0.48	1.51±0.32*	2.30±0.92	2.4	4.5
Cantaloupe	89.7	0.06±0.01	0.21±0.02*	0.15±0.08	3.59±0.30	2.51±0.20*	2.97±0.62	3.1	4.8
Plum	86.7	0.24±0.08	0.10±0.03	0.17±0.10	58.96±18.17	65.47±24.02	62.22±20.22	62.4	0.3
Watermelon	91.7	0.16±0.04	0.22±0.04	0.19±0.04	1.10±0.11	1.37±0.07*	1.23±0.17	1.4	13.4
Kiwi	83.1	0.34±0.04	0.14±0.03*	0.24±0.12	8.84±2.52	8.31±1.38	8.58±1.90	9.2	2.6
Navel orange	85.8	0.18±0.02	0.39±0.10*	0.29±0.13	15.10±1.93	20.61±3.08*	17.85±3.79	18.1	1.6
Grapefruit	88.3	0.41±0.09	0.29±0.07	0.35±0.10	12.19±0.90	18.06±1.58*	15.13±3.36	15.5	2.3
Strawberry	90.6	0.57±0.13	0.14±0.06*	0.36±0.25	33.69±3.95	37.13±4.29	35.41±4.24	35.8	1.0
Blueberry	83.9	0.52±0.10	0.21±0.05*	0.36±0.18	62.29±6.01	61.38±10.18	61.84±7.75	62.2	0.6
Rasperry	85.1	2.01±0.13	0.79±0.39*	1.62±0.66	48.85±8.79	45.24±2.92	47.65±7.18	49.3	3.3
Avocado	71.5	4.20±0.92	6.77±1.69*	5.52±1.85	13.41±5.06	14.22±1.98	13.81±3.58	19.3	28.6
<i>Vegetables</i>									
Tomato	94.5	0.26±0.07	0.22±0.07	0.24±0.01	2.95±0.21	3.26±0.94	3.13±0.69	3.4	7.1
Radish	95.6	0.36±0.09	0.18±0.06*	0.26±0.12	8.43±1.38	9.92±0.94	9.28±1.31	9.5	2.7
Letuce, Iceberg	95.7	0.29±0.10	0.36±0.11	0.33±0.10	7.12±0.45	1.98±0.67*	4.18±2.80	4.5	7.3
Celery	95.0	0.45±0.04	0.36±0.05*	0.41±0.07	5.37±0.85	5.29±3.01	5.33±2.05	5.7	7.1
Potato, Russet	78.3	0.42±0.16	0.60±0.02	0.51±0.14	11.86±2.42	13.57±2.61	12.72±2.28	13.2	3.9
Carrot	88.0	0.59±0.14	N/A ^c	0.59±0.14	11.56±1.79	N/A	11.56±1.79	12.2	4.9
Baby carrot	90.0	0.74±0.14	0.86±0.28	0.81±0.22	4.50±1.84	2.84±0.74	3.55±1.48	4.4	18.6
Broccoli	90.5	1.68±0.13	1.77±0.33	1.72±0.24	13.85±2.45	14.51±1.86	14.18±2.04	15.9	10.8
<i>Nuts</i>									
Almond	N/C ^d	2.06±0.18	1.38±0.49*	1.72±0.50	41.64±11.23	44.01±6.88	42.82±8.71	44.5	3.9
Macadamia	N/C	2.15±0.53	2.88±0.37	2.52±0.57	14.86±3.37	14.01±0.75	14.43±2.31	17.0	14.9
Pecan	N/C	4.26±1.34	4.07±0.64	4.16±0.98	172.39±7.62	178.26±13.08	175.24±10.36	179.4	2.3
Pistachio	N/C	3.01±0.40	5.18±1.21*	4.25±1.46	72.00±4.80	78.26±13.51	75.57±10.50	79.8	5.3
Cashew	N/C	4.16±1.82	5.17±1.02	4.74±1.38	15.08±2.96	15.34±1.56	15.23±2.04	20.0	23.7
<i>Dried fruits</i>									
Date	N/C	0.46±0.14	0.22±0.04*	0.32±0.16	37.47±2.11	39.50±3.91	38.63±3.21	39.0	0.8
Raisin	N/C	0.28±0.09	0.42±0.14	0.35±0.13	33.34±4.98	26.69±3.09	30.02±5.23	30.4	1.2
Prune	N/C	2.28±0.27	1.30±0.11*	1.79±0.56	82.01±14.68	85.98±20.35	83.99±16.56	85.8	2.1
<i>Grain brans</i>									
Rice bran	N/C	154.70±3.58	N/A	154.70±3.58	88.17±1.52	N/A	88.17±1.52	242.9	63.7

^aDifferences between Pass 1 and Pass 2 (sampling times) were significantly different ($P < 0.05$).

^bData were expressed as fresh weight (FW) or usual form of consumption.

^cMean values for Pass 1 and Pass 2 represent duplicate analyses of samples from 4 different regions of the US.

^dN/A, not available.

^eN/C, not calculated.

2.9.3 *In vivo* effects of antioxidants

The excess production of oxygen-derived free radicals is implicated in the onset and prognosis of many diseases such as cancer, rheumatoid arthritis atherosclerosis and degenerative disease associated with aging process (Vives Corrons *et al.*, 1995: 328). Reactive oxygen species have also been implicated as the cause of damage to the red blood cell's (RBC) in sickle cell anaemia, β -thalassemia and other haemoglobinopathies (Scott *et al.*, 1993: 1707; Vives Corrons *et al.*, 1995: 328; Rice-Evans *et al.*, 1986: 266).

Though enough evidence from human epidemiological and animal studies supporting the beneficial effects of consuming polyphenol rich beverages exists, only recently have studies been published indicating the presence of polyphenols in human plasma at concentrations that range from 0.5 to 1.6 μ M (Paganga & Rice-Evans, 1997: 78). The chemical structure of polyphenols influences their metabolism, absorption and hence their bioavailability. The human RBC's being the oxygen carriers with high

concentrations of haemoglobin and polyunsaturated fatty acid contents in their membranes are at risk of oxidative damage. Methemoglobin and ferylhemoglobin, oxidized forms of haemoglobin produced from a reaction with hydrogen peroxide, are potent promoters of oxidative damage involved in the aging process and in pathological conditions (Santos-Silva *et al.*, 2001: 119).

The information on bioavailability, absorption, distribution, metabolism, and excretion of polyphenols is sketchy, as few *in vivo* examinations have been completed. Of the majority of polyphenols ingested, about 75% does not appear in the urine. It is postulated that they are moreover absorbed via the gastro intestinal tract, absorbed and excreted in bile, or metabolized by the colonic microflora or other body tissues (Brannon, 2007: 4). It is acknowledged that to sustain high plasma levels, polyphenols need continual intake over time (Scalbert & Williamson, 2000: 2074).

Ruel *et al.* (2005: 860) in their study on changes in plasma antioxidant capacity after short term cranberry juice consumption showed that consumption of the juice was linked with considerable rise in plasma antioxidant capacity and reduction of circulating LDL concentration. Fruit juice consumption has been reported to improve the antioxidant status, reduce the levels of oxidative DNA damage in lymphocytes and enhance immune functions in a healthy human intervention study (Bub *et al.*, 2002: 90).

In another study on the effect of green tea on oxidative stress, Coimbra *et al.* (2006: 793) found a significant reduction in serum levels of malondialdehyde (MDA) an important biomarker of lipid peroxidation. They also found a reduction in oxidative stress within RBC'S, indicated by significantly lower membrane bound haemoglobin (MBH) and a rise in plasma antioxidant capacity. This suggested an overall beneficial effect, by reducing development or enhancement of oxidative stress and thus protection against oxidative stress associated diseases.

Henning *et al.*, (2004: 1560) have also shown that flavanol absorption is improved when tea polyphenols were administered as green tea supplements and led to a little yet important increase in plasma antioxidant activity when compared to tea polyphenols consumed as black or green tea. This was an intervention study comparing the pharmacokinetic disposition of tea polyphenols and their effect on the antioxidant capacity of plasma 4 hours after consumption of either green tea, black tea or green tea extract supplement.

Another study to examine the association between plasma levels of polyphenols and

antioxidant activity of red and white wine in twenty health subjects showed a significant reduction in urinary Prostaglandin F-2 α -III, a marker of oxidative stress. There was a higher percentage decrease in subjects consuming red wine than those consuming white wine. The subjects taking red wine displayed higher plasma polyphenols levels than those consuming white wine (Pignatelli *et al.*, 2005: 80).

In a study on the impact of the ingestion of 300 mL of black and green tea, alcohol-free red wine, alcohol-free white wine, or water on plasma total antioxidant capacity in five healthy volunteers, Serafini *et al.* (2000: 587) showed an increase in plasma antioxidant capacity values in those who drank green tea at 30 minutes and a peak of 50 minutes for those who consumed black tea and red wine. They also indicated that red wine and green tea were the most effective in protecting low density lipoproteins from oxidation by peroxy and ferril radicals respectively.

In a crossover intervention study to investigate whether the non-alcoholic constituent of wine intensify plasma antioxidant capacity (measured as total radical trapping antioxidant parameter or TRAP) and whether such an effect is associated with the presence of phenolics compounds in plasma, Serafini *et al.* (1998:1005) showed that the intake of alcohol-free red wine significantly increased plasma TRAP values and polyphenol concentrations 50 minutes after ingestion and decreasing after 2 hours. Alcohol-free white wine and water had no effects on either of the plasma values.

Modun *et al.* (2008: 250) using red wine, de-alcoholised red wine, polyphenol-stripped red wine, ethanol-water solution and water investigated the role of wine polyphenols and induction of plasma urate elevation on plasma antioxidant capacity in a crossover human intervention study. After plasma antioxidant capacity, ethanol, catechins and urate concentrations were determined before and at 30, 60, 90, 120 and 180 minutes after the ingestion of the beverage. De-alcoholised red wine and polyphenol-stripped red wine showed similar increases in ferric reducing antioxidant potential (FRAP) values which was almost half the effect the original red wine showed. The results indicated that consumption of red wine involves two separate mechanisms in increasing plasma FRAP values and both wine and plasma urate contribute to the increase in FRAP values effect (Modun *et al.*, 2008: 253).

Only a few studies have been done to investigate the antioxidant effects of coffee consumption in humans. Natella *et al.* (2002: 6213) showed that drinking 200 mL of coffee significantly increased the plasma antioxidant capacity measured as TRAP. In another study on 10 healthy male participants, Nardini *et al.*, (2002: 5738), showed that

coffee consumption also significantly increased total plasma caffeic acid concentration.

It is imperative to note that polyphenols that are common in human diets are not necessarily the most active and bioavailable within the body. This can be attributed to their poor absorption in the intestines, metabolism, excretion and their intrinsic activity. The metabolites that are found in the blood and target organs and that result from digestive or liver activity may differ from the original substances in terms of biological activity. Thus knowledge of the bioavailability of polyphenols is vital if their putative health effects are to be understood. Polyphenols are capable of penetrating tissues especially those in which they are metabolised, but their ability to accumulate within specific tissues need to be investigated further. After consumption of polyphenols, the plasma concentration differs highly depending on the type and the source of food (Brannon, 2007: 4).

The few studies that have measured the *in vivo* effect of various antioxidant rich beverages on antioxidant capacity of plasma have produced conflicting findings. These findings range from immediate effects, no effects to mild immediate effects on antioxidant capacity after ingestion of various beverages (Serafini *et al.*, 2000: 586; McAnlis *et al.*, 1998: 202; Hodgson *et al.*, 2000: 1103). However, the universal observation is a rise in antioxidant capacity during the first or the second hour after intake, and then a decline in antioxidant capacity four to five hours after ingestion of the beverage (Serafini *et al.*, 1996: 29).

The absolute *in vitro* antioxidant capacity of compounds in beverages is often interpreted as their *in vivo* antioxidant function in the tissue. Such interpretations are more often than not misleading as have no direct correlation and hence are not very significant (Papas, 1999: 1005). This study proposed to investigate this phenomenon by establishing the effect if any the beverages has on the antioxidant capacity of plasma and erythrocytes on human volunteers. At the moment and to the best of my knowledge there is no study that has looked into the short term *in vivo* effects of rooibos on the plasma antioxidant capacity in South Africa.

CHAPTER THREE

RESEARCH DESIGN AND METHODOLOGY

3.1 Study design

This chapter describes the research design and methodology used for the research project. It also describes the assays used in the analysis, the sampling methods, preparation of the samples as well as the recruitment criteria for the participants of the short term pilot study.

This research was experimental based, and was divided into two phases. The first phase involved the measurement of the antioxidant content and total antioxidant capacity (TAC) of South African beverages and compilation of a database. The database was compiled according to the guidelines provided by Greenfield and Southgate (2003). The second phase of the study was a short term human intervention study involving the investigation of the *in vivo* effect of rooibos on plasma. The study protocol for the second phase of the study was approved by the Ethics Committee of the Health and Wellness Sciences Faculty of the Cape Peninsula University of Technology, and written informed consent was obtained from each volunteer. Figure 3.1 summarises the analytical methods used in the *in vitro* and *in vivo* phases of the study.

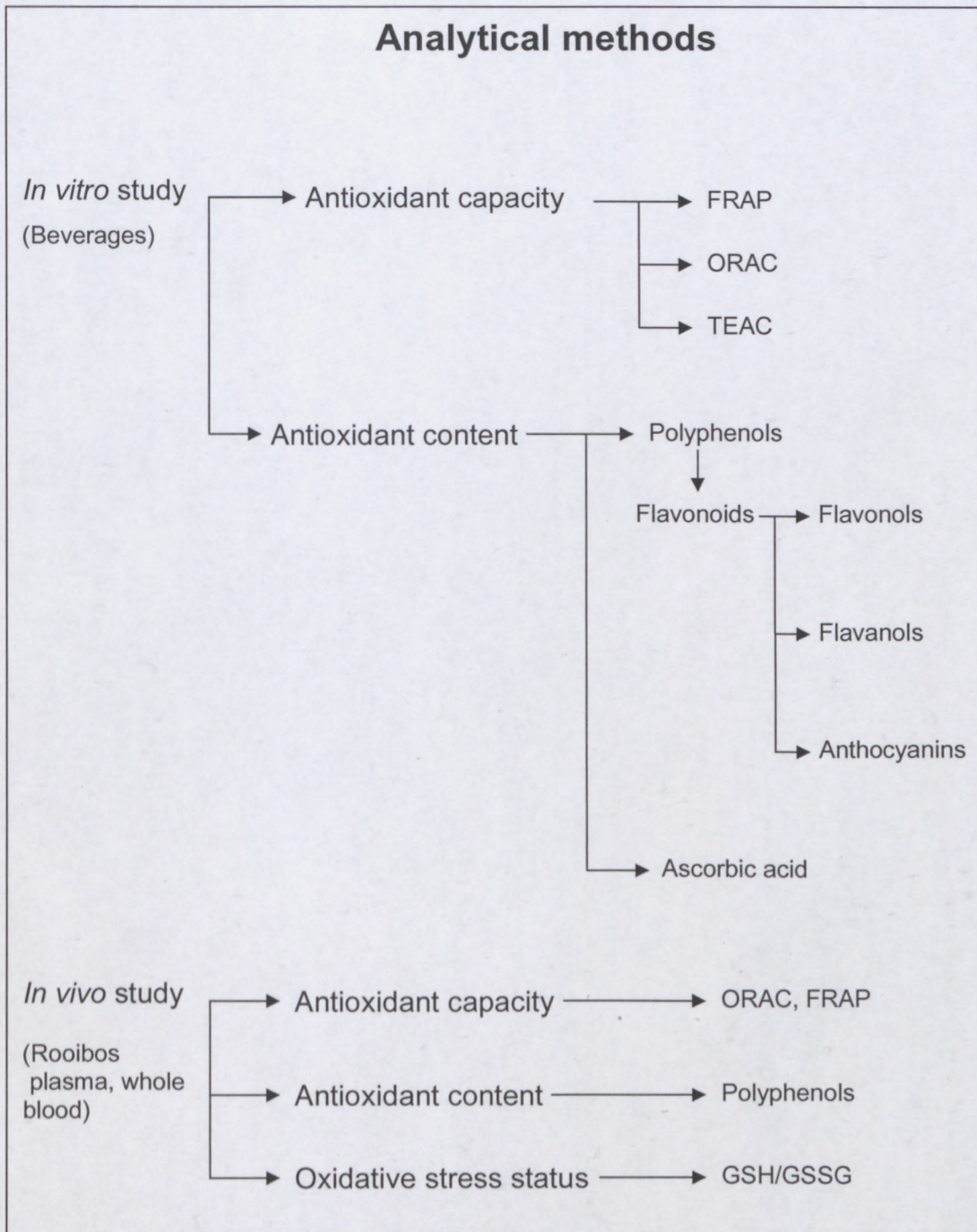


Figure 3.1: Flow diagram of study design.

3.1.2 *In vitro* study area, sampling and preparation of samples

Fruit juices were purchased from supermarkets (depending on their availability) in the Cape Town region. All the varieties of fruit juices (48 in total) from four leading brands and ice teas (six in total) from one leading brand were collected. Thirteen red wines and seven white wines were sampled from four farms (two wineries in Paarl, one in Stellenbosch and one in Franschhoek) in the Western Cape wine region. Three leading instant coffee brands as well as one pure coffee brand were purchased from supermarkets. Four leading black tea brands as well as one rooibos brand were also purchased from these supermarkets.

Tea was prepared by adding 180 mL boiling water to 2.5 g tea leaves and steeping it for three or five minutes. Coffee was prepared by adding 180 mL boiling water to 2.5 g coffee. Fruit juices and wines were used as purchased. In order to ensure that the beverages retained their antioxidant capacity, all samples were aliquoted and stored in a -40°C freezer to preserve the antioxidant capacity and content and to prevent autoxidation.

3.1.3 Database development

3.1.3.a Procedure

The database was compiled according to the guidelines provided by Greenfield and Southgate (2003). When opened, the wines were frozen at -40°C to preserve the phenolic content until analysis could be done. The samples were diluted appropriately and their total antioxidant capacity measured using the FRAP, ABTS and ORAC_{FL} assays. The antioxidant content of the individual antioxidants was measured using various assays including total polyphenols, flavanols, flavonols, vitamin C, and anthocyanins.

Various researchers make use of a predetermined reaction endpoint whereas others use the reaction kinetics when measuring the total antioxidant capacity of samples. Consequently there is no agreement regarding the most favourable time (Papas, 1999: 1005). This research proposed to use three different assays, taking into consideration these factors. ORAC_{FL} assay was chosen since it combines both delay

of oxidation and amount of inhibition of oxidation into one parameter, which is measured as the area under the curve. The sub groups of polyphenols in the sample were characterised as much as was possible using specific assays for the individual antioxidants content.

The absolute *in vitro* antioxidant capacity of compounds in beverages is often interpreted as their *in vivo* antioxidant function in the tissue (Papas, 1999: 1005). Such interpretations are usually inaccurate and have no direct correlation or are not very meaningful. This study proposed to investigate this phenomenon by establishing the effect that consumption of a polyphenol-rich beverage has on certain chemical parameters in plasma and erythrocytes of human volunteers.

3.2 Measurement of antioxidant capacity

3.2.1 Oxygen radical absorbance capacity (ORAC) assay

3.2.1.a Introduction

Cao and co-workers (1993: 303) set up a method to directly measure the oxygen radical absorbance capacity of antioxidants. The ORAC method was further modified in 2001 using a fluorescein salt instead of β -phycoerythrin (PE) (Ou *et al.*, 2001: 4619). The ORAC method is performed using a fluorescence spectrophotometer until zero fluorescence occurs. The results are reported as the ORAC value, which refers to the net protection area under the quenching curve of the fluorescein in the presence of an antioxidant.

The fluorescein-based ORAC assay which has been automated by Huang *et al.* (2005: 4437) is the most flexible method with respect to the number of samples that can be tested as it can be used with a fluorometric microplate reader using 96-well plates to perform simultaneous kinetic analysis of many samples and to reduce the amount of sample required. It is also superior in its manner of data analysis (McAnalley *et al.*, 2003: 7).

3.2.1.b Principle of ORAC

ORAC is based on the fact that the fluorescence of an oxidisable substrate e.g. fluorescein changes with respect to time upon damage caused by a peroxy or hydroxyl attack. This assay involves a prooxidant produced by a generator such as 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) and an oxidisable substrate capable of fluorescence. The free radical induces oxidative damage which is inhibited in the presence of an antioxidant (Huang *et al.*, 2005: 1845). This inhibition is measured (in terms of fluorescence) and related to the antioxidant capacity of the antioxidant.

ORAC measures the net protection area under the quenching curve of the PE or fluorescein in the presence of the antioxidant. The ORAC method is a simple, sensitive and reliable way to measure the peroxy radical absorbing capacity (with AAPH) of antioxidants in plasma, serum and other biological fluids (Prior & Cao, 1999: 1178).

ORAC value is calculated by dividing the area under the sample curve by the area under the Trolox curve with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as net protection area provided by 1 μM Trolox in the final concentration. It takes into account inhibition time and degree of inhibition into a single quantity by measuring the area under the curve. ORAC is not affected by dilution (Huang *et al.*, 2005: 1846).

3.2.1.c Chemicals and Equipments

Monobasic sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) were purchased from Merck Chemicals (South Africa). Fluorescein sodium salt ($\text{C}_{20}\text{H}_{10}\text{Na}_2\text{O}_5$), 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH) and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (South Africa). The fluorescence was read on a Fluoroskan plate reader (Thermo Electron Corporation).

The phosphate buffer (75mM, pH 7.4) was prepared by initially dissolving 1.04 g

monobasic sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) in 100 mL ddH₂O and 2.52 g dibasic sodium phosphate in 100 mL ddH₂O ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$). The two solutions were then mixed together until a pH of 7.4 was obtained. Fluorescein sodium salt stock solution (120 μM) was prepared by dissolving 0.0225 g fluorescein sodium salt in 50 mL phosphate buffer. It was stored at 4°C in a dark container. The peroxy radical, AAPH (25 mg/mL, 9.22mM), was prepared by weighing 125 mg of AAPH into a 15 mL conical centrifuge tube. This was prepared fresh on the day of the analysis. Trolox (500 μM stock solution) was prepared by dissolving 0.00625 g Trolox in 50 mL phosphate buffer. This solution was then diluted to make a series of standard solutions (5, 10, 15, 20 and 25 μM) for the construction of the standard curve (Huang *et al.*, 2005: 1846).

3.2.1.d Assay procedure

From the fluorescein stock solution, 10 μL was added to 2 mL phosphate buffer, mixed by inversion and then 240 μL of this solution diluted to 15 mL with phosphate buffer to give a fluorescein working solution of 9.6 nM. A multichannel pipette was used to transfer 138 μL of the working solution into each well of a black 96- well micro plate. This was followed by the addition of 12 μL of either the blank (phosphate buffer), standards or samples in separate wells. The blank, as well as all the standards and samples were assayed in triplicate. The AAPH solution was prepared by adding 5 mL of phosphate buffer to the AAPH weighed earlier and mixed on a vortex for 20 seconds. In order to initiate the reaction, 50 μL of the AAPH solution was transferred with a multichannel pipette to each well. The multiwell plate was then inserted into the fluorometer of which the excitation wavelength had been set at 485 nm and the emission wavelength at 530 nm. The fluorescence intensity was measured at intervals (every 5 minutes for 2 hours, pH 7.4, and 37°C). As the reaction progressed, fluorescein was consumed and its intensity decreased. In the presence of antioxidants, the fluorescein decay was inhibited (Huang *et al.*, 2005: 1846).

3.2.1. e Data analysis and calculations

The area under the curve (AUC) was calculated using the following equation:

$$\text{AUC} = (0.5 + f_2/f_1 + f_3/f_1 + f_4/f_1 + \dots + f_i/f_1) \times \text{CT}$$

Equation 3.1

where f_1 = initial fluorescence reading at cycle 1,
 f_i = fluorescence reading at cycle i ,
and CT = cycle time in minutes.

A standard curve was obtained by plotting the concentration of the Trolox standards and the AUC (linear quadratic fit between 0 and 25 μM Trolox). The blank AUC was subtracted from the sample and standard AUC values. The ORAC values of the samples were calculated using a regression equation ($Y = a + bX + cX^2$) between Trolox concentration (X) (μM) and the net area under the fluorescence decay curve (Y). The data was expressed as micromoles of Trolox equivalents (TE) per litre (Ou *et al.*, 2001: 4620). If any of the sample ORAC values was greater than 25 μM , the assay was repeated after the sample was diluted 10 fold. All the calculations were performed using a Microsoft Excel® spreadsheet.

3.2.2 Trolox equivalent antioxidant capacity (TEAC assay or ABTS assay)

3.2.2.a Introduction

The TEAC method was first developed by Miller *et al.* in 1993. Although the TEAC value for antioxidants do not show clear correlation between TEAC values and the number of electrons an antioxidant can give away, the assay has been applied to study antioxidant capacity due to its operational simplicity and its ability to detect the contribution of other components (Huang *et al.*, 2005: 1849). Due to the aforementioned reasons the assay can be used for both nutritional studies and pharmacological studies (McAnalley *et al.*, 2003).

3.2.2.b Principle of the assay

This assay is based on the observation that when ABTS (radical generator) is incubated in the presence of a peroxidase and hydrogen peroxide or hydroxyl, peroxy, alkoxy and inorganic radicals, the slightly stable $\text{ABTS}^{\cdot+}$ radical is generated which increases the absorbance (Prior & Cao, 1999: 1175). Addition of an antioxidant delays the formation of the ABTS radical, thus increasing inhibition of the absorbance. The absorbance is measured as a TEAC value. TEAC is also affected by the dilution being tested, yielding an increase in TEAC values at lower concentrations of the

sample. The chemicals being tested could also absorb at 734 nm yielding a higher absorbance. The samples are measured at a fixed time which does not take into account the length of inhibition time which is an important component of TAC (Villano *et al.*, 2004: 502).

3.2.2.c Chemicals and Equipments

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) diammonium salt (ABTS) and Trolox (6-Hydrox-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (South Africa). Potassium-peroxodisulphate and ethanol (EtOH) were purchased from Merck (South Africa). The absorbance was read in a Multiskan plate reader (Thermo Electron Corporation).

The ABTS solution was prepared by dissolving 0.0192 g of ABTS in 5 mL distilled water and vortex mixing well until it dissolved. Potassium-peroxodisulphate solution was prepared by dissolving 0.1892 g $K_2S_2O_8$, in 5 mL distilled water and vortex mixing until it dissolved. The ABTS working solution was prepared by adding 88 μ L of the potassium-peroxodisulphate solution to 5 mL of the ABTS solution mixing it well. This solution was left in the dark at room temperature for 24 hours before use. The working ABTS solution was obtained by dilution in EtOH to an absorbance of 2 ± 0.1 at 734 nm. Trolox (500 μ M stock solution) was prepared by dissolving 0.0125 g Trolox in 50 mL of EtOH. This was prepared fresh on the day of analysis and then diluted appropriately to make a series of standard concentrations (50, 100, 150, 250 and 500 μ M) for the construction of the standard curve (Re *et al.*, 1999: 1231).

3.2.2.d Assay procedure

Ethanol was used as a blank and 25 μ L was added to the designated blank wells of a clear 96-well plate. Twenty five microliters of the Trolox standard solutions and 25 μ L of the samples were added to designated wells. The ABTS working solution (300 μ L) was then added to each well using a multichannel pipette. After 30 minutes at room temperature, the 96-well microplate was inserted into the plate reader and the absorbance read at 734 nm (Re *et al.*, 1999: 1231).

3.2.2.e Data analysis and calculations

A standard curve was obtained by plotting the concentration of the Trolox standards and the absorbance readings. The blank absorbance value was subtracted from the sample and standard absorbance values. The TEAC values of the samples were calculated using a regression equation ($Y = mX + c$) between Trolox concentration (X) (μM) and the absorbance (Y). The data was expressed as micromoles of Trolox equivalents (TE) per litre. If any of the sample TEAC values was greater than $500 \mu\text{M}$, the assay was repeated after the sample was diluted 10 fold. All the calculations were performed using a Microsoft Excel® spreadsheet.

3.2.3 Ferric reducing antioxidant power assay (FRAP assay)

3.2.3.a Introduction

The FRAP assay was first developed and published by Benzie and Strain in 1996. The assay is based on electron transfer reactions where antioxidants are used as reductants in a redox-linked colorimetric method. The change in absorbance is directly proportional to the concentration of antioxidants. One FRAP unit has been randomly defined as the reduction of 1 mole of Fe (III) to Fe (II) by Benzie & Strain (1996: 70). The FRAP assay is fast, reproducible with single antioxidants in pure solutions and with mixtures in aqueous solutions. However in FRAP there are no free radicals. It is simply a redox reaction to measure the ability of a sample to reduce ferric iron (III) to ferrous iron (II) hence it is not a direct measure of the antioxidant capacity of a potential antioxidant. Since there are no radicals in the assay there is no way of comparing antioxidant potential towards different kinds of radicals. FRAP cannot measure antioxidant capacity of certain antioxidants because the iron (II) does not react with SH (sulphur and hydrogen) group containing antioxidants (e.g. GSH). It does not take into account the quantity of inhibition and thus leaves out an important component of TAC (McAnalley *et al.*, 2003: 5). The reaction is non-specific, in that any half-reaction that has a lower redox potential, under reaction conditions, than that of the ferric/ferrous half-reaction will drive the ferric iron (III) to ferrous iron (II) reaction.

3.2.3.b Principle of the assay

The FRAP assay uses an oxidation/reduction reaction to measure the ability of antioxidants in a sample to reduce ferric iron (III) to ferrous iron (II). At low pH, reduction of a ferric-tripyridyltriazine (Fe^{III} -TPTZ) complex to the ferrous form, which has an intense blue colour, can be monitored by measuring the absorption at 593 nm (Benzie & Strain, 1996: 70).

3.2.3.c Chemicals and Equipments

Sodium acetate, glacial acetic acid, 2,4,6-tri [2-pyridyl]-s-triazine (TPTZ), L-ascorbic acid and iron (III) chloride salt were all purchased from Sigma-Aldrich (South Africa). Hydrochloric acid (HCl) was purchased from Merck Chemicals (South Africa). The absorbance was read using a Multiskan plate reader (Thermo Electron Corporation, Germany).

The acetate buffer (300 mM, pH 3.4) was prepared by dissolving 1.627 g sodium acetate in 16 mL glacial acetic acid and making up with distilled water to 1 litre. The pH was checked and the solution stored at room temperature. A dilute solution of HCl (40 mM) was prepared by mixing 1.46 mL concentrated HCl (32% HCl) and making it up to 1 litre with distilled water and stored at room temperature. The TPTZ solution (10 mM) was prepared by dissolving 0.0093 g TPTZ in 3 mL of the 40 mM HCl solution. This was prepared fresh on the day of analysis. Iron (III) chloride hexahydrate (20 mM) was prepared by dissolving 0.054 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mL distilled water. This solution was also prepared fresh on the day of analysis. Ascorbic acid (1.0 mM) standard stock solution was prepared by dissolving 0.0085 g in 50 mL distilled water. The solution, which was prepared fresh on day of analysis, was mixed well and used as the stock standard solution. The FRAP working reagent was prepared by adding 30 mL acetate buffer, 3 mL TPTZ solution, 3 mL FeCl_3 solution and 6.6 mL distilled water in a 50 mL conical tube. The solution which should be straw coloured was prepared fresh on the day of analysis. The stock ascorbic acid solution was diluted with water to make a series of standard solutions (50, 100, 200, 500 and 1000 μM) and used to construct a standard curve (Benzie & Strain, 1996: 72).

3.2.3.d Assay procedure

From the ascorbic acid standard solutions, 10 μL of each was added to the designated standard wells including a blank (water). In the designated sample wells, 10 μL of each sample in triplicate was added, followed by the addition of 300 μL of the FRAP working reagent to each of the wells with a multichannel pipette. The final volume in each well was 310 μL . The clear 96-well micro plate was incubated for 30 minutes at room temperature after which the plate was inserted into the plate reader for the measurement. The absorbance was read at 593 nm (Benzie & Strain, 1996: 72).

3.2.3.e Data analysis and calculations

A standard curve was obtained by plotting the concentration of the ascorbic acid standards and the absorbance readings. The blank absorbance value was subtracted from the sample and standard absorbance values. The FRAP values of the samples were calculated using a regression equation ($Y = mX + c$) between ascorbic acid concentration (X) (μM) and the absorbance (Y). The data was expressed as micromoles of ascorbic acid equivalents per litre (Benzie & Strain, 1996: 71). If any of the sample FRAP values was greater than 1000 μM , the assay was repeated after the sample was diluted 10 fold. All the calculations were performed using a Microsoft Excel® spreadsheet.

3.3. Measurements of antioxidants: Antioxidant content

3.3.1. Total polyphenols assay using the Folin-Ciocalteu method

3.31.a Introduction

This assay was originally designed for the analysis of proteins but was later extended to the analysis of total phenols in wine by Singleton and Rossi (1965) and since then, the assay has been applied extensively. The Folin method suffers from a number of interfering substances such as sugars, aromatic amines, sulphur dioxide, ascorbic acid and other enediols and reductones, organic acids and Fe (II).

3.3.1.b Principle of the assay

The assay involves a probe, in this case the Folin reagent (molybdotungstophosphoric

heteropolyanion) that abstracts an electron from the antioxidants, causing colour change of the probe. The degree of the colour change is proportional to the polyphenol concentrations. The reaction end point is achieved when the change in colour stops. The change in absorbance is monitored spectrophotometrically at 765 nm (Huang *et al.*, 2005: 1848).

3.3.1.c Chemicals and Equipments

Folin-Ciocalteu phenol reagent, sodium carbonate (Na_2CO_3) salt and Gallic acid were all purchased from Sigma-Aldrich (South Africa). Ethanol was purchased from Merck Chemicals (South Africa). The absorbance was read in a Multiskan Plate reader (Thermo Electron Corporation, Germany). Ethanol (10%) was prepared by mixing 100 mL of ethanol with 900 mL water in a 1 litre media bottle. The Folin working solution was prepared fresh on the day of analysis by mixing 2 mL Folin-Ciocalteu phenol reagent with 18 mL water. Sodium carbonate (7.5%) was prepared by dissolving 7.50 g Na_2CO_3 in 100 mL water, mixing it well until it dissolved and storing it at room temperature. Gallic acid standard was prepared fresh on the day of analysis by dissolving 40 mg gallic acid in 50 mL 10% ethanol to give a stock standard concentration of 800 mg/L. The standard stock solution was diluted appropriately to make a series of standards (20, 50, 100, 250 and 500 mg/L) to construct a standard curve (Singleton & Rossi, 1965: 144).

3.3.1.d Assay procedure

In the designated wells of a clear micro well plate which included a blank, 25 μL of the gallic acid standards and 25 μL of the samples were added in triplicate. This was followed by addition of 125 μL Folin working solution to all the wells using a multichannel pipette. After 5 minutes, 100 μL Na_2CO_3 was added to each well using a multichannel pipette. The plate was left for 2 hours at room temperature after which the plate was inserted into the plate reader for the measurement. The absorbance was read at 765 nm (Singleton & Rossi, 1965: 144).

3.3.1.e Data analysis and calculations

A standard curve was obtained by plotting the concentration of the gallic acid

standards and the absorbance readings. The blank absorbance value was subtracted from the sample and standard absorbance values. The total polyphenol values of the samples were calculated using a regression equation ($Y = mX + c$) between gallic acid concentration (X) (mg/L) and the absorbance (Y). The data was expressed as milligram gallic acid equivalents (GAE) per litre (Singleton & Rossi, 1965: 144). If any of the sample total polyphenol values was greater than 500 mg/L, the assay was repeated after the sample was diluted 10 fold. All the calculations were performed using a Microsoft Excel® spreadsheet.

As previously mentioned, ascorbic acid present does interfere with the Folin assay resulting in an overestimation of the total polyphenols present in a sample. As ascorbic acid could be present in significant amounts in fruit juices, a correction factor was applied to compensate for this interference. The ascorbic acid contribution to the absorbance in the Folin assay is consistent in a weight-to-weight ratio of 0.64:1.0. This means that every 1 mg/mL ascorbic acid present in the sample contributes 0.64 mg/mL gallic acid equivalents in the total polyphenols assay (Asami *et al.*, 2003: 1237).

3.3.2 Assay for flavonols/flavones

3.3.2.a Principle of the assay

Flavonols and flavones are subclasses of flavonoids with a 3-hydroxyflavone and a 2-phenylchromen-4-one backbone respectively. The assay is based on the observation that in an acidic solution, flavonols and flavones absorb UV light maximally at 360 nm (Andersen & Markham, 2006). This is an effective way of measuring these two subclasses as they are the only polyphenol/flavonoid subclasses that absorb at this wavelength.

3.3.2.b Chemicals and Equipments

Absolute ethanol and HCl were purchased from Merck (South Africa). Quercetin was purchased from Sigma-Aldrich (South Africa). The absorbance was read on a Multiskan plate reader (Thermo Electron Corporation, Germany). Ethanol (10%) was prepared by mixing 50 mL ethanol with 450 mL of distilled water. Hydrochloric acid (0.1% in 95% ethanol) solution was prepared by adding 1.667 mL 30% HCl into a 500

mL volumetric flask and filling to the mark with 95% EtOH (950 mL EtOH mixed with 50 mL H₂O). HCl (2%) solution was prepared by pipetting 33.33 mL 30% HCl into a 500 mL volumetric flask containing 466.67 mL H₂O. Quercetin standard was prepared fresh on the day of analysis by dissolving 4 mg of quercetin in 50 mL 95 % ethanol (80 mg/L). The standard stock solution was diluted appropriately to make a series of standard solution (5, 10, 20, 40 and 80 mg/L) to construct a standard curve.

3.3.2. c Assay procedure

In the designated wells of a clear UV 96-well microtitre plate, 12.5 µL blank (water), the quercetin standards and the samples were added in triplicate. This was followed by the addition of 12.5 µL of HCl (0.1% in 95% ethanol) solution to each of the wells with a multichannel pipette followed by 225 µL of HCl (2%). The 96- well micro well plate was incubated for 30 minutes at room temperature after which the plate was inserted into the plate reader for measurement. The absorbance was read at 360 nm.

3.3.2.d Data analysis and calculations

A standard curve was obtained by plotting the concentration of the quercetin standards and the absorbance readings. The blank absorbance value was subtracted from the sample and standard absorbance values. The flavonol/flavone values of the samples were calculated using a regression equation ($Y = mX + c$) between quercetin concentration (X) (mg/L) and the absorbance (Y). The data was expressed as milligram quercetin equivalents (QE) per litre. If any of the sample flavonol/flavone values was greater than 80 mg/L, the assay was repeated after the sample was diluted 10 fold. All the calculations were performed using a Microsoft Excel® spreadsheet.

3.3.3 Assay for flavanols

3.3.3.a Principle of the assay

Flavanols (flavan-3-ols or catechins) are a subclass of flavonoids with a 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton. This group includes well known compounds such as catechin, EC, ECG, EGCG, EGC, GC, and GCG (Bors & Michel, 1999: 1413). The flavanol assay is based on the discovery that when flavanols and proanthocyanidins

react with 4-dimethylaminocinnamaldehyde (DMACA) it forms a characteristic light blue colour which can be measured at 640 nm. The intensity of the blue colour is proportional to the amount of flavanols (Treutter, 1989: 185).

3.3.3.b Chemicals and Equipments

Hydrochloric acid, DMACA, and methanol (MeOH) were purchased from Merck chemicals (South Africa). Catechin was purchased from Sigma-Aldrich (South Africa). The absorbance was read using a Multiskan plate reader (Thermo Electron Corporation, Germany).

Methanol-HCl buffer was prepared by mixing 250 mL HCl with 750 mL methanol. 4-dimethylaminocinnamaldehyde was prepared by dissolving 0.25 g DMACA in 500 mL HCl-MeOH mixture. This was prepared fresh on the day of analysis. Catechin stock standard was prepared by dissolving 0.005 g catechin in 50 mL MeOH (100 mg/L). The catechin standard stock solution was diluted appropriately to make a series of standards (5, 10, 20, 50 and 100 mg/L) for the construction of a standard curve.

3.3.3.c Assay procedure

In designated wells of a clear 96-well microplate, 50 μ L blank (water), the catechin standards and the samples were added (all in triplicate). The reaction was initiated by adding 250 μ L of DMACA to all the wells using a multichannel pipette. The plate was inserted into the plate reader for measurement after 30 minutes incubation at room temperature. The absorbance was read at 640 nm (Treutter, 1989: 185).

3.3.3.d Data Analysis and Calculations

A standard curve was obtained by plotting the concentration of the catechin standards and the absorbance readings. The blank absorbance value was subtracted from the sample and standard absorbance values. The flavanol values of the samples were calculated using a regression equation ($Y = mX + c$) between catechin concentration (X) (mg/L) and the absorbance (Y). The data was expressed as milligram catechin equivalents (CAE) per litre (Treutter, 1989: 185). If any of the sample flavanol values was greater than 100 mg/L, the assay was repeated after the sample was diluted 10

fold. All the calculations were performed in a Microsoft Excel® spreadsheet.

3.3.4 Assay for Ascorbic acid

3.3.4.a Principle of the assay

The assay is a colorimetric method which is based on the ability of ascorbic acid to reduce the tetrazolium salt, MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] in the presence of the electron carrier PMS (5-methylphenazinium methosulfate) at pH 3.5 to a formazan which is measured at 578 nm. Other reducing agents such as polyphenols will however also reduce MTT. Therefore the assay is performed in two parts. In the first part, total reducing substances (this will include ascorbic acid) of a sample are determined (reaction 1). In the second part, the ascorbic acid in the sample is converted to dehydroascorbic acid with ascorbate oxidase (reaction 2). The reducing substances remaining in the sample (now excluding ascorbic acid) are then determined using the same reaction as described for the first part. The difference in the absorbance between the two parts is calculated to determine ascorbic acid content of the sample.

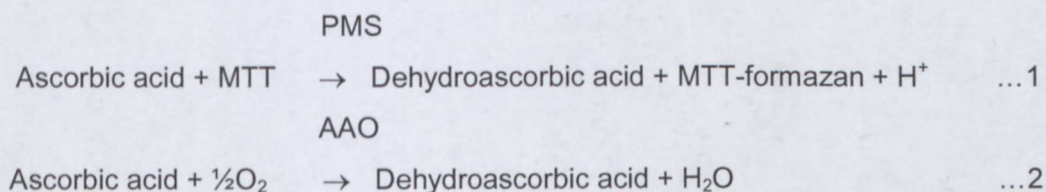


Figure 3.2

3.3.4.b Chemicals and Equipments

The ascorbic assay test kit was purchased from Roche (South Africa). The absorbance was read in a Multiskan plate reader (Thermo Electron Corporation, Germany). The test kit (Roche) consisted of three solutions. Solution 1 contained sodium phosphate/citrate buffer (pH approximately 3.5) and MTT. Solution 2 contained ascorbate oxidase (AAO) and solution 3 contained PMS.

3.3.4.c Assay procedure

Ascorbic acid standard stock solution was prepared by dissolving 0.01 g of ascorbic acid in 50 mL of water and mixing until it dissolves. The ascorbic acid standard stock solution was diluted appropriately to make a series of standards (10, 20, 50, 100 and 200 mg/L) for the construction of a standard curve. For each of the samples, a sample blank was included as described in Table 3.1. The assay procedure was set according to the table below:

Table 3.1: Procedure for vitamin C assay

Pipette into 96-well plate	sample blank	sample
Solution 1	100 μ L	100 μ L
Distilled water	140 μ L	150 μ L
Sample	10 μ L	10 μ L
Solution 2	10 μ L	
Mix and incubate for 15 minutes at 37°C. Read the absorbance of the sample blank and the sample (A_1) at 578nm.		
Solution 3	10 μ L	10 μ L
Mix and allow the solutions to stand for 10 minutes at 37°C. Read the absorbance of the sample blanks and samples (A_2) at 578 nm.		

3.3.4. d. Data analysis and calculations

The absorbance differences ($A_2 - A_1$) for both the sample and sample blank were calculated. The absorbance difference of the sample blank was subtracted from the absorbance difference of the sample ($\Delta A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{sample blank}}$). A standard curve was obtained by plotting the concentration of the ascorbic acid standards and the absorbance readings (ΔA). The ascorbic acid values of the samples were calculated using a regression equation ($Y = mX + c$) between ascorbic acid concentration (X) (mg/L) and the absorbance (Y). The data was expressed as milligram ascorbic acid per litre. If any of the sample ascorbic acid values was greater than 200 mg/L, the assay was repeated after the sample was diluted 10 fold. All the calculations were performed using a Microsoft Excel® spreadsheet.

3.4 Statistical analysis

Pearson correlation coefficients (r) were used to analyse the degree of association between two methods. Data were tested for normal distribution using the D'Agostino-Pearson test which computes a single P-value for the combination of the coefficients of Skewness and Kurtosis. Data were log transformed if found not to be normally distributed. Methods were also compared by calculating an inter-rater agreement statistic (Kappa). Agreement is quantified by the Kappa (K) or Weighted Kappa (Kw) statistic: K is 1 when there is perfect agreement between the classification system; K is 0 when there is no agreement better than chance; K is negative when agreement is worse than chance. The K value can be interpreted as listed in Table 3.2 (Altman, 1991).

Table 3.2: Interpretation of the K-Value

Value of K	Strength of agreement
< 0.20	Poor
0.21 - 0.40	Fair
0.41 - 0.60	Moderate
0.61 - 0.80	Good
0.81 - 1.00	Very good

The program used to perform the statistical calculations was MedCalc® Version 9.4.2.0.

3.5 *In vivo* effect of rooibos on plasma and erythrocytes

The aim of this pilot study was to investigate the short term changes in various blood markers of antioxidant capacity and oxidative stress after healthy male volunteers have consumed 500 mL of rooibos. The antioxidant capacity of polyphenols and their bioavailability has not been clearly established *in vivo* in humans after consumption of foods and/or beverages that contain polyphenols. The study's objective was to establish whether consuming a health beverage such as rooibos does improve the antioxidant capacity and or reduce oxidative stress in the blood.

As participants were their own controls, both experimental and control groups was the same and thus the strategy for recruitment was the same. The participant recruitment

strategies included poster advertisements on the Cape Town and Bellville campuses and residences of CPUT, as well as a general electronic notice to all CPUT staff and students. These posters served as an information poster as well as containing the specific inclusion criteria each potential participant needs to qualify as a successful participant. The study was approved by the Health and Applied Sciences Research Ethics Committee.

The study population included 8 apparent healthy males, aged between 20 and 35. The selection criteria [apparent health as described by the American Heart Association (2002)] included apparent health (blood pressure between 120-139/80-89 mmHg), relative stable body mass index (20-29 kg/m²), non-smoking, not using any chronic medication nor antioxidant supplements. Participants with known renal, hepatic, endocrine or gastrointestinal disorders, undesirable alcohol consumption (>2 drinks per day), unusual dietary habits (e.g. vegetarian and vegan diets), using chronic/life sustaining medication, antioxidant supplementation, minerals and vitamins supplements, aspirin or any other drugs e.g. anti-inflammatory, with established antioxidant properties, were excluded from the study. The volunteers were asked to abstain from any alcohol/tea/fruit juice, vigorous exercise for 24 hours and from food and drink (fasting) for 12 hours before the start of the study which was the following morning. Subjects (n=8) were required to report to the treatment room (equipped with chairs, air conditioner, reading material and drinking water) at CPUT (Cape Town Campus) at 8h00 in the morning on the day of study. They were seated for at least 10-15 minutes before blood pressure measurement and phlebotomy were performed. An 18 gauge intravenous cannula, with a resealing injection site (butterfly), was inserted into an antecubital vein in the forearm of the participants by the study phlebotomists to allow for serial blood sampling.

A blood sample was drawn from each volunteer (20 mL, comprising 10 mL EDTA blood and 10 mL clotted blood) before the beverage challenge. This sample was designated time 0 hours. Each subject was tested on one occasion in a pilot intervention experimental design with regards to rooibos consumed (500 mL). After ingestion, blood samples were drawn with minimum stasis at 45, 90 and 180 minutes (a total amount of 80 mL). The participants were advised to refrain from taking anything else apart from water for the period of the study. After completion of the study all participants were served a warm meal with a soft drink.

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The blood samples were centrifuged the same day and serum/plasma samples stored at -80°C until analyzed for various biochemical and antioxidant parameters. The samples for the oxidised glutathione analysis was mixed with M2VP and stored at -80°C .

3.5.1 Glutathione redox analysis (GSH/GSSG)

3.5.1. a Introduction

Glutathione is a tripeptide of glycine, glutamate (glutamic acid) and cystein. The abundance or deficiency of cystein, a sulphur containing amino acid regulates the body's ability to produce GSH (Kidd, 1997:155). The ability of the sulphur (thiol) group of GSH to donate electrons to reduce free radicals confers its antioxidant capacity. GSH has five major functions in the body which includes: being the most powerful naturally occurring antioxidant in cells, a recycler of other antioxidants that have been oxidised, prevention of disease, detoxification, immune system support, and protection from radiation. Glutathione exists in the body in two forms; the active (reduced) form often referred to as GSH and the inactive form (oxidized) referred to as GSSG. GSH is oxidised to GSSG and recycled back to GSH by glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH) in the glutathione peroxidase (GPx) catalysed reduction of peroxides to water and alcohol:

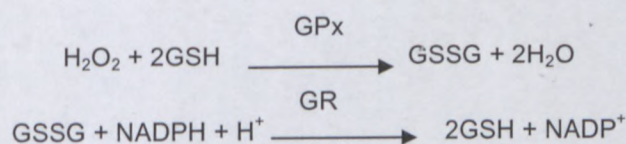


Figure 3.3

Under normal conditions the percentage of GSH is about 90% to 10% GSSG in humans. A change in this ratio or decrease in GSH indicates an increase in GSSG which in turn indicates lipid peroxidation (Sen, 1997: 660). The glutathione redox analysis was done according to Asensi *et al.*, (1999: 267).

3.5.1. b Principle of the assay

In this assay, the thiol reagent, 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB) reacts with GSH to form a spectrophotometrically detectable product at 412 nm, 5-thionitrobenzoic acid (TNB) and GS-TNB. The GS-TNB is then reduced by GR and NADPH, releasing a second TNB molecule and recycling the GSH; thus intensifying the reaction. Any GSSG initially present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly converted to GSH and is detectable in the assay.

3.5.1. c Chemicals and equipments

Sodium di-hydrogen orthophosphate dehydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), di-sodium hydrogen orthophosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), Metaphosphoric acid (MPA), Trichloric acetic acid, 5,5' Dithiobis-(2-nitrobenzoic acid) were purchased from Merck Chemicals. Nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), oxidised glutathione (GSSG) and glutathione reductase (GR) were purchased from Sigma-Aldrich (South Africa). The absorbance was read in a Multiskan plate reader (Thermo Electron Corporation, Germany).

Phosphate buffer was prepared by adding 16 mL of solution 1 (prepared by adding 2.78 g of sodium di-hydrogen orthophosphate dehydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) in 100 mL of distilled water) to 84 mL of solution 2 (7.17 g di-sodium hydrogen orthophosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) added to 100 mL distilled water). The mixture was then adjusted to pH 7.5 and 0.0372 g EDTA added. DTNB was prepared by dissolving 0.12mg/ml of 5,5'-dithiobis-2-nitrobenzoic acid to make a 0.3Mm solution. A 0.004 U/ μl solution of GR was prepared by adding 0.78 μl of the enzyme to 49.22 μl assay buffer. A 1mM solution of NADPH was prepared by dissolving 0.833 mg/ml in the assay buffer.

The samples were kept on ice during the analysis as GSH and GSSG are stable in intact resting cells for up to 24 hours at 4°C.

3.5.1. d GSSG sample preparation

A 100 μL portion of EDTA-treated whole blood was added to 10 μL M2VP and mixed gently and frozen at -80°C . The samples were thawed and mixed immediately and incubated at room temperatures for 2-10 minutes whereafter, 290 μL of cold 5% MPA was added to the tube and vortexed for 15-20 seconds. The mixture was centrifuged at 10 000 x g for 10 minutes. A 25 μL portion of this MPA extract was added to the buffer in Eppendorf tubes and the extracts were placed on ice until use.

3.5.1. e GSH sample preparation

A 50 μL portion of whole EDTA-treated blood was aliquoted into tubes and frozen at -80°C . On the day of analysis, the samples were thawed and mixed immediately with 350 μL of 5% cold MPA and vortexed for 15-20 seconds, centrifuged at 10 000 x g for 10 minutes. A 5 μL portion of the MPA extract was added to 300 μL of buffer and placed on ice until use.

3.5.1. f Assay procedure

A series of GSH or GSSG standards were prepared and 50 μL of the standards, blank or samples was added to the 96-wells plate to which 50 μL of the DNTB solution was added using a multichannel pipette. A 50 μL portion of the enzyme solution (GR) was then added using a multichannel pipette. The samples were mixed and incubated for 5 minutes at 25°C in a preheated BioTek microplate reader. In order to initiate the reaction, 50 μL of NADPH was added to each well.

3.5.1. g Data analysis and calculations

The standard stock solutions of GSH and GSSG were diluted appropriately to make a series of standard solution (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 μM) to construct a standard curve. The absorbance was read at 412 nm for 5 minutes and the linear standard curve was used to calculate the concentrations as follows:

$$\text{GSH}_t = \mu\text{M} \times \text{dilution factor} (488) \quad (\text{GSH} = \text{GSH}_t - 2\text{GSSG})$$

$$\text{GSSG} = \mu\text{M} \times \text{dilution factor} (60)$$

$$\text{Ratio} = \frac{\text{GSH}_t - (2 \times \text{GSSG})}{\text{GSSG}}$$

$$\text{GSSG}$$

Equation 3.2

All the calculations were performed using a Microsoft Excel® spreadsheet.

3.5.2 Assay for anthocyanins

3.5.2 a Introduction

Anthocyanins are relatively unstable and often undergo degradative reactions during storage and processing. Anthocyanins have a typical absorption band in the 490 to 550 nm region of the visible spectra. However interference from anthocyanin degradation products or melanoidins necessitates the use of differential and/or subtractive methods to quantify anthocyanins and their degradation products (Francis, 1982). The pH differential method is a fast and easy for the quantification of monomeric anthocyanins (Wrolstad *et al.*, 1995).

3.5.2 b Principle of the assay

The differential assay method for anthocyanins is based on measuring the absorbance at two different pH values. It is based on the structural transformations of the anthocyanin chromophore as a function of pH. In order to determine the total monomeric anthocyanin content the absorbance at pH 1.0 and 4.5 is measured at the $I_{\text{vis-max}}$ and at 700nm.

3.5.2 c Chemicals and equipments

The Potassium metabisulfite ($K_2S_2O_5$), Sodium acetate and Potassium chloride buffers were purchased from Sigma-Aldrich (South Africa). The bisulfite solution was prepared by dissolving 1 g of $K_2S_2O_5$ in 5 ml. The 0.025M pH 1.0 Potassium chloride buffer was prepared by mixing 1.86 g KCL and 980 ml distilled water and adjusting it to 1.0 with concentrated HCl.

3.5.2 d Assay procedure

The appropriate dilution factor for the sample was determined by diluting with potassium chloride buffer pH 1.0, until the absorbance of the sample at the $\lambda_{\text{vis-max}}$ is within the linear range of the spectrophotometer and the final volume is divided by the

initial volume to obtain the dilution factor. Two dilutions of the sample were prepared, one with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5, diluting each other by the previously determined dilution factor. The absorbance of each dilution at the $\lambda_{\text{vis-max}}$ and at 700 nm against a blank cell filled with water. The absorbance of the diluted samples is the calculated appropriately.

3.5.2 e Data analysis and calculations

The absorbance of the diluted samples was calculated as follows:

$$A = (A_{\lambda_{\text{vis-max}}} - A_{700})_{\text{pH 1.0}} - (A_{\lambda_{\text{vis-max}}} - A_{700})_{\text{pH 4.5}}$$

Equation 3.3

All the calculations were performed using a Microsoft Excel® spreadsheet.

3.5.3 Preparation of plasma samples for total polyphenols and ORAC assay

The total polyphenols were estimated using a modified Folin-Ciocalteu method by Serafini *et al.* (1998: 1003) to eliminate interference from proteins. For hydrolysis of the conjugated proteins in the samples, 1 mL of 1.0M HCl was added to 500 μL of the sample and vigorously vortexed for 10 seconds. The samples were then incubated at 37°C for 30 minutes after which, 1.0 mL of 2.0M NaOH in 75% methanol was added, and the mixture vortexed again for 3 minutes and incubated at 37°C for 30 minutes. This was to break the links of the polyphenols with lipids and provide the first extraction of the polyphenols. After mixing for 3 minutes, 1 mL of MPA was added to precipitate the plasma proteins and the sample centrifuged at 1500 x g for 10 minutes. The supernatants were assayed for the total polyphenols and ORAC values as described previously.

3.5.4 Statistical analysis

Data obtained from the *in vivo* study was presented as mean \pm standard deviation. Where more than two sets of data were compared for statistically significant differences, ANOVA (analysis of variance) was used. A p-value of less than 0.05 was considered significant. The program used to perform the statistical calculations was MedCalc® Version 9.4.2.0.

Table 4.1: Total antioxidant capacity of fruit juices

Brand Name	Flavour	ORAC ($\mu\text{mole TE/L}$)	FRAP ($\mu\text{mole/L}$)	TEAC ($\mu\text{mole/L}$)	Rank ORAC	Rank FRAP	Rank TEAC
Lipton	Lemon	10940 \pm 454	3653 \pm 1031	3241 \pm 726	17	11	15
	Green	15977 \pm 1101	6749 \pm 1486	3707 \pm 890	4	2	9
	Raspberry	8724 \pm 2059	3106 \pm 336	3148 \pm 568	29	19	16
	Mango	7493 \pm 2395	2281 \pm 1549	2397 \pm 1938	37	29	28
	Peach	10539 \pm 456	3176 \pm 189	3346 \pm 462	18	18	13
	Red rooibos	8896 \pm 1605	2197 \pm 546	2375 \pm 762	28	30	30
Liqui Fruit	Breakfast punch	12422 \pm 279	6485 \pm 575	4573 \pm 66	12	4	2
	Apricot	9072 \pm 1405	2032 \pm 220	2883 \pm 130	26	33	23
	Clear apple	11076 \pm 1822	2453 \pm 353	3107 \pm 64	16	25	18
	Berry blaze	10142 \pm 169	4715 \pm 545	4146 \pm 427	20	9	3
	Red grape	7620 \pm 1310	2919 \pm 478	3017 \pm 162	35	20	20
	Passion power	7917 \pm 665	1893 \pm 195	2373 \pm 535	33	36	31
	Litchi	4997 \pm 324	2420 \pm 440	1082 \pm 28	46	26	43
	Cranberry	9539 \pm 2081	2544 \pm 485	2076 \pm 540	23	23	37
	Peach & orange	7502 \pm 943	3317 \pm 1054	2703 \pm 1409	36	16	24
	Mango & orange	7066 \pm 741	3412 \pm 769	2318 \pm 330	38	14	32
	Orange	13419 \pm 295	3507 \pm 276	3117 \pm 261	10	12	17
	Summer pine	7901 \pm 365	2887 \pm 803	2914 \pm 397	34	21	22
Ceres	Mango	8160 \pm 504	5379 \pm 450	3903 \pm 1091	31	6	5
	Hanepoot	3217 \pm 943	1435 \pm 945	392 \pm 386	48	40	44
	Apple	5396 \pm 1138	2180 \pm 320	2422 \pm 335	44	31	27
	Secrets of the valley	4060 \pm 1908	1302 \pm 63	1488 \pm 62	47	42	42
	Peach	9800 \pm 1099	3208 \pm 514	3684 \pm 205	22	17	10
	Medley of fruits	12144 \pm 426	6335 \pm 992	3805 \pm 953	14	5	6
	Litchi	5791 \pm 527	1308 \pm 312	390 \pm 87	42	41	45
	Orange	14288 \pm 422	5125 \pm 632	3717 \pm 304	7	8	8
	Cloudy apple & pear	12531 \pm 2799	6655 \pm 105	3914 \pm 507	11	3	4
	Whispers of summer	6853 \pm 991	2358 \pm 168	2446 \pm 303	39	27	26
	Cranberry & kiwi	8540 \pm 1056	2521 \pm 294	2379 \pm 289	30	24	29
	Tomato	19673 \pm 2426	2036 \pm 121	3495 \pm 744	1	32	11
	Ruby grape fruit	18874 \pm 1639	3968 \pm 69	3460 \pm 65	2	10	12
Minute Made	Breakfast punch	9248 \pm 299	2010 \pm 616	2271 \pm 209	24	35	34
	Apple	14420 \pm 3504	1776 \pm 387	2528 \pm 223	6	37	25
	White grape	6420 \pm 175	678 \pm 34	346 \pm 91	42	46	47
	Naartjie/orange	10508 \pm 197	2024 \pm 52	2240 \pm 140	19	34	35
	Orange/peach	17255 \pm 2276	3457 \pm 619	3805 \pm 448	3	13	7
	Mango	8994 \pm 151	1590 \pm 222	2086 \pm 88	27	39	36
	Orange	13933 \pm 134	2871 \pm 64	3083 \pm 34	8	22	19
	Guava	15228 \pm 1177	7808 \pm 516	5242 \pm 379	5	1	1
Quali	Orange	11159 \pm 2742	2344 \pm 436	2061 \pm 238	15	28	38
	Litchi	6526 \pm 2429	565 \pm 126	365 \pm 35	41	47	46
	Tropical punch	9803 \pm 314	1599 \pm 73	2277 \pm 293	21	38	33
	Pineapple & kiwi	5353 \pm 1245	532 \pm 103	317 \pm 1	45	48	48
	Guava	12197 \pm 305	5223 \pm 164	3270 \pm 172	13	7	14
	Apple	8010 \pm 1147	988 \pm 125	1635 \pm 417	32	45	39
	Granadilla	9162 \pm 612	1292 \pm 251	1492 \pm 252	25	43	41

Brand Name	Flavour	ORAC	FRAP	TEAC	Rank	Rank	Rank
		($\mu\text{mole TE/L}$)	($\mu\text{mole/L}$)	($\mu\text{mole/L}$)	ORAC	FRAP	TEAC
Quali	Mango	6773 \pm 228	1007 \pm 95	1629 \pm 100	40	44	40
	Loganberry	13767 \pm 1273	3398 \pm 214	2996 \pm 82	9	15	21

Between FRAP and ORAC ($r = 0.5533$) as well as TEAC and ORAC ($r = 0.6920$) only moderate positive correlation coefficients are apparent. Another way to perform method comparison is to calculate an inter-rater agreement statistic (Kappa) (see Section 3.4) using a ranking system (highest value rank as 1 with the lowest as 48) as set out in Table 4.1. These results show a fair agreement ($K = 0.376$) between ORAC and FRAP, a moderate agreement ($K = 0.462$) between ORAC and TEAC and a good agreement ($K = 0.698$) between FRAP and TEAC. The statistical results highlight the importance of using more than one analytical method to measure the antioxidant capacity of complex samples. This is also the recommendation by Cao and Prior (1998: 1309) after comparing different analytical methods to measure *in vivo* total antioxidant capacity. In that study, a weak correlation was shown between serum ORAC and serum FRAP, with no correlation between serum ORAC and serum TEAC as well as no correlation between serum FRAP and serum TEAC. Although fruit juices are complex mixtures, the antioxidants that are predominant would be polyphenols and to a lesser extent ascorbic acid. This would probably explain the high correlation and agreement between the FRAP and TEAC methods compared to those found by Cao and Prior (1998) in serum. Serum, in addition to polyphenols and ascorbic acid, also contains reduced glutathione, methionine, uric acid, bilirubin and lipophilic antioxidants such as α -tocopherol, β -carotene and ubiquinol-10. Thaipong *et al.* (2006) found a correlation of 0.74 between ORAC and FRAP, 0.82 between ORAC and TEAC and 0.97 between FRAP and TEAC on methanolic extracts of guava fruit extracts.

In the report (ORAC of selected foods - 2007) prepared by the Nutrient Data Laboratory and the United States Department of Agriculture (USDA, 2007), the ORAC values of 13 fruit juices are listed. Eleven of these juices were similar to those tested in this study, the exceptions being blueberry juice and pomegranate juice. The average ORAC value of the 11 juices are $10703 \pm 6124 \mu\text{mole TE/L}$ compared to $9986 \pm 3737 \mu\text{mole TE/L}$ for the 48 fruit juices tested in this study. There are some fruit juices that are common to three or more of the brands tested in this study. If for example, the ORAC results of these were compared, litchi juice (Liqui fruit, Ceres and Quali juice) ranked consistently in the lower half (average position: $43 \pm 3 (\mu\text{mole TE/L})$), orange juice (all brands except Lipton) in the top half (average position: 9 ± 4) and mango juice (all brands except Liqui fruit) also in the lower half (average position: 35 ± 5). This is so despite

various factors such as growth region, cultivar, climate, storage atmosphere, harvest maturity, shipping, storage conditions and processing that are known to affect the chemical composition of fruit juices (Eisele & Drake, 2005: 213).

Spectrophotometric methods are often used to estimate the amount of similar structured chemical compounds in a sample. These methods are easy to perform, inexpensive and not as time consuming as chromatographic techniques. However, spectrophotometric methods are not sensitive and non-specific, which can lead to an underestimation or overestimation of the amount of the targeted molecules. It is well documented that the Folin-Ciocalteu method is influenced by a number of interfering substances such as sugars, ascorbic acid, aromatic amines, organic acids, sulphur dioxide and other non phenolic organic substances (Singleton & Rossi, 1965: 144). The effect of ascorbic acid (AA) was subtracted from the total polyphenol values for fruit juices as seen in Table 4.2. The method used in this study to measure flavanols (DMACA reagent) has been shown (apart from catechins) to also react with resorcinol, orcinol, phloretin and phloroglucinol (McMurrough & McDowell, 1978: 96). The UV spectrophotometric method to measure flavonols/flavones at 360 nm is also affected by other compounds that have the same maximum absorption at 360 nm such as the polyphenol subgroup, xanthenes (e.g. mangiferin) (Gómez-Zaleta *et al.*, 2006: 1002).

Total polyphenol values (including ascorbic acid influence) for the fruit juices ranged from 48.6 mg/L (Quali litchi) to 1118 mg/L (Minute made guava). When the interfering effect of ascorbic acid in the total polyphenols assay was removed, the total polyphenol range changed from 48.6 mg/L (Quali litchi) to 964 mg/L (Lipton green tea). The flavonol/flavone values range from 3.1 mg/L (Ceres hanepoot) to 193 mg/L (Minute made Orange/Peach) while the flavanol values ranged from 0.5 mg/L (Ceres hanepoot) to 84.0 mg/L (Lipton green tea). Anthocyanins were only detected in those fruit juices that contained red grape or any type of red berries. Values ranged from 2.9 mg/L (Liqui fruit cranberry) to 8.0 mg/L (Ceres secrets of the valley). Ascorbic acid values ranged from values below the detection limit of the assay (ND) in several of the fruit juices to 779 mg/L (Ceres cloudy apple & pear) (Table 4.2).

Adding the amount of flavonols/flavones, flavanols and anthocyanins for each fruit juice and calculating this new value (estimated total flavonoid content) as a percentage of the total polyphenols (excluding interference by ascorbic acid), gives an average of 24.3%. The range for total flavonoid content varied from as low as 3.1% (Ceres apple), 4.7% (Liqui fruit clear apple), 5.9% (Ceres cranberry & kiwi) to as high as 97.5% (Quali litchi), 50.1% (Minute made mango) and 44.6% (Minute made naartjie & orange). The

difference in percentage between total polyphenols and total flavonoids will be made up by flavonoid subgroups not measured in this study (e.g. flavanones such as naringenin in orange juice) and other polyphenol subgroups such as phenolic acids and xanthenes (e.g. mangiferin in mangoes).

When the ascorbic acid values for the 25 Ceres and Liqui fruit juices tested in this study (other brands do not list the ascorbic acid content) were compared to those printed on the label of the respective fruit juices, significant differences were found. The average ascorbic acid value for the above mentioned brands found was 288 mg/L while on the labels the average was shown as 461 mg/L. In only five of the fruit juices (Liqui fruit breakfast punch and berry blaze as well as Ceres medley of fruits, cloudy apple & pear and cranberry & kiwi) the tested ascorbic acid values were higher than those printed on the labels. The Pearson correlation coefficient between the measured ascorbic acid values and those printed on the labels was only 0.3596. The spectrophotometric method used in this study only measured L-ascorbic acid and not dehydroascorbic acid. In a study done by Mokady *et al.* (1983: 452) it was found that the ascorbic acid content of several fruit and fruit blends decreased over a 3 hour incubation period at room temperature. However, the decrease in ascorbic acid was accompanied by an equal increase in the level of dehydroascorbic acid. This instability of L-ascorbic acid may therefore explain the lower values obtained in this study. The higher ascorbic acid values found in some fruit juices may be caused by seasonal and other environmental factors and the fact that the printed values on the labels are not updated frequently. It is also possible that the ascorbic acid content printed on the labels includes both the L-ascorbic acid and dehydroascorbic acid forms, although the official ascorbic acid AOAC method (2,6 dichloroindophenol titration 967.21) used for nutrition labelling also only measures the reduced form of ascorbic acid.

Table 4.2: Antioxidant content of fruit juices

Brand Name	Flavour	Polyphenols (mg/L)	Polyphenols (mg/L excl. AA)	Flavonols (mg/L)	Flavonols (mg/L)	Anthocyanins (mg/L)	Ascorbic acid (mg/L)
Lipton	Lemon	517±23	469±32	36.2±12	16.6±8	N.D	73.6±13
	Green	1024±151	964±152	39.8±6	84.0±31	N.D	91.5±2
	Raspberry	346±52	283±33	43.3±18	18.5±8	N.D	96.4±30
	Mango	209±236	184±201	44.6±18	12.5±11	N.D	38.2±54
	Peach	348±83	287±76	74.7±23	15.1±12	N.D	94.1±10
	Red rootibos	296±10	248±11	60.3±5	1.8±2	N.D	73.2±2
	Breakfast punch	1029±8	618±4	126±25	16.8±5	N.D	633±7
	Apricot	417±31	322±34	83.1±10	6.3±0	N.D	146±100
	Clear apple	453±95	350±82	15.3±4	1.3±1	N.D	158±20
	Berry blaze	680±51	470±11	36.2±4	8.9±5	3.5±8	323±61
Liqui Fruit	Red grape	436±25	303±38	22.0±1	4.3±2	7.7±15	205±20
	Passion power	388±181	297±152	98.1±54	3.1±1	N.D	140±44
	Litchi	308±16	160±47	13.8±2	1.8±0	N.D	229±48
	Cranberry	429±6	317±50	16.7±6	2.8±1	2.9±6	171±68
	Peach & orange	549±150	355±151	68.2±16	11.2±6	N.D	297±1
	Mango & orange	463±7	254±7	75.5±12	5.3±1	N.D	322±23
	Orange	580±144	341±134	124±33	5.2±1	N.D	368±15
	Summer pine	457±35	282±47	35.2±23	2.4±1	N.D	270±18
	Mango	793±8	312±10	113.8±1	9.5±0	N.D	739±28
	Hanepoot	95.7±106	N.D	3.1±1	0.5±1	N.D	260±8
Ceres	Apple	351±54	278±36	7.9±3	0.7±1	N.D	113±28
	Secrets of the valley	215±16	197±10	10.9±1	1.7±2	8.0±16	27.6±9
	Peach	599±68	411±66	61.2±4	7.9±4	N.D	289±2
	Medley of fruits	956±98	633±92	123±1	16.8±8	N.D	497±9
	Litchi	145±78	122±84	17.2±2	2.6±0	N.D	35.2±10
	Orange	694±81	399±74	124±12	8.3±2	N.D	455±10
	Cloudy apple & pear	1029±37	523±57	53.7±6	12.1±3	N.D	779±31

Brand Name	Flavour	Polyphenols (mg/L)	Polyphenols (mg/L excl. AA)	Flavonols (mg/L)	Flavonols (mg/L)	Anthocyanins (mg/L)	Ascorbic acid (mg/L)	
Ceres	Whispers of summer	332±27	205±21	61.5±4	6.6±2	N.D	196±10	
	Cranberry & kiwi	284±77	191±90	8.9	2.4±3	N.D	143±20	
	Tomato	636±78	636±78	133±22	5.8±1	N.D	N.D	
	Ruby grape fruit	758±27	495±36	148±18	3.8±1	N.D	405±14	
Minute Made	Breakfast punch	344±65	321±39	109±21	5.7±5	N.D	35.9±39	
	Apple	450±89	450±89	46.8±8	3.2±1	N.D	N.D	
	White grape	83.6±50	83.6±50	7.9±6	0.8±0	N.D	N.D	
	Naartjie & orange	339±9	289±2	124±22	4.8±0	N.D	77.3±18	
	Orange & peach	613±122	562±91	193±15	24.9±9	N.D	77.8±47	
	Mango	224±17	201±10	96.3±19	4.5±1	N.D	34.5±12	
	Orange	493±39	366±33	137±24	5.9±2	N.D	195±10	
	Guava	1118±18	921±261	122±19	22.7±12	N.D	303±374	
	Quali	Orange	342±34	309±5	128±43	6.4±0	N.D	50.8±45
		Litchi	48.6±21	48.6±21	43.6±1	3.8±0	N.D	N.D
		Tropical punch	251±36	249±38	46.5±12	5.2±1	N.D	2.4±2
		Pineapple & kiwi	55.2±8	55.2±8	15.3±2	1.3±0	N.D	N.D
Guava		708±54	542±102	42.9±7	9.1±6	N.D	255±74	
Apple		128±31	128±31	17.2±2	1.8±1	N.D	N.D	
Granadilla		232±3	232±3	57.1±8	4.2±1	N.D	N.D	
Mango		125±2	125±2	39.2±5	4.0±0	N.D	N.D	
Loganberry		611±75	611±75	40.0±6	4.0±0	6.05±1.2	N.D	

N.D.: None detected

4.3 Wines

A total of 13 red wines and 7 white wines were purchased for the analysis of the antioxidant capacity and content. Different cultivar wines from three different areas of the Western Cape (Paarl, Stellenbosch and Franschhoek) were analysed. The red wine cultivars and/or blends included Shiraz, Shiraz/Cabernet Sauvignon, Cabernet Sauvignon, Lanoy, Merlot and Pinotage. The white wine cultivars included Chardonnay, Chenin Blanc and Sauvignon Blanc. Appendix B summarises the variety (cultivar), the area and a short description of these wines.

The results for the antioxidant capacity and content for the wines are summarised in Table 4.3. The average red wine ORAC value (50080 $\mu\text{mole TE/L}$) was 8.21 times higher than that of the white wine (6102 $\mu\text{mole TE/L}$). Cao *et al.* (1995: 1738) found that red wine's ORAC value was 5.3 times that of white wines; however the ORAC_{PE} method was used. The average FRAP value for red wine in this study (22521 $\mu\text{mole/L}$) was 12.20 times that of the white wine (1847 $\mu\text{mole/L}$). The average TEAC value for red wine (11618 $\mu\text{mole/L}$) was 8.54 times that of the white wine (1360 $\mu\text{mole/L}$), which was very similar to that of the ORAC ratio. De Beer *et al.* (2003: 905) obtained higher values for South African red wine (14916 $\mu\text{mole/L}$) but slightly lower values for South African white wine (939 $\mu\text{mole/L}$) using the TEAC assay. There was a high positive correlation coefficient between FRAP and TEAC ($r = 0.9577$) between FRAP and ORAC ($r = 0.9866$) and between ORAC and TEAC ($r = 0.9264$) (Table 4.7). The red wines, in accordance with cited literature are rich in anthocyanins, catechins, flavones, flavonols, phenolic acids, proanthocyanidins and tannins. Singleton (1982: 215) reported a 20-fold difference between red wine polyphenols and white wine polyphenols which corresponds well to the 22-fold difference found in this study. Therefore, as expected, the antioxidant capacities and contents for the red wines were higher than those of the white wines. The high anthocyanin, flavanols, flavonols accounted for the high total polyphenols and thus the overall TAC value of red wines. The longer duration of maturation for Shiraz, and Lanoy (12 months) as compared to the others (9 months) could account for their slightly higher TAC values (Appendix 2) although samples sizes were too small to draw any significant conclusions. The white wine, which lacks anthocyanins and since red wine phenolic compounds are derived

from the skin, seeds and stems of grapes in which the contact between pomace and juice fermentation is prolonged (thus transferring more polyphenols to the juice during the first stage of wine making), had lower polyphenols content than the red wines and thus a lower antioxidant capacity (Pellegrini *et al.*, 2003: 2816). The differences in wineries where the wines were purchased, vinification techniques, wood maturation, differences in climate and soil type where the grapes were grown may have influenced the variation of phenolic contents within the cultivars. This was also noted by De Beer *et al.* (2004: 574) in a study on antioxidant activity of South African red and white cultivar wines and selected phenolic compounds.

Table 4.3: Antioxidants capacity and content of red and white wines

Colour	Cultivar	ORAC ($\mu\text{mole TE/L}$)	FRAP ($\mu\text{mole/L}$)	TEAC ($\mu\text{mole/L}$)	Polyphenols (mg/L)	Flavonols (mg/L)	Flavonols (mg/L)	Anthocyanins (mg/L)
Red	Shiraz 1	45777	22439	12700	3254	265	163	222
	Shiraz 2	52801	21738	12434	2967	200	133	186
	Shiraz 3	53122	21454	8144	2988	215	110	114
	Shiraz 4	44231	20084	12191	3114	183	112	134
	Shiraz/Cabernet Sauvignon	59462	24494	11223	3538	259	182	147
	Cabernet Sauvignon 1	53899	24661	13893	3416	204	173	189
	Cabernet Sauvignon 2	51390	22373	9325	3092	162	131	74.1
	Cabernet Sauvignon 3	49664	22857	14341	3401	225	109	71.1
	Lanoy	62596	25830	13507	3645	232	186	97.2
	Merlot	49613	21329	8252	3244	188	120	65.1
	Pinotage 1	45495	24377	12103	3303	219	177	225
	Pinotage 2	41731	20318	9683	2813	162	127	121
	Pinotage 3	41265	20819	13235	3014	188	136	168
White	Chardonnay 1	6932	1949	1310	176	34	35	N.D
	Chardonnay 2	7979	2164	1583	191	40	71	N.D
	Chenin Blanc 1	6516	1824	1360	137	25	31	N.D
	Chenin Blanc 2	5652	1738	1378	123	30	29	N.D
	Chenin Blanc 3	5158	1630	1184	122	29	19	N.D
	Sauvignon Blanc 1	5526	1875	1423	149	28	50	N.D
	Sauvignon Blanc 2	4953	1746	1283	124	34	18	N.D

N.D: None detected

4.4 Coffees and Teas

The antioxidant capacity and contents of the coffees and teas are summarised in Table 4.4. The teas were extracted in freshly boiled water for 3 and 5 minutes and then analysed. The duration of extraction affected the antioxidant capacity of the teas. Extraction for five minutes resulted in increased antioxidant content as well as increased antioxidant capacity. The average increase for the four black teas from 3 to 5 minutes was 36% in the ORAC assay, 46% in the FRAP assay and 58% in the TEAC assay. Flavanol content of the black teas was much higher compared to that of the coffees and rooibos. Pure (100%) coffee (Nescafe) showed a higher antioxidant capacity and polyphenolic content than the blended instant coffees and this is in agreement with the results of Pellegrini *et al.* (2003: 2812). The black teas were high in flavanols and flavonols which accounted for their high polyphenols levels and overall TAC as has been reported in the literature review. The black teas on average had a 50.5% higher antioxidant capacity than rooibos (using the ORAC assay and 5 minutes brewing time as the reference method), while the instant coffees had a 26.8% higher antioxidant capacity when compared to the rooibos.

As with the wine, the Pearson correlation coefficients for the coffees and teas were high ($r = 0.9417$) for FRAP and TEAC, ($r = 0.9727$), ($r = 0.9727$) for ORAC and FRAP. The only low correlation coefficients was for flavanols against the antioxidant capacity assays (FRAP, ORAC, TEAC). Pellegrini *et al.* (2003: 2817) found a correlation of $r = 0.997$ for TEAC and FRAP on alcoholic beverages, teas and coffees which was in agreement with what this study acquired.

4.4 Coffees and teas

Table 4.4: Antioxidant capacity and content of coffees and teas

Beverage	Brand	Brewing time (min)	ORAC (μ mole TE/L)	FRAP (μ mole/L)	TEAC (μ mole/L)	Polyphenols (mg/L)	Flavonols (mg/L)	Flavanols (mg/L)
Coffee	Ricoffi	-----	11496	4113	3011	663	157	3.5
	Frisco	-----	9700	2930	2364	408	148	2.1
	Koffiehuis	-----	9267	3368	1975	408	148	2.7
	Nescafe	-----	23172	9979	6819	1726	416	10.4
Tea	Glen	3	7158	2972	2126	664	43.0	93.2
		5	9884	4811	4015	901	83.6	156
		Change from 3 to 5 minutes	+38.08%	+61.88%	+88.85%	+35.69%	+94.42%	+67.38%
	Trinco	3	9034	4086	3466	456	70.4	125
		5	11257	5295	4767	722	102	170
		Change from 3 to 5 minutes	+24.61%	+29.59%	+37.54%	+58.33%	+44.89%	+36.00%
	Joko	3	11829	5701	4431	955	74.5	188
		5	15019	7685	6484	1133	119	266
		Change from 3 to 5 minutes	+26.97%	+34.80%	+46.33%	+18.64%	+59.73%	+41.49%
	5 Roses	3	7403	3751	3065	778	59.2	118
	5	12043	6294	5458	1109	121	205	
	Change from 3 to 5 minutes	+62.68%	+67.80%	+78.08%	+42.54%	+104.39%	+73.73%	
Rooibos	3	6897	1872	624	170	120	4.2	
	5	8007	2271	996	230	140	7.1	
	Change from 3 to 5 minutes	+16.09%	+21.31%	+59.62%	+35.29%	+16.67%	+69.05%	

Table 4.5: Pearson correlation coefficients (r) for fruit juice assays

Fruit juices	FRAP*	TEAC	ORAC	Polyphenols	Flavonols	Flavanols*
FRAP	---	0.8888	0.5533	0.9036	0.4180	0.6747
TEAC	0.8888	---	0.6920	0.8819	0.5341	0.7067
ORAC	0.5533	0.6920	---	0.6878	0.6638	0.5562
Polyphenols	0.9036	0.8819	0.6878	---	0.4851	0.6680
Flavonols	0.4180	0.5341	0.6638	0.4851	---	0.5087
Flavanols	0.6747	0.7067	0.5562	0.6680	0.5087	---

* Data log transformed to obtain a normal distribution before correlations were calculated.

Table 4.6: Pearson correlation coefficients (r) for coffees and teas assays

Fruit juices	FRAP	TEAC	ORAC*	Polyphenols	Flavonols*	Flavanols
FRAP	---	0.9417	0.9727	0.9784	0.5478	0.3585
TEAC	0.9417	---	0.8699	0.9331	0.2557	0.6277
ORAC	0.9727	0.8699	---	0.9345	0.6834	0.1805
Polyphenols	0.9784	0.9331	0.9345	---	0.4901	0.3750
Flavonols	0.5478	0.2557	0.6834	0.4901	---	-0.5522
Flavanols	0.3585	0.6277	0.1805	0.3750	-0.5522	---

* Data log transformed to obtain a normal distribution before correlations were calculated.

Table 4.7: Pearson correlation coefficients (r) for wine assays

Fruit juices	FRAP	TEAC	ORAC	Polyphenols	Flavonols	Flavanols
FRAP	---	0.9577	0.9866	0.9974	0.9719	0.9393
TEAC	0.9577	---	0.9264	0.9594	0.9424	0.9056
ORAC	0.9866	0.9264	---	0.9846	0.9611	0.9215
Polyphenols	0.9974	0.9594	0.9846	---	0.9740	0.9278
Flavonols	0.9719	0.9424	0.9611	0.9740	---	0.9290
Flavanols	0.9393	0.9056	0.9215	0.9278	0.9290	---

* Data log transformed to obtain a normal distribution before correlations were calculated.

The differences in the composition of fruit juices where some are made up of single juice, or a mixture of two or more juices where one is a base juice and or a mixture of two or more with a puree could explain why their antioxidant capacity poorly correlated unlike in the wines. The clarity of the beverage solutions before the analysis may also account for the poor correlation.

4.5 *In vivo* short term rooibos study

The effect of drinking rooibos on the antioxidant parameters in plasma was evaluated by measuring the plasma ORAC values, FRAP, total polyphenols, and GSH/GSSG ratio over a period of three hours. After a baseline sample of blood was taken (0 minutes), eight healthy male participants ingested 500 mL rooibos tea and blood samples were taken at 45, 90 and 180 minutes and analysed. Table 4.8 summarises the time trend results of the plasma ORAC, FRAP, polyphenols and GSH/GSSG ratio.

Table 4.8: Effect of Rooibos tea on blood antioxidant parameters

Antioxidant parameter	Time (0 min)	Time (45 min)	Time (90 min)	Time (180 min)
FRAP ($\mu\text{mole/L}$)	575 \pm 98 ^a	610 \pm 105 ^a	580 \pm 113 ^a	584 \pm 107 ^a
ORAC ($\mu\text{mole TE/mL}$)	31.4 \pm 2.7 ^a	32.4 \pm 4.3 ^a	32.1 \pm 2.8 ^a	32.5 \pm 1.7 ^a
Polyphenols ($\mu\text{g/mL}$)	174 \pm 10 ^a	169 \pm 8 ^a	175 \pm 8 ^a	172 \pm 8 ^a
GSH (μM)	801 \pm 127 ^a	851 \pm 145 ^a	809 \pm 119 ^a	815 \pm 84 ^a
GSSG (μM)	97 \pm 53 ^a	109 \pm 76 ^a	81 \pm 43 ^a	83 \pm 44 ^a
GSH:GSSG ratio	6.23 \pm 5.0 ^a	5.81 \pm 7.5 ^a	7.98 \pm 6.0 ^a	7.81 \pm 6.8 ^a

* Values in columns are means \pm S.D. of 8 participants done in triplicate. Means followed by the same letter (in each of the rows) do not differ significantly. If letters differ, then $P < 0.05$.

After the consumption of rooibos, the plasma FRAP increased to reach a peak (610 $\mu\text{mole/L}$) at 45 minutes (an increase of 6.10%) and then decreased to 584 $\mu\text{mole/L}$ after 180 minutes. The plasma ORAC values also increased slightly from a baseline value of 31.4 $\mu\text{mole TE/mL}$ to 32.4 $\mu\text{mole TE/mL}$ 45 minutes (increase of 3.18%) after the ingestion of the rooibos. It remained at this level after 180 minutes. The total polyphenols decreased slightly (decrease of 2.87%) after 45 minutes (from a baseline value of 174 $\mu\text{g/mL}$ to 169 $\mu\text{g/mL}$), and then returned to the initial baseline value after 90 minutes. The GSH levels increased from an initial baseline of 801 μM to reach a peak of 851 μM at 45 minutes (increase of 6.24%) after which it returned to the baseline value. The plasma GSSG levels also increased from an initial baseline value of 97 μM to reach a peak of 109 μM at 45 minutes (increase of 12.37%) after which it decreased to below the baseline value at 90 and 180 minutes. The GSH:GSSG ratio decreased from a baseline value of 6.23 to 5.81 but increased to reach a peak of 7.98 at 90 minutes and then remained at this level at 180 minutes. None of the increases or decreases observed over the 3 hour time period in any of the antioxidant parameters tested was statistically significant.

The *in vivo* effect of flavonoids remains a controversial topic as is their absorption and metabolism (Walle, 2004: 829). Several short term studies to show *in vivo* benefits of flavonoid rich beverages have been carried out. Whitehead *et al.* (1995: 32) investigated the effect of red and white wine ingestion on the antioxidant capacity of serum. Nine subjects ingested 300 mL of red or white wine and blood was collected at one and two hour intervals. The antioxidant capacity (using a chemiluminescent assay) of the serum increased by 18% after 1 hour and by 11% after 2 hours with red wine consumption. The white wine resulted in a 4% and 7% increase over the same time periods. Nardini *et al.* (2003: 49) tested the effect on plasma antioxidant capacity of 10 volunteers after ingestion of 200 mL brewed coffee. Using the total radical trapping antioxidant parameter assay they found an increase of 5.5% after 1 hour, maintaining at 4% after 2 hours. Benzie *et al.* (1999: 83) showed that the antioxidant capacity (using the FRAP assay) in human plasma increased by 4%, 40 minutes after ingesting green tea. Also using the FRAP assay, Duthie *et al.* (1998: 733) showed a significant increase in the antioxidant capacity of plasma 30 minutes after consumption of 100 mL of red wine. They also found a significant increase in the total polyphenol content of the plasma. Interestingly, Lotito and Frei (2004: 251) ascribed the increase of the antioxidant capacity in human plasma after apple consumption not to apple flavonoids, but to the effect of apple fructose on plasma uric acid. Although no statistically significant increases in the antioxidant parameters were found in this study after consumption of rooibos, the increases were of similar magnitude to those found in the other antioxidant rich beverage studies mentioned above.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Although several spectrophotometric and fluorometric assays have been employed to analyse the antioxidant capacity and content of the beverages, it is important to remember that although beneficial, these assays have their limitations. Data from this study was used to compile the most current database for antioxidant capacity and content of South African beverages. This database will not only be of importance to consumers seeking the most antioxidant rich beverages, but also for researchers investigating the health effects of dietary antioxidants, manufacturers of the beverages and functional food formulators searching for the best antioxidant health benefits for their products.

Since there are different types of antioxidant compounds in most beverages, the total antioxidant capacity of a given beverage may be the integrated action of different compounds instead of any single compound. Thus in order to evaluate antioxidant capacity of given beverages and their health promoting effects accurately, the possible interaction and synergistic effects of the components in their contribution to the antioxidant capacity must be considered. From the results it seems clear that a number of factors including, the type of assay used, method and duration of extraction (in the case of the teas), influence of additives (e.g. vitamin C), and sampling have an impact on the levels of antioxidant capacity of the beverages. Thus in order to compile any meaningful and reliable compositional database for these beverages, the aforementioned factors must always be considered.

Of the fruit juices analysed, the guava, green tea, breakfast punch and cloudy apple & Pear had the highest antioxidant capacities. The high antioxidant capacities of these fruit juices were as a result of their high polyphenol content. Results also showed that red wines have a higher antioxidant capacity than white wines and that black tea and coffee have a higher antioxidant capacity than rooibos.

High positive correlations were evident when comparing the three antioxidant capacity assays in wines as well as the teas. Lower correlations were however obtained when the fruit juice results were compared. This was probably caused by the high variety of polyphenols and other antioxidants present in each fruit juice (composed of, in some cases, only one fruit juice and in other cases a combination of fruit juices) while wines are only composed of the antioxidants of grapes and the tea from the antioxidants of just one plant (*Camellia sinensis*). Another reason for the lower correlation could be the analytical variation due to clouding agents in the juice or suspended solids which may have caused a variation in the analysis of the juices which was absent in the wines. The high positive correlation coefficient between antioxidant capacity and polyphenols content in all the beverages indicated that this class of compounds were the major contributing factor to their antioxidant capacity. The presence of Vitamin C in some of the beverages also contributed to the overall antioxidant capacity of the beverages. This study has shown that it is probably better to do a battery of antioxidant capacity assays when samples of high variable antioxidant content are to be compared.

Results of the *in vivo* study showed that, 45 minutes after the consumption of rooibos, there was an increase in the antioxidant capacity of the plasma as measured by the FRAP and ORAC assays, however no increase was evident in the total polyphenol content of the plasma. Therefore, it was possible that the increase in antioxidant capacity was caused by an increase in uric acid as described by some investigators (Section 4.5). Although not significant, the GSH:GSSG ratio also increased from the baseline value after 90 and 180 minutes.

5.2 Recommended future work

A database is a continuous and evolving system and should therefore be updated on a regular basis. New products on the market and seasonal factors are just some of the aspects contributing to this. However, in the case of beverages, the sheer number of brands and products already on the market will make this extremely challenging. For example, of the 560 wineries in South Africa in 2007, only 4 were tested in this study. In 1998, this figure was 315. The wine regions of South Africa are divided into 20 regions/districts/wards. Only 2 (Paarl and Stellenbosch/Fransshoek) of these were investigated in this study.

In order to obtain more statistically significant data, it is recommended that a similar *in vivo* study involving rooibos be performed, but on a larger number of volunteers. More information on the *in vivo* effect of beverages on plasma and red blood cells will be required to substantiate the putative increase in plasma antioxidant capacity. The bioavailability of antioxidants in the beverages, their metabolism and interaction with other dietary components, *in vivo* stability and retention of antioxidants by tissues needs to be investigated further.

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APPENDIX A
Fruit juice label information

FRUIT JUICES LABEL INFORMATION	
LIQUI-FRUIT	DETAILS /INGREDIENTS
1) CRANBERRY	Cranberry Juice, Apple juice, blended with Grape and/or Pear juice Vitamin C per 250 ml = 153mg or 204% of RDA
2) APRICOT	Apricot Puree blended with Grape and/or Apple and Pear juice Vitamin C per 250 ml = 150mg or 200% of RDA
3) MANGO & ORANGE	Mango puree, Orange juice, blended with Grape and/or Pear Juice Vitamin C per 250 ml = 328mg or 473% of RDA
4) PEACH & ORANGE	Peach puree, Orange juice, blended with Grape and/or Apple and Pear juice Vitamin C per 250 ml = 175mg or 233% of RDA
5) SUMMER PINE	Pineapple juice blended with Grape and/or Apple and/or Pear juice Vitamin C per 250 ml = 163mg or 217% of RDA
6) CLEAR APPLE	Apple juice Vitamin C per 250 ml = 75mg or 100% of RDA
7) ORANGE	Orange juice, blended with Grape and/or Apple and/or Pear juice Vitamin C per 250 ml = 195mg or 260% of RDA
8) RED GRAPE	Red grape juice Vitamin C per 250 ml = 60mg or 80% of RDA
9) BREAKFAST PUNCH	Orange juice, Guava puree, Granadilla juice, blended with Grape and/or Apple and/or Pear juice Vitamin C per 250 ml = 93mg or 124% of RDA
10) LITCHI	Litchi puree, blended with Grape juice Vitamin C per 250 ml = 153mg or 204% of RDA
11) BERRY BLAZE	Black currant juice, Strawberry juice, Guava puree, Raspberry juice, blended with Grape and/or Apple and/or Pear juice Vitamin C per 250 ml = 55mg or 73% of RDA
12) PASSION POWER	Passion fruit juice, Orange juice, blended with Grape and/or Apple and/or Pear juice Vitamin C per 250 ml = 173mg or 231 % of RDA
CERES	
1) APPLE	Pure Apple juice Vitamin C per 200 ml = 35mg or 58% of RDA
2) MEDLEY OF FRUITS	Guava puree, Pineapple juice, Papaya puree, Mango puree, Peach puree, Passion fruit juice, Apple and/or Grape and/or Pear juice. Vitamin C per 200 ml = 91mg or 152 % of RDA
3) HANEPOOT	Pure hanepoot grape juice Vitamin C per 200 ml = 55mg or 92% of RDA
4) WHISPERS OF SUMMER	Granadilla juice, Orange juice, Peach puree, Mango puree, Grape and/or Apple and/or Pear juice & Vitamins Vitamin A per 200 ml = 135µg or 15% of RDA Vitamin E per 200 ml = 2.25mg or 15% of RDA

	Vitamin C per 200 ml = 45mg or 60% of RDA
5) CRANBERRY & KIWI	Cranberry juice, Kiwi juice, Apple and/or Grape and/or Pear juice Vitamin C per 200 ml = 24mg or 40% of RDA
6) LITCHI	Litchi puree, Grape and/or Pear juice Vitamin C per 200 ml = 51mg or 85% of RDA
7) TOMATO COCKTAIL	Tomato juice, Salt, Worcestershire sauce Vitamin C per 200 ml = 40mg or 53% of RDA
8) CLOUDY APPLE/PEAR	Cloudy apple juice, Pear juice, Grape juice Vitamin C per 200 ml = 78mg or 130% of RDA
9) MANGO	Mango puree, Apple and/or Grape and/or Pear juice Vitamin C per 200 ml = 157mg or 262% of RDA
10) SECRETS OF THE VALLEY	Apple juice, cherry juice, blackcurrant juice grape and/or pear juice Vitamin C per 200 ml = 64mg or 107 % of RDA
11) PEACH	Peach puree, apple and/or grape and/or pear juice Vitamin C per 200 ml = 70mg or 117 % of RDA
12) ORANGE	Orange juice, apple and or grape and/or pear juice Vitamin C per 300 ml = 91mg or 152 % of RDA
13) RUBY GRAPE FRUIT	Ruby grape fruit juice, grape and/or pear juice Vitamin A per litre = 2.25mg or 15 % of RDA Vitamin C per litre = 85mg or 116% of RDA
MINUTE MAID	
1) GUAVA	Guava puree, sugar, citric acid and flavouring
2) NAARTJIE ORANGE	Grape juice and/or Pear juice, Naartjie juice, Orange juice, calcium citrate
3) ORANGE	Orange juice, Pear juice and/or Apple juice and Grape juice
4) ORANGE PEACH	Orange juice, Grape juice and/or Pear juice, Peach puree, calcium citrate
5) MANGO	Orange juice, sugar, Mango puree, flavouring and citric acid
6) WHITE GRAPE	Grape juice and Apple juice
7) APPLE	100% pure Apple juice
8) BREAKFAST BLEND	Grape and/or Pear juice, Orange juice, Granadilla juice, Guava puree, calcium citrate
QUALI JUICE	
1) ORANGE	100% Orange juice, Apple juice, citric acid, permitted flavour, natural colorant Preservatives: Sodium benzoate, potassium sorbate & Pimaricin
2) GRANADILLA	Apple juice, Granadilla juice, permitted flavour Preservatives: Sodium benzoate, potassium sorbate & Pimaricin
3) LOGANBERRY	Apple juice, Logan berry juice, permitted flavours and colorants Preservatives: Sodium benzoate, potassium sorbate & Pimaricin
4) APPLE	Apple juice, acidulates Preservatives: Sodium benzoate, potassium sorbate & Pimaricin
5) PINEAPPLE & KIWI	Apple juice, Pineapple juice, Kiwi juice,

	Preservatives: Sodium benzoate, potassium sorbate & Pimaricin
6) TROPICAL PUNCH	Apple juice, Mango puree, Orange juice, Guava puree, stabilizer, permitted flavours and colorants Preservatives: Sodium benzoate, potassium sorbate & Pimaricin
7) GUAVA	Apple juice, guava puree, stabilizer, citric acid, permitted flavour and colorants Preservatives: Sodium benzoate, potassium sorbate & Pimaricin
8) MANGO	Apple juice, mango juice permitted flavour and natural colorants Preservatives: Sodium benzoate, potassium sorbate & Pimaricin

APPENDIX B

Description of wines

KWV WINE EMPORIUM		
RED WINES		
VARIETY	AREA	DESCRIPTION
Golden Kaan Pinotage	Paarl	Pinotage at its fruity best. Sweet plums and raspberries on the nose with soft fruit and wood tannins which are well integrated and friendly. Matured for 9 months.
Shiraz	Paarl	Matured for 12 months, elegant, suggesting ripe berries overlaid with green pepper and spicy flavours.
Cabernet Sauvignon	Paarl	A complex, elegant wine with cassis and plums which is in perfect balance with oaky, vanilla and nutty flavours. Excellent follow through on the palate. Matured for 12 months.
Cathedral Cellar Merlot	Paarl	Deep, dark and intense. Filled with flavours reminiscent of ripe berry, mintiness. Matured in New French oak barrels which give way to a smokiness and balancing the wine.
WHITE WINES		
Chenin Blanc	Paarl	Pale straw appearance with excellent varietal expressions. Intense aromas which range from country ray, tropical fruit, apricots and spice. Best enjoyed young. Semi-dry.

NEDERBURG		
RED WINES		
VARIETY	AREA	DESCRIPTION
Pinotage '05	Paarl	Ripe berry flavours with soft compact fruit tannins. Very good maturation potential.
Cabernet Sauvignon '06	Paarl	Classic varietal taste – soft wood mellows the palate.
Shiraz '06	Paarl	Soft tannins, subtle berry and spicy flavours on the palate- good maturation potential.
WHITE WINES		
Sauvignon Blanc '07	Paarl	Crispy, easy drinking with a grassy palate.
Chardonnay '06	Paarl	Slightly wooded. Delicate balance of fruit and oak.

HIDDEN VALLEY		
RED WINES		
VARIETY	AREA	DESCRIPTION
Cabernet Sauvignon	Stellenbosch	Dark, intense mulberry in colour. Ripe, creamy, well concentrated and structured. Full bodied, finely balanced wine with tannins. Will benefit from careful cellaring for up to 6 years from vintage.
Shiraz 2005	Stellenbosch	Deep ruby red colour with purple hue. Enticing aromas of Turkish delight, violet and liquorice. Ripe sweet fruit flavours of plums and chocolate with a soft lingering black cherry finish.
Pinotage 2005	Stellenbosch	Deep red ruby with a crimson hue. Big bold style with surprising softness on palate. Arousing aromas of plum, mint, and mocca, chocolate and vanilla. Palate surprisingly and appealingly non pinotage. Rich seductive aromas of mulberry, with a ripe spicy, blackberry.
WHITE WINES		
Sauvignon Blanc 2007	Stellenbosch	Intense goose berry, lime, passion fruit and peach skin character explode from the glass. Mouth filling flavours of smoky fig and gooseberry and a long, lingering flinty finish.
Chenin Blanc 2006	Stellenbosch	Chenin blanc 86%, Viognier 10%, Sauvignon Blanc 4%. Intense litchi and lime characters with subtle guava undertones. Ripe smoky, pineapples and pear flavours on the palate with along creamy citrus finish.

BOSCHENDAL		
RED WINES		
VARIETY	AREA	DESCRIPTION
Lanoy 2004/2005	Franschhoek	Vinification: Picked at optimum maturity, juice fermented and left on the skins for two weeks before pressing to soften the fruit tannins. Blended after maturation. Wine made from Cabernet Sauvignon, Merlot and Shiraz. 25% of the wine matured in new French oak and the balance in 2 nd and 3 rd fill barrels for 12 months. Well integrated medium-bodied wine with good depth and fruit flavours. Multi-layered peppery spice dominating the berry fruits. Rewards with ageing. Ideal choice for rare roast fillet, casseroles and hard cheeses.
Shiraz 2006	Franschhoek	
Shiraz/ Cabernet Sauvignon 2005/2006	Franschhoek	Vinification: Grapes picked at optimum ripeness from three vineyards. After malolactic fermentation 40% of the wine is matured for 12 months in new French oak and the balance in 2 nd and 3 rd fill barrels. Has deep ruby colour with typical cabernet Sauvignon flavours. Cigar box with hints of mints and herbs. Earthy warmth on palate with good red berry fruit. Medium bodied wine fine, silky tannins.
WHITE WINES		
Chardonnay 2006/2007	Franschhoek	None
Chenin Blanc 2007	Franschhoek	None

