Extraction of grape seed to produce a proanthocyanidin rich extract

By

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Submitted in fulfilment of the requirements for the degree of

Master of Science

Department of Paraclinical Sciences
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Promoter: Prof J.N. Eloff

Preface

The experimental work described in this thesis was conducted in the Programme for Phytomedicine, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Prof J.N Eloff.

These studies have not been submitted in any form to any other University and the results represent work done by Havanakwavo Chikoto, except where the work of others is acknowledged.

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Havanakwavo Chikoto

Acknowledgements

I am deeply indebted to my supervisor, my wife, my supervisor's wife, my colleagues and my family back at home.

My supervisor has expertly guided me through this work, and together with his wife, Christna, made my two years comfortable, imparting lessons, implied and expressed, which I shall always cherish and use. Thank you.

My wife, Pelagia, my friend and confidante, who has been (and still is!), very loving, patient, and supportive through out the duration of this endeavour.

My colleagues who have been a great family away from home.

My family back in Zimbabwe who have been very supportive through this period I have been away from home

I use "my" with such great pride!

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Abbreviations used

AE	Antiradical efficiency
BAW	Butan-2-ol/acetic acid/water (14/1/5)
BEA	Benzene/ethanol/ammonium hydroxide (90/10/1)
C	Procyanidin product
CEF	Chloroform/ethyl acetate/formic acid (5/4/1)
DCM	Dichloromethane
DNA	Deoxy ribonucleic acid
DPPH	1,1 dipheny-2-picryl hydrazine
$DPPH_{t1/2}$	Time taken to reduce DPPH concentration by 50 %
EC_{50}	Concentration required to reduce initial DPPH concentration by 50%
EMW	Ethyl acetate/methanol/water (40/5.4/4)
GBE	Ginkgo biloba extract
GDP	Gross Domestic Product
HCl	Hydrochloric acid
^{1}H NMR	Proton nuclear magnetic resonance
I	Immunochoice product
LP1-LP5	Different stages of Laboratory product
LDL	Low density lipoproteins
NAPQ1	N-acetyl-p-benzoquinonemine
O	Pro-oxidin product
OEGS	Oil expressed grape seed
OPC	Oligomeric proanthocyanidins
Ppm	Parts per million
RGS	Red grape seed
ROS	Reactive Oxygen species
SOD	Superoxide dismustase
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TMS	Trimethylsilane
UVA	Ultraviolet A
WC	Warren Chemical product
WAFE	Water/acetic acid/formic acid/ethyl acetate (20/2/3/70)

Summary

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The aim of this study was to develop a cost-effective process to produce a grape seed extract of high quality using only non-toxic extractants. When this study was started no grape seed extract was produced in South Africa. Large quantities were imported to supply the local demand in the human and animal herbal medicine industry. Grape seed extract is mainly used to boost the immune system of humans and animals based on its antioxidant activity.

Initial work with different extractants established the polarity of the compounds with antioxidant activity. Antioxidant related activity was determined with five analysis techniques. Parameters such as the type, preparation and pre-treatment of grape seed, ratio of extractant to grape seed, composition of extractant, extraction time, extraction temperature, the interaction between temperature and time, drying temperature and subsequent treatment of extracts to remove compounds without antioxidant activity were evaluated. In all cases the cost implications of different methods used were kept in mind.

Not only the quality but also the quantity extracted is important in establishing a viable extraction plant. According to the patent literature most techniques used to date produce yields of 0.5 to 2.5 %. The laboratory product went through five stages of development. The percentage extracted for our five laboratory products decreased from 12.0, 10.1, 6.0, 5.9 to 5.5 % whereas antioxidant activity for our product increased from 30, 55, 67 78 to 172 % compared to the best available commercial product.

An important reason for the success of the procedure developed, is that we analyzed the different products developed with sophisticated procedures that gave information about the chemical composition of the extract. From this information procedures could be developed to increase the yield and activity.

The procedure has been licensed to a private company that is in the process of establishing a factory for the large-scale production of grape seed extract. The detail regarding the procedure is confidential to protect the intellectual property and industrial exploitation of the process.

Opsomming

The extraction of grape seed to produce a proanthocyanidin rich extract

Deur

Havanakwavo Chikoto

Voorgele ter vervulling van die vereistes vir graad
Magister Scientiae

Leier: Prof. J.N Eloff

in die
Department of Paraclinical Studies
Fakulteit Veeartsenykunde
Universiteit van Pretoria

Die doel van die studie was om deur die gebruik van nie-toksiese ektraheermiddels, ‘n koste-effektiewe proses te ontwikkel om ‘n hoë-kwaliteit druiwepitekstrak te ontwikkel. Met die aanvang van hierdie studie is geen druiwepitekstrak in Suid-Afrika vervaardig nie, ten spyte van die groot hoeveelhede wat ingevoer word om in die behoeftes van die kruiemedisynebedryf te voorsien. Druiwepitekstrak word grotendeels gebruik vir stimulering van die immuunstelsel as gevolg van die antioksidantaktiwiteit.

Aanvanklik is ‘n verskeidenheid ekstraheermiddels gebruik. Hieruit het dit duidelik geword wat die polariteit van die antioksidantaktiewe verbindings was. Antioksidantverwante aktiwiteit is volgens vyf verskillende metodes bepaal. Parameters soos die tipe, voorbereiding en voorafbehandeling van druiwepitte, verhouding ekstraheermiddel tot druiwepitte, samestelling van die ekstraheermiddel, ekstraksieperiode, ekstraksietemperatuur, interaksie tussen tyd en temperatuur, droogtemperatuur, en finale behandeling van ekstrakte om nie aktiewe verbindings te

verwyder, is ondersoek. Die kosteimplikasies van verskillende behandelings is voortdurend in aanmerking geneem.

Nie net die kwaliteit van die produk nie, maar ook die hoeveelheid geekstraheer is belangrik. Volgens die patent literatuur, het die meeste prosesse ‘n opbrengs van 0.5 tot 1.5%. Die laboratoriumproduk het deur vyf fases van ontwikkeling gegaan met die persentasieopbrengs wat verander het van 12.0, 10.1, 6.0, 5.9 tot 5.5%. Vir hierdie fases het die antioksidantaktiwiteit invergelyking met die beste kommersiële produk gewissel van 30, 55, 67, 78 to 172%.

Die sukses van die proses is grotendeels moontlik gemaak omdat ons die verskillende produkte se chemiese samestelling met gesofistikeerde prosedures ondersoek het. Hieruit het moontlikhede om die opbrengs en aktiwiteit te verhoog, na vore gekom.

Die proses is gelisensieer aan ‘n private maatskappy wat besig is om ‘n vervaardigingsaanleg te vestig. Die besonderhede oor die proses is vertroulik om intellektuele eiendom en die industriële ontginning van die proses te beskerm.

1 Introduction

Our research group was approached by Bioextracts (Pty) Ltd to develop an economically viable procedure to extract antioxidant compounds from grape seeds, with the aim of producing a proanthocyanidin rich herbal medicine.

The use of grape seeds, as a source of antioxidants is attractive because the seed is a waste product of wine making, a vibrant and economically important industry in South Africa. In 1999 the South African Wine Industry Information and Systems (SAWIS) commissioned a study, which found that the wine industry contributes R14 557 million to the annual GDP of the country (Anon, 2001).

In 1998 grape seed related extract was the eighth most popular herbal product in the United States of America, recording sales volumes of US$12.114 million, a 22% increase from the previous year. In South Africa, products such as Procyanidin®, Pro-oxidin®, Life extension®, OPC grape gold® and OPC gold blend® are readily available. When this research started there was no grape seed extraction plant in South Africa, consequently the grape seed extract to produce herbal medicines were imported from overseas.

1.1 Oxidative stress

Life on this planet uses oxygen and oxygen metabolites as electron acceptors in energy conversion. The constant generation of pro-oxidants, for example, oxygen free radicals is an essential part of aerobic life (Sies, 1991). Every cell in the living organism can generate reactive oxygen species and some cell types are specialized to do so continually (Sies, 1991). Free radical challenge also comes from external sources such as ionising radiation, toxins, drugs, chemicals and environmental pollutants. These challenges are met by a system of antioxidants, which maintain a steady state, and if disturbed leads to oxidative stress.

Oxidative stress is defined as a disturbance in the pro-oxidant - antioxidant balance in favour of the former leading to potential damage in a physiological system (Sies, 1991). A pro-oxidant is a toxic substance that can cause oxidative damage to lipids,

proteins and nucleic acids, resulting in various pathological events and/or diseases. Pro-oxidant is a synonym for reactive oxygen species. Thus, chemically a pro-oxidant is an oxidant of pathological importance. An antioxidant can be defined as a substance that, when present in low concentrations compared with those of an oxidizable substrate significantly delays or prevents a pro-oxidant initiated oxidation of the substrate (Prior & Cao, 1999). An antioxidant can efficiently reduce a pro-oxidant resulting in a system with little or no toxicity (Prior & Cao, 1999).

1.1.1 Occurrence and nature of free radicals

Reactive oxygen species (ROS) are oxygen containing free radicals. These include the superoxide anion ($O_2^{\cdot-}$), and hydroxyl radical ($^{\cdot}OH$). Free radicals contain an unpaired electron and because of the tendency of atoms to attain their normal valence shells, any free radical is a highly reactive species that easily donates electrons and reduces susceptible metabolites. The reactivity of free radicals ranges from relatively low as in the case of hydrogen peroxide to very high in the case of the hydroxyl radical (Packer & Glazer, 1990). Oxygen and ROS are some of the major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* (Wettasinghe & Shahidi, 2000).

Oxygen has a lone pair of electrons. Reactions with hydrogen atoms and molecules containing unpaired electrons such as transition metal complexes and free radicals result in triplet state oxygen. The triplet state oxygen can react with other molecules to yield ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (Wettasinghe & Shahidi, 2000). Two processes, which produce free radicals *in vivo*, have been identified and named the Fenton reaction and the Haber-Weiss reaction (Fig 1.1). It was suggested that traces of soluble iron or copper can catalyse these two reactions (Chevion *et al*, 1998).

$$O^{\cdot-}_2 + H_2O_2 \longrightarrow O_2 + OH + {}^{\cdot}OH \qquad \text{Haber-Weiss reaction}$$

$$Fe^{3+}/Cu^{2+} + O^{\cdot-}_2 \longrightarrow Fe^{2+}/Cu^{+} + O_2 \qquad \text{metal reduction}$$

$$Fe^{2+}/Cu^{+} + H_2O_2 \longrightarrow Fe^{3+}/Cu^{2+} + OH + {}^{\cdot}OH \qquad \text{Fenton Reaction}$$

Figure 1.1: The Fenton and Haber Weiss equation (Halliwell & Gutteridge, 1985).

These metal ions are insoluble under physiological conditions, and remain in solution only by becoming complexed to low or high molecular weight components. Consequently, they serve as catalytic centres for free radical production. For example copper forms complexes with many proteins and iron forms stable complexes with nucleotide diphosphates and triphosphates (Chevion *et al*, 1998). Hydroxyl radicals are highly reactive and have a short cellular half life of 10^{-9} sec, resulting in their action being limited to their site of formation.

Reactive oxygen species are generated in specific organelles under normal physiological conditions (Haraguchi, 2001). Peroxisomes generate hydrogen peroxidases as a by-product in the process of the β-oxidation of fatty acids. The reduction of molecular oxygen to water in the mitochondria proceeds by a series of single electron transfers resulting in highly reactive oxygen species (Cadenas, 1995). Under normal metabolic conditions, 2-5 % of oxygen consumed by mitochondria is converted to reactive oxygen species (Boveris & Chance, 1973). Some microsomal cytochrome P-450 reduce oxygen to the superoxide radical directly (Goeptar *et al*, 1995).

Although largely considered toxic, free radicals are beneficial to the organism in the stimulation of T-lymphocytes, in oxidative bursts and in apoptosis, under normal physiological conditions (Lopaczynski & Zeisel, 2001). Hydrogen peroxide at a micromolar concentration acts as a potent activator of T lymphocyte functions by activating transcription factors, inducing early gene expression as well as cell proliferation (Los *et al*, 1995).

Phagocytic cells can overproduce ROS, a process referred to as oxidative burst (Badwey & Karnovsky, 1980b). Oxidative bursts are important in killing bacteria by producing a variety of damaging toxic species of free radicals, which include the superoxide anion, nitric oxide and hypochlorite (Badwey & Karnovsky, 1980b).

The apoptotic cell death process is a basic biological phenomenon that occurs when a genetically encoded signalling programme is activated leading to the cell committing suicide (Korsmeyer, 1995). Apoptosis results in the orderly elimination of cells whilst minimizing tissue damage without inducing inflammation (Lopaczynski & Zeisel, 2001). It is involved in development, normal tissue turnover, negative selection in the immune system and cell death, (Wyllie, 1998). ROS regulate apoptotic signal transduction and may activate apoptotic pathways (Jabs, 1999).

1.1.2 Causes of oxidative stress

Pollution, ionising radiation, drugs, toxins and chemicals result in a high incidence of pro-oxidants, and hence cause oxidative stress.

1.1.2.1 Pollution

Important pollutants can be divided into natural and anthropogenic emissions (Stocker & Frei, 1991b).

Anthropogenic emissions are man made and are a result of using fossil and mineral fuel. Carbon monoxide, sulphur dioxide and nitrogen oxides are pollutants present in these emissions.

Natural emissions on the other hand are from swamps, volcanoes and sediments and release carbon dioxide, sulphur dioxide and hydrochloric acid into the atmosphere. Volatile organic compounds like methane are released from the intestinal contents of animals and these represent important air pollutants (Hippeli & Elstner, 1991).

- Combustion of organic materials, especially coal, results in the production of sulphur dioxide. Sulphur dioxide or hydrogen sulphate can react with the superoxide radical resulting in the formation of the highly reactive bisulphite radical that is a powerful initiator of several radical chain processes (Hippeli & Elstner, 1991).
- Large quantities of nitrogen oxides are also formed as by products of combustion processes. These oxides are free radicals themselves and due to their unpaired electrons they can initiate several reactions. The attacks at alkenic structures, resulting in the formation of free radicals are characteristic of these oxides

(Hippeli & Elstner, 1991). Nitrogen oxide and hydrogen peroxide, both components of cigarette smoke, can react to form the superoxide radical.

- Ozone is formed from oxygen and nitrogen oxides, catalysed by volatile organic compounds in a chain of atmospheric events. Ozonization of alkenes yield addition products, which decompose into ketones, and products that eventually yield peroxyl products. In the presence of aromatic compounds, ozone is decomposed into hydroxyl radical like compounds (Hippeli & Elstner, 1991).
- Petroleum soot and other soot particles have been shown to contain napthoquinones and nitroaromatic compounds. Napthoquinones and nitroaromatics undergo redox cycling resulting in the formation of superoxide and hydrogen peroxide (Hippeli & Elstner, 1991).
- Asbestos particles are airborne and they are highly toxic, especially in enclosed areas (Hippeli & Elstner, 1991). The fibres are able to generate hydroxyl and oxygen radicals from hydrogen peroxide via iron catalyses. They may act as immobilised catalysts for Fenton type or Haber-Weiss reactions (Weitzman & Graceffa, 1984).
- Halogenated compounds are widely used for industrial and agricultural purposes. Reductive dehalogenation of carbon tetrachloride, catalysed by cytochrome P450 in microsomes generates trichloromethyl and trichloromethylperoxyl radicals which initiate lipid peroxidation (Recknagel *et al*, 1989).

1.1.2.2 Ultraviolet radiation

For biological purposes the ultraviolet component of sunlight incident on the earth's surface is divided into two wavelength regions, which are termed UVA (320-380nm) and UVB (290-320nm) (Tyrell, 1991). The UVB region is generally believed to be responsible for the most damaging effect of sunlight including skin cancer.

Wavelengths in the UVA are also potentially damaging and can cause a wide variety of biological effects. Human exposure to UVA is increasing due to the longer exposure times permitted by the use of primarily UVB-absorbing sunscreens. The longer wavelength UVA seems to exert its effect through reactive oxygen species generation whereas UVB irradiation produces mostly pyrimidine dimers by direct action (Coohill *et al*, 1987). The biological actions of UVA that depend on the

presence of oxygen are attributed to DNA damage, membrane alterations, inactivation of enzymes, inactivation of mammalian cells and skin damage.

1.1.2.3 Drugs

Some drugs have been shown to result in the production of free radicals and are consequently potential sources of oxidative stress. These drugs include paracetamol, phytomenadione, adriamycin and carboplatin.

Paracetamol was introduced in the 1950s as an antipyretic and analgesic drug (Bergman *et al*, 1996). Today paracetamol is one of the most popular over the counter drugs for the alleviation of acute and chronic pain. Adverse effects of paracetamol taken at recommended therapeutic doses are rare, however when overdosed it may cause severe and sometimes fatal hepatic necrosis (Prescott, 1983). Paracetamol requires metabolic activation to exert its hepatotoxic effects. Liver microsomal cytochrome P450 can catalyze the conversion of paracetamol to a reactive electrophile and oxidising agent identified as N-acetyl-p-benzoquinoneimine (NAPQI) (Dahlin *et al*, 1984). Hepatotoxicity is more likely due to oxidative stress induced directly by NAPQ1 or via co-production of the superoxide radical and hydrogen peroxide through redox cycling between the paracetamol semiquinone radical and NAPQI (Bergman *et al*, 1996).

Phytomenadione, a quinone compound, is a synthetic analogue of Vitamin K. The drug is necessary for prothrombin biosynthesis in the liver, the enhancement of prothrombin in blood plasma and blood coagulability. The one electron reduction of menadione results in the formation of a semiquinone radical which can be oxidised by oxygen resulting in the production of a superoxide radical (Chiou *et al*, 1997a). Adriamycin is an anthracycline antibiotic that is one of the most frequently used in the treatment of human malignancies.

Adriamycin is however cardiotoxic. A dominant hypothesis to explain this involves the production of free radicals. It was shown that treatment of erythrocytes with adriamycin leads to protein oxidation, an essential marker of oxidative stress (DeAtley *et al*, 1999).

Carboplatin is a second-generation platinum containing anticancer drug. It is currently being used against a variety of cancer such as ovarian cancer and small cell lung cancer (Husain *et al*, 2001). Carboplatin may however induce an excess amount of nitrogen oxide and reactive oxygen species which in turn causes oxidative impairment of the cochlea leading to hearing loss (Husain *et al*, 2001).

1.1.3 Effects of oxidative stress

Pro-oxidants are involved in a number of disease conditions, including asthma, cancer, and cardiovascular disease (Alan & Miller, 2000). Their role in disease is manifested by various modes that may involve the damage and consequent mutation of chromosomes, inhibition of key enzymes and initiation of the peroxidation of lipid membranes (Haraguchi, 2001).

1.1.3.1 DNA damage

Damaging of DNA can be through the oxidation of bases, strand breaks, sister chromatid exchanges and the formation of micronuclei resulting in mutagenicity and/or carcinogenesis (McCall & Frei, 1999). DNA strand breaks occur when the deoxyribose chain interacts with hydroxyl radicals. Ferrous atoms that are bound to DNA can interact with the highly diffusible but relatively unreactive hydrogen peroxide in a metal catalysed Fenton reaction.

1.1.3.2 Lipid peroxidation

Lipid peroxidation is one of the major outcomes of free radical mediated injury to tissue. The process can be divided into three separate phases: initiation, propagation and termination.

ROS initiate the lipid peroxidation process by the abstraction of a hydrogen atom from a methylene group of a fatty acid containing two or more separated double bonds resulting in the formation of a conjugated stabilized carbon centered radical (Figure 1.2). The radical formed reacts with oxygen at a diffusion controlled rate (Maillard *et al*, 1983), giving rise to a peroxyl radical.

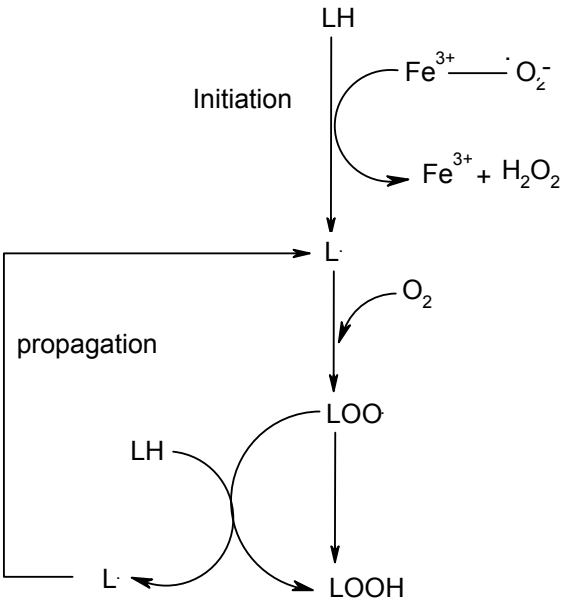


Figure 1.2: Lipid peroxidation process. LH, polyunsaturated fatty acid; L·, carbon centered radical, Fe^{3+}--$O_2^{\cdot-}$, perferryl radical, LOO·, lipid peroxyl radical; LOOH, lipid hydroxyperoxides (Andree et al 1997).

Propagation involves the peroxyl radicals removing a hydrogen atom from another fatty acid to form lipid hydroxyperoxides which can react with ferrous ions producing the alkoxyl radical. The alkoxyl radical is more reactive than peroxyl radical and it can reinitiate lipid peroxidation by abstraction of a hydrogen atom from an adjacent polyunsaturated fatty acid with the formation of a carbon centered radical and an alcohol as the end product. Lipid hydroperoxides cause reversible alterations to membrane structure and function. They are a source of highly reactive aldehydes (eg 4-hydroxynononal) that are capable of modifying both DNA and proteins resulting in mutagenic, genotoxic and cytotoxic events (Lopaczynski & Zeisel, 2001).
The reaction terminates when two radicals react to form non-radical products.

Cellular damage due to lipid peroxidation has been linked to serious health effects, which include, ischemia-reperfusion injury (Omar *et al*, 1991), coronary arteriosclerosis (Jackson *et al*, 1993), diabetes mellitus (Sugawara *et al*, 1988) and neurodegenerative diseases.

1.1.3.3 Protein modification

Protein oxidation may involve propagating radicals (e.g. alkoxyl radicals) and results in a variety of ROS (e.g. protein hydroperoxides) and stable products. Protein oxidation products include protein carbonyl derivatives, oxidised amino acids, side chain protein fragments and formation of advanced glycation products (McCall & Frei, 1999). Oxidation of proteins can result in loss of protein structure and function. The oxidation and nitration of intracellular proteins and the formation of protein aggregates may underlie the loss of cellular function and reduced ability of animals to withstand physiological stress. Protein modification can thus be linked to neurodegenerative diseases and cardiovascular disease (McCall & Frei, 1999).

1.1.4 Combating oxidative stress

The body has evolved mechanisms to deal with the deleterious effects of oxygen species. It does this by using *in vivo* defence munitions, which are either enzymatic or non-enzymatic.

1.1.4.1 Enzymatic Defence

Enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, phospholipid hydroperoxide glutathione peroxidase and glutathione S-transferase. Superoxide dismutase (SOD) catalyses the dismutation of the superoxide anion to hydrogen peroxide and oxygen. Hydrogen peroxide is further converted to water and oxygen. The activities of SOD increase the reaction by several orders of magnitude at physiological pH (Larson, 1988). It has however been noted that enzymatic antioxidants are present in very low concentrations in the plasma (Stocker & Frei, 1991b).

1.1.4.2 Non enzymatic Defense

Non enzymatic antioxidants are divided into metal binding proteins and small molecular antioxidants.

1.1.4.2.1 Metal Binding Proteins

Ceruloplasmin, transferrin, lactoferrin, albumin, haptoglobin and haemopoxin can tightly bind metal ions, mainly iron and copper. The metals will thus not be available

for reactions with primary oxygen reduction products which results in the formation of more reactant oxidants like the hydroxyl radical (Stocker & Frei, 1991b).

Ceruloplasmin is a copper transporting protein that has the ability to scavenge superoxide radicals. Ceruloplasmin can catalyse the oxidation of ferrous to ferric ions thereby inhibiting both iron stimulated lipid peroxidation and the Fenton reaction (Samokyszyn *et al*, 1989).

Most of the iron in human plasma is bound to transferrin and is thus not available for catalysis of lipid peroxidation. Transferrin can take up iron released from ferritin resulting in the inhibition of ferritin dependent lipid peroxidation (Aruoma & Halliwell, 1987).

Albumin is capable of binding copper tightly and iron weakly. Albumin bound copper can however still act as a reagent and may take part in Fenton reactions (Stocker & Frei, 1991b).

Haemopexin and haptoglobin are effective inhibitors of lipid peroxidation catalysed by haem and haemoglobin respectively, through their iron binding capabilities (Stocker & Frei, 1991b).

1.1.4.2.2 Small Molecular antioxidants

Water and lipid soluble antioxidants make up the non-enzymatic small molecular antioxidants

1.1.4.2.2.1 Water soluble antioxidants

Water-soluble antioxidants include, ascorbic acid, uric acid, pyruvic acid, bilirubin glutathione and glucose (Stocker & Frei, 1991b).

Ascorbic acid (Vitamin C) has a strong reducing potential, which makes it a suitable antioxidant. Ascorbic acid has been shown to scavenge superoxide radical, hydrogen peroxide and hydroxyl, aqueous peroxyl radicals and singlet oxygen effectively (Stocker & Frei, 1991b). During its antioxidant action ascorbic acid undergoes a two electron oxidation to dehydroascorbic acid with intermediate formation of the

relatively unreactive ascorbyl radical (Figure 1.3) (Bielski *et al*, 1975). Dehydroascorbic acid is relatively unstable and hydrolyses readily to diketogulonic acid. Dehydroascorbic acid can however be reduced back to ascorbic acid in erythrocytes and other blood cells

(a) (b)

Figure 1.3: Ascorbic acid (a) and its oxidation product, dehydroascorbic acid (b)

Uric acid efficiently scavenges free radicals (Figure 1.4) (Ames *et al*, 1981) and in addition has been shown to react with oxygen, ozone and nitrogen dioxide (Cadenas, 1998). Uric acid stabilises ascorbic acid in human serum possibly through the formation of stable complexes with iron ions (Davies *et al*, 1986).

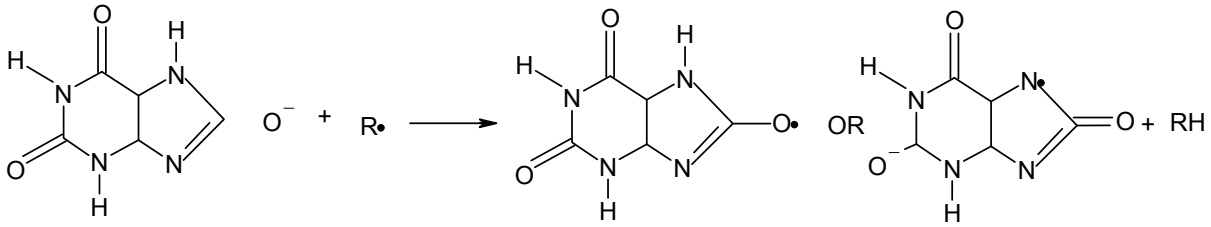


Figure 1.4: Antioxidant activity of uric acid

It is well established that glutathione is important in the reduction of cellular oxidative stress and detoxification of electrophiles. Glutathione can also act on the preventative level by spontaneously reacting with and scavenging a number of ROS (Sen, 1995). Glutathione disulphide, a cytotoxin, is a major by-product of these antioxidative reactions. The disulphide is usually reduced to glutathione, but when produced in large quantities the reducing enzyme fails to cope and the glutathione disulphide is detected in the serum (Sen, 1995). High levels of gluthathione disulphide therefore reflect high oxidative stress. Glutathione is thought to regenerate ascorbate from its oxidised by product.

Albumin bound bilirubin protects albumin bound linoleic acid from peroxyl radical induced oxidation *in vitro* (Stocker *et al*, 1987).

Human plasma also contains glucose and pyruvate, metabolites that can react with hydroxyl radicals and hydrogen peroxide respectively (O'Donnell-Tormey *et al*, 1985). Glucose does not react efficiently with radicals that are less reactive than the hydroxyl radical.

1.1.4.2.2.2 Lipid Soluble antioxidants

Lipid Soluble antioxidants include α-tocopherol (Vitamin E), ß-carotene (Vitamin A) and ubiquinol-10.

Vitamin E occurs in eight different forms in the diet and of the eight forms d-α-tocopherol and d-α-tocotrienol have the highest antioxidant activity in low density lipoproteins (LDL) (Traber, 1994). They act by inactivation of peroxyl radicals, resulting in the termination of two potential radical chain reactions (Figure 1.5) (Larson, 1988). The reaction results in the formation of an α- tocopheroxyl radical which in high concentrations becomes toxic, by producing a pro-oxidant effect. The water soluble antioxidants, ascorbic acid and reduced glutathione may be involved in regenerating α-tocopherol from its radical by product (Constantinescu *et al*, 1993).

Carotenoids prevent the formation of superoxide and peroxyl radicals. The carotenoids remain intact during quenching and hence can be used again. The most efficient antioxidant carotenoid is the open ring carotenoid lycopene, which contributes about a third of the total carotenoids in the body (Burton & Ingold, 1984). Carotenoids are also chain breaking antioxidants and thus inhibit lipid peroxidation. Lipid peroxidation activity of carotenoids appears to depend on the formation of radical adducts forming a resonance stabilized radical (Burton & Ingold, 1984). An *in vitro* synergistic effect of Vitamin E and carotenoids has also been observed in the inhibition of peroxyl radical induced lipid peroxidation (Palozza & Krinsky, 1992).

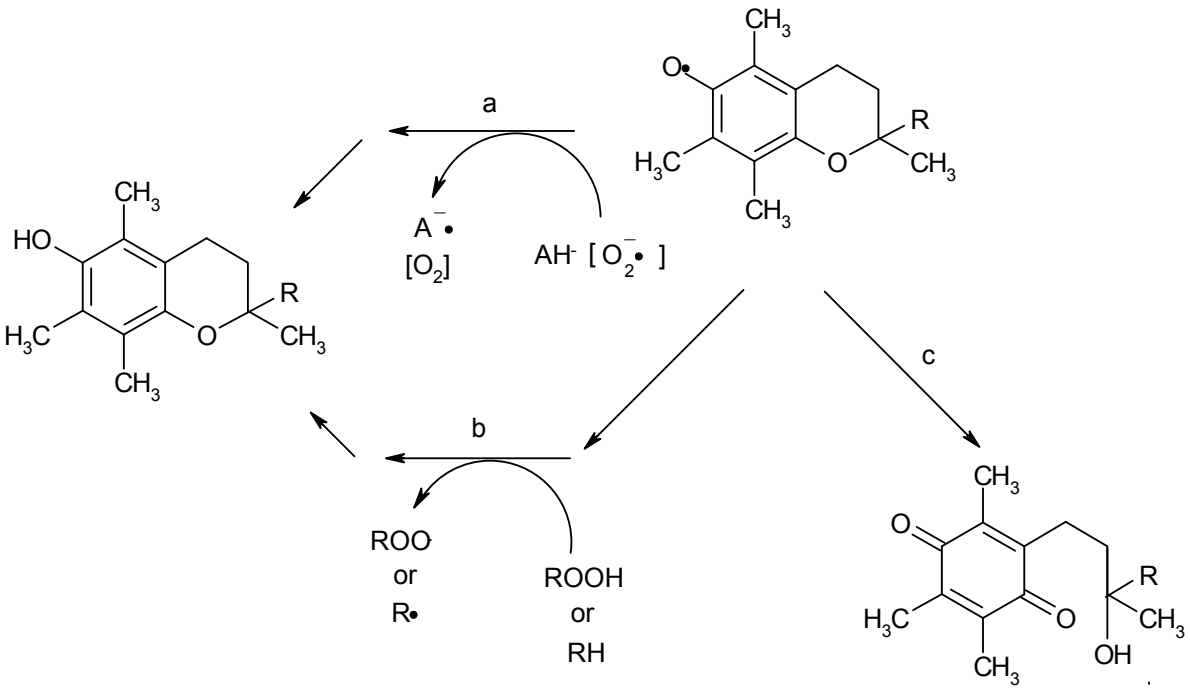


Figure 1.5: Reductive and oxidative decay pathways for the α-tocopherol to a quinone. Abbreviations: A, ascorbic acid; ROO·,lipid peroxyl radical; R·, pherol radical, a-recovery by ascorbate, b- reaction with RH, ROOH in LDL, c- oxidation lipid alkyl radical, R, C16 (Cadenas, 1995).

Ubiquinol 10 (reduced Co-enzyme Q10) is an effective lipid soluble chain breaking antioxidant present in human blood plasma. Ubiquinol 10 scavenges lipid peroxyl radicals with slightly higher efficiency than α-tocopherol (Stocker & Frei, 1991a) and appears to regenerate membrane bound α-tocopherol from the α-tocopherol radical (Figure 1.6).

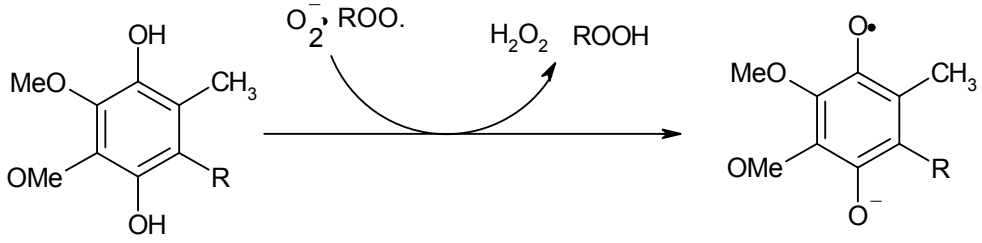


Figure 1.6: Formation of the ubiquinol antioxidant radical (Cadenas, 1995)

1.2 Phytochemical antioxidants

There is currently substantial interest in phytochemicals as bioactive components of food. The role of fruit, vegetables and red wine in disease prevention has been attributed to the antioxidant properties of their phenolic constituents. The literature is however not clear on what is required for a compound to exert antioxidant activity. Recent studies have shown that many dietary phenolic constituents derived from plants are more effective antioxidants *in vitro* than a-tocopherol or ascorbic acid and thus might contribute significantly to the protective effects *in vivo* (Rice-Evans *et al*, 1996a).

1.2.1 Phenolics

Antioxidant activity in plants has being attributed mainly to phenolic compounds. Phenolic compounds are widely distributed in nature and can be divided into four groups (Ribereau-Gayon, 1972).

- Benzoic acids, cinnamic acids and coumarins
- Flavones, flavonols and related compounds
- Anthocyanins
- Chalchones, dihydrochalconnes and aurones

In vitro studies have shown that phenolic compounds reduce oxidation of low density lipoprotein (LDL), particularly those phenolics with multiple hydroxyl groups which are generally the most efficient in the prevention of lipid and LDL oxidation (Meyer *et al*, 1998). Polymeric polyphenols are more potent antioxidants than simple monomeric phenolics. (Hagerman *et al*, 1998) demonstrated the higher antioxidant ability of condensed and hydrolysable tannins at quenching peroxyl radicals over simple phenols. Factors affecting antioxidant activity of monomerics are extended conjugation, the number and arrangement of phenolic constituents and molecular weight.

1.2.1.1 Flavonoids

The last three groups in the classification above constitute the flavonoids. Flavonoids are a widespread group of water-soluble phenolic derivatives (Goodwin & Mercer, 1972). They possess a diphenylpropane skeleton, where a C3 group links two benzene rings (C6-C3-C6) (Figure 1.7).

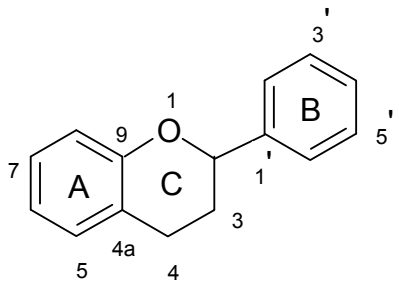


Figure 1.7: General structure of flavonoids

The individual groups are classified according to the state of oxidation of the C3 unit (C-2,3,4) in the molecule (Goodwin & Mercer, 1972)(Table 1.1). Differences also arise from the variation in number and arrangement of hydroxyl groups as well as the nature and extent of alkylation and/ or glycosylation of these groups. The preferred glycosylation site on flavonoids is the 3-position and less frequently the 7 position. Glucose is the most usual sugar residue but others include galactose, rhamnose, xylose (Rice-Evans *et al*, 1996a).

Table 1.1: Flavonoid classes

Flavonoid Class	**Example of class members**
Flavan-3-ols	Catechins
Flavones	Apigenin
Flavonols	Kaempferol
Flavanones	Naringenin
Dihydroflavonols	Taxifolin
Flavan-3,4-diols	Isoteracacidin
Anthocyanins	Cyanidin
Isoflavones	Genistein
Neoflavones	Dalbergin

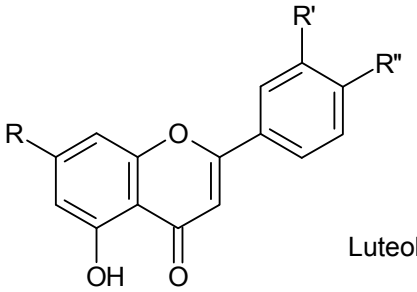
The free radical scavenging activities can be predicted from the chemistry of the flavonoids. This is because the reduction potentials of the flavonoid radicals are lower than those of alkyl peroxyl radicals and the superoxide radical, which implies that flavonoids may inactivate these ROS (Jovanovic *et al*, 1992).
Using free radicals generated in the aqueous phase, (Rice-Evans *et al*, 1996a), described three criteria important for effective radical scavenging activities by flavonoids,

(1) The presence of an *O*-dihydroxy structure in the B-ring confers higher stability to the radical form and participates in electron delocalisation. Quercetin and morin are both flavonols, morin however possesses dihydroxy groups which are arranged *meta* to each other in the B-ring, this results in a two fold decrease in antioxidant activity compared to quercetin.

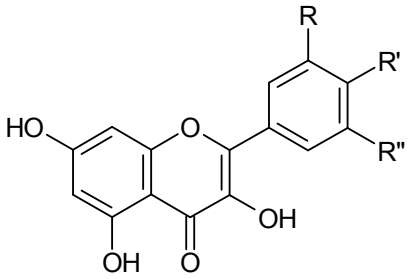
(2) The 2,3 double bond in conjugation with a 4-oxo function in the C-ring is responsible for electron delocalisation from the B-ring. Where these compounds react with free radicals the phenoxyl radicals produced are stabilised by the resonance effect of the aromatic nucleus. In this regard quercetin is two times more potent than catechin a flavan-3-ol.

(3) The 3 and 5-OH groups with 4-oxo function in the A and C rings are required for maximal radical scavenging potential. Luteolin a flavone lacks

the 5-OH and is thus a less effective antioxidant compared to quercetin (Figure 1.8).



	R	R'	R"
Luteolin	OH	OH	OH
Luteolin-4-glucoside	OH	OH	Glc
Luteolin-3'-7'-glucoside	Glc	Glc	OH



	R	R'	R"
Quercetin	OH	OH	H
Morin	OH	OH	H

Figure 1.8: Structure of some flavonoids

Flavonoid antioxidant activity in lipophilic systems may be through the chelation of copper ions via the *ortho* dihydroxy phenolic structure. Two points of metal attachment have been proposed, the *o*-diphenolic group in the 3',4'-dihydroxy position in the B-ring and the ketol structure, 4-oxo, 3-OH or 4-oxo, 5-OH in the C-ring of the flavonoids. Chelation is however, a process that is specific to different metal ions (Rice-Evans *et al*, 1996b). Flavonoids may also act as chain breaking antioxidants by scavenging lipid alkoxyl and peroxyl radicals or they may regenerate α-tocopherol through the reduction of the α-tocopheroxyl radical (Rice-Evans *et al*, 1996a). According to Cholbi et al (1991), free hydroxyl groups in the A-ring participate in the inhibition of lipid peroxidation. They also concluded that the presence of hydroxyl groups on the B-ring is not necessary although it increases the activity with some differences according to the structure type.

The radical formed by the association of a flavonoid antioxidant with a lipid radical is stabilised by the delocalisation of unpaired electrons around the aromatic ring. In an aqueous system, the *O*-dihydroxy structure in the B-ring plays an important role in stabilising the resultant free radical (Rice-Evans *et al*, 1996a).

Glycosylation of flavonoids seems to reduce their antioxidant activity when compared to the corresponding aglycones (Shahidi & Wanasundara, 1992). Rice-Evans *et al.* (1996) noted that in luteolin the antioxidant activity decreased with increasing number of glucose moieties. The mono-glucoside was one and a half times less potent than luteolin whilst the di-glucoside was two and a half times less potent than luteolin (Figure 1.8).

Flavonoids with the most hydroxyl groups are most easily oxidised whilst for simple flavonoid oligomers, the ability to scavenge free radicals is correlated to the degree of polymerization (Hagerman *et al*, 1998).

Catechins and their derivatives are discussed separately below because of their significance to this study.

1.2.1.1.1 Proanthocyanidins

Catechin and epicatechin make up the flavan-3-ol class and are among the most common flavonoids known. Proanthocyanidins are oligomers or polymers of monomeric flavan-3-ols units linked by carbon-carbon bonds (Bombardelli & Morazzori, 1995). These compounds are also referred to as procyanidins, procyanidolic oligomers or oligomeric proanthocyanidins (OPC), leucoathocyanidins or pycnogenols (Bombardelli & Morazzori, 1995). When proanthocyanidins are heated in the presence of acid the interflavan carbon-carbon bonds are broken and the flavan units released are converted by aerial oxidation to give anthocyanidins. The most common members of the group differ only in the number of hydroxyl groups in the phenyl B-ring (Figure 1.9) and these groups are never methylated (Gayon-Ribereau, 1972).

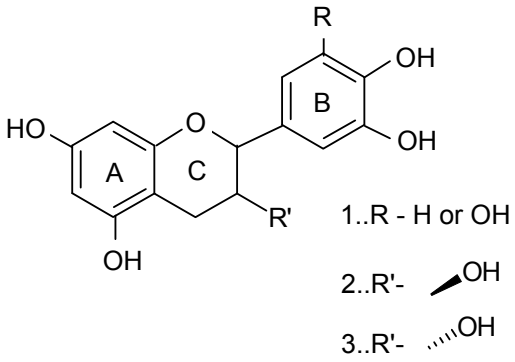


Figure 1.9: Basic flavan-3-ol structure. 2, (+)-catechin with R=OH. 3, (-)-epicatechin, R=OH

The most commonly reported flavan-3- ols in plants are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin. Gallic acid esters occur, with the acid moiety attached to the OH group at C-3 (Figure 1.10).

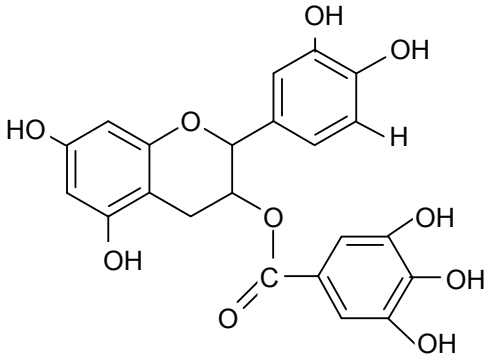


Figure 1.10: catechin gallate

Proanthocyanidin formation is thought to occur during the biosynthesis of flavan-3-ols. Formation of the oligomer is said to be through stereospecific capture of an intermediate carbocation by nucleophilic attack of the end product flavan-3-ol. Binding of flavan-3-ols characteristically takes place in the 4 position of the original flavan-3-ol (Figure 1.11), whilst esterification with gallic acid takes place at position 3 of the flavan-3-ol nucleus (Figure 1.10). OPC can consist entirely of flavan-3-ols structural units or units in which the flavan-3-ol oligomers are bound to saccharide structures (Haslam, 1989). According to (Ricardo da Silva *et al*, 1991)*et al.* (1991) tetramers or greater are referred to as polymers and the astringency of the molecule would increase. Oligomeric PCs are therefore less astringent, bind less strongly to proteins and are more soluble and mobile in the body (Ricardo da Silva *et al*, 1991).

Catechins do not have electron delocalization between the A and B rings due to the saturation of the heterocyclic ring, this makes them less potent antioxidants than flavonoids such as quercetin which has a 2,3 double bond and a 4-oxo function (Figure 1.8). However ester linkage via the 3-OH and the addition of a 5'-OH to form epigallocatechin gallate results in the enhancement of the antioxidant activity (Rice-Evans *et al*, 1996b). Proanthocyanidins have specificity for the hydroxyl free radical, trap lipid peroxides and free radicals, markedly delay the onset of lipid peroxidation and chelate to free iron molecules resulting in inhibition of iron induced lipid peroxidation (Rice-Evans *et al*, 1996b).

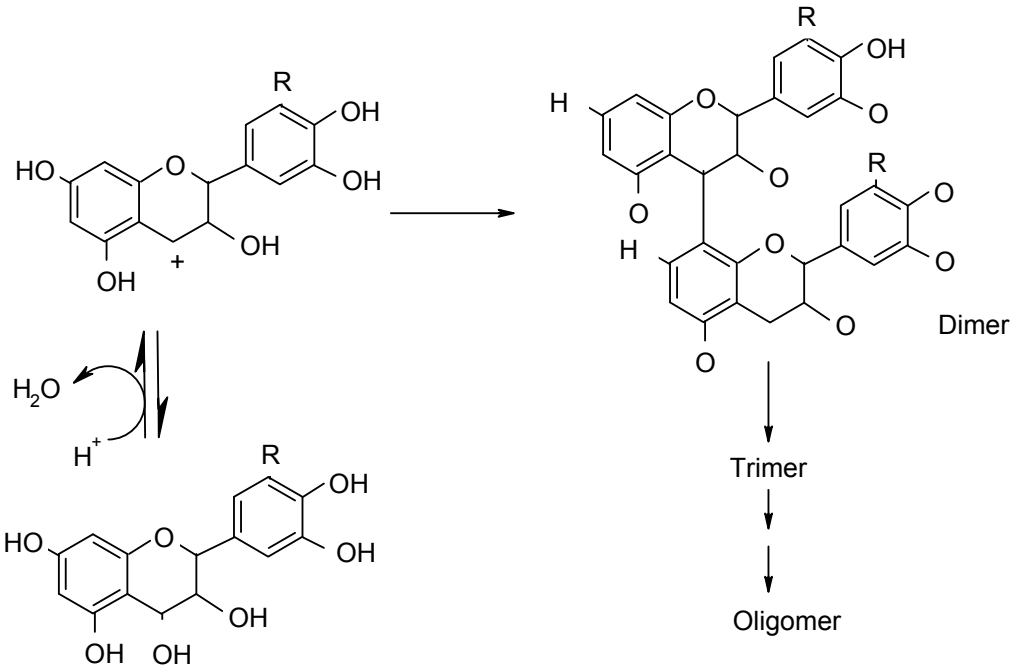


Figure 1.11: Formation of Oligomers

The interaction between iron or copper with proanthocyanidins extracted from *Vitis vinifera* has been studied by (Goeptar *et al*, 1995). They observed a procyanidin to iron complexing ratio of 1:2 and a procyanidin to copper ratio of 1:4. The stability of the resultant complexes is very high (Goeptar *et al*, 1995). Metal ions complexed to procyanidins are not available for the Fenton and Haber-Weiss reaction.

Low molecular proanthocyanidins are also known as sustained release antioxidants. They can remain in the plasma and tissue for up to seven to ten days while exerting

their antioxidant effects (Bagchi *et al*, 2000). Evidence exists that catechin is bio-available in humans (Packer *et al*, 1999). Forty six percent of radio-labeled 3-O-methyl catechin was recovered in urine as glucoronides and sulphates (Badwey & Karnovsky, 1980a). The sulphated forms of epicatechin and epigallocatechin, but not of their gallate esters have also been observed in the urine of human volunteers. All of these compounds with the exception of epicatechin gallate were also detected in plasma after ingestion (Lee & Jarwoski, 1987).

On the other hand high molecular weight proathocyanidins are not absorbed. They therefore exert their antioxidant effect in the digestive tract resulting in the sparing of other absorbable antioxidants and protection of lipids and proteins in the digestive tract (Hagerman *et al*, 1998).

Oligomeric procyanidins have a higher antioxidant activity than the monomerics due to the accumulative effect of the monomeric units. Polymeric procyanidins are 15 to 30 times more efficient at quenching free radicals than the monomers (Hagerman *et al*, 1998).

1.2.1.2 Coumarins

Coumarins are lactones which can be considered to be derived from o-hydroxycinnamic acid (Goodwin & Mercer, 1972). Coumarins have the C6-C3 conFig.uration. The C3 chain is in the form of an oxygen heterocycle.
Paya *et al.* (1992), investigated the effects of lipid peroxidation and found fraxetin, esculitin and daphnetin to be effective inhibitors of Fe^{3+} - ascorbate induced microsomal lipid peroxidation. Minami *et al.* (1994), investigated hydroxyxanthones and their antioxidant properties were effective in preventing lipid peroxidation in rat brain homogenates.

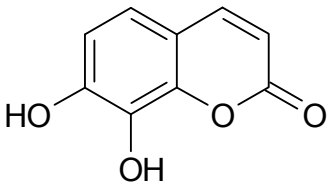


Figure 1.12: Structure of a coumarin

1.2.1.3 Phenylpropanoids

Phenylpropanoids are derived from trans cinnamic and *p*-coumaric acids (Figure 1.13).

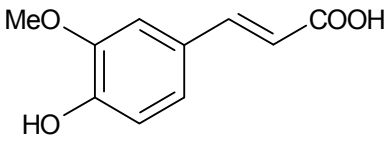


Figure 1.13: Ferulic acid, a phenylpropanoid

Hydroxy cinnamic acids are among the most widely distributed phenylpropanoids. Toda *et al.* (1991), investigated the effects of these phenol carboxylic acids in the generation of superoxide anion, ferulic acid scavenged superoxide anion. Caffeic acid and ferulic acid inhibited lipid peroxidation. In a study of the scavenging effects of propanoid glycosides, the compounds significantly inhibited both ascorbate-Fe^{2+} and Fe^{3+} -ADP/NADPH induced lipid peroxidation in rat liver microsomes. The number of phenolic hydroxyl groups in the molecule increased the antioxidant activity (Wang *et al.*, 1996).

1.2.2 Non-phenolic antioxidants

Non-phenolic antioxidants have been described as having antioxidant activity, an example being the terpenoids. Although some terpenoids may contain a phenolic group, not all terpenoids do.

1.2.2.1 Terpenoids

Terpenoids are compounds with varying number of carbon atoms that are clearly derived from isoprene units (Figure 1.14) (Goodwin & Mercer, 1972). It is believed that more individual terpenes and terpenoids exist than any other group of plant metabolites.

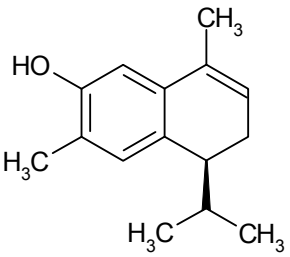


Figure 1.14: Sesquiterperne from *Heterotheca inuloides*

Lipophilic terpenoids have antioxidant activity and have been seen to have protective effects against oxidative stress in mitochondria (Haraguchi, 2001).

Re *et al.* quantified the activity of different antioxidants using the spectrophotometric-based Trolox equivalent antioxidant capacity decolourisation assay. In this assay there is a preformed radical 2.2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) that is generated by the oxidation of ABTS with potassium persulphate and is reduced in the presence of hydrogen donating antioxidants. The extent and rate of decolourisation is related to the potency of the antioxidants and the values obtained are compared to a Vitamin E water-soluble analogue, Trolox (Table 1.1).

Table 1.2: Comparison of antioxidant activity of different antioxidants using the TEAC method. Values represent activity relative to Trolox expressed as mM concentrations.

Compounds	**TEAC**
p-Coumaric acid	1.84 ± 0.06
Quercetin	3.03 ± 0.02
Luteolin	1.76 ± 0.03
Naringenin	0.89 ± 0.05
Cyanidin	2.48 ± 0.22
Ascorbic acid	1.05 ± 0.02
α-Tocopherol	0.97 ± 0.06
Glutathione	1.28 ± 0.04
Uric acid	1.01 ± 0.06
β-carotene	2.57 ± 0.03

1.3 Botanical sources of antioxidants

A number of botanical sources have been exploited for their antioxidant activity; these include pine bark, *Ginkgo biloba*, green tea and grape seed.

1.3.1 Pine Bark

The *Pinus maritima* blend of extract is patented under the trade name Pycnogenol ®. It is widely used as a nutritional supplement and as a phytochemical remedy for various diseases ranging from chronic inflammation to circulatory dysfunction. Pine bark extract is obtained by water extraction of the raw bark of the *P. maritima* followed by washing with ethyl acetate to remove some of the fat soluble substances (Packer *et al*, 1999). Monomers of catechin and taxifolin make up about 8% of the weight whilst condensed flavonoids make up 85% of the weight (Guo *et al*, 1999). The extract also contains phenolic acids such as caffeic acid and p-hydroxy benzoic acids as minor constituents. The flavonoid mixture has high antioxidant activity, inhibits enzyme activity and modulates the expression of some genes (Packer *et al*, 1999).

1.3.1 Green Tea

Tea leaves are considered to be important sources of polyphenols, they contain more than 35% of their dry weight in polyphenols (Serafini *et al*, 1996). Non fermented Green Tea Extract, *Camella sinesis*, contains catechin-based flavonoids, including catechin and epicatechin and their gallic acid esters. Epigallocatechin gallate occurs in the highest proportion (Alan & Miller, 2000). Black tea, produced by the fermentation of green tea, contains more free gallic acid, which makes it a strong antioxidant. According to Alan & Miller (2000) epidemiological studies of people who drink a lot of green tea indicates that it protects against gastrointestinal cancers.

1.3.2 Ginkgo biloba

The leaves of *Gingko biloba* were used in ancient China as evident in Chinese medical books dating back to 1436 AD (Wyllie, 1998). Standardised *Ginkgo biloba* Extract (GBE) contains 24% ginkgo flavone glycosides and 6% terpenes (Alan, 2000). Flavonoids, particulary quercetin and myricertin from GBE have been

identified as the compounds responsible for preventing free radical induced neuronal damage (Ginter *et al*, 1982).

Diabetics show signs of oxidative stress in the retina, resulting in thickened basement membrane and altered retinal vessel permeability. GBE improved functioning by decreasing retinal oxidative stress (Doly *et al*, 1992).

1.3.3 Grape seed

Vitis vinifera is a perennial woody vine belonging to the Vitaceae family, whose seeds are 2-4 in number and pyriform or ovoid in shape (Bombardelli & Morazzori, 1995). *V. vinifera* is a native of the Caspian Sea region in Asia Minor. Cultivation of *V. vinifera* probably began in this area and from here it spread both towards West and East (Bombardelli & Morazzori, 1995). *In vivo* studies have shown that grape seed proanthocyanidin extract is a better free radical scavenger and inhibitor of oxidative tissue damage than ß- carotene, α-tocopherol, ß-carotene and α-tocopherol combined (Bagchi *et al*, 1998). Grape seeds are a rich source of catechins and procyanidins, compounds known to exert an antioxidant effect (Prieur *et al*, 1994). In addition to the monomers (+)-catechin, (-)-catechin and (-)-epicatechin-3-O-gallate, dimeric, trimeric and tetrameric procyanidin have been identified in grape seed. About half of the procyanidin in grape seed consisted of more than five monomer units (Prieur *et al*, 1994). Several gallolyl procyanidins which are most commonly the gallate esters of the dimeric procyanidins and some free gallic acid are present (Ricardo da Silva *et al*, 1991).

1.4 Methods that have been used for extraction of grape seed

A number of methods that describe the extraction of proanthocyanidins have been developed and patented.

Franc *et al.* (1998) in US patent number 5 804 192 describes a process for obtaining procyanidol oligomers. The finely ground grape seed was extracted at temperatures between 55^{o}C and 60 ^{o}C in a mixture of deionized water, sodium chloride and acetone. The acetone was removed and the proanthocyanidin in the sample dissolved by adding ethyl acetate. The mixture was then concentrated and

precipitated by adding dichloroethane, an organic solvent in which the oligomers are not soluble.

Frangi et al (1996) describes a method which is almost free from monomers by subjecting grape seed extract to ultra-filtration on membranes of cut-off from 3 000 to 600 or to a selective extraction with ethers, or esters or mixtures of ethyl acetate and aromatic hydrocarbons.

The method of Nafisi-Movaghar et al (1999), involves preparing an aqueous mixture of proanthocyanidins by heating initial material under increased pressure and reduced oxygen. The resultant mixture is filtered through a polymeric membrane to remove larger molecular weight polymers and particulates. The permeate is then contacted with an adsorbent material which reversibly retains the procyanidins. The proanthocyanidins are then washed off and concentrated by reverse osmosis to produce a concentrated proanthocyanidin extract.

These are only a few of the large number of patented procedures described in the literature. Some methods make use of solvents, which are not safe for human consumption, for example dichloromethane and acetone, these solvents are volatile and hence can be removed by evaporation but trace amounts remain behind. Other methods are complex and would result in a very expensive product, for example the use of ultra-filtration and high performance liquid chromatography (HPLC) in the procedures.

1.5 Aim of study

The aim of this study is to use grape seed obtained from a local source to make a low cost and effective product, which will be able to compete with products already in the market. To do this various ways of extracting the active fraction will be investigated. The extraction of the antioxidant compounds from grape seed will be carried out with non-aggressive and non-toxic solvents and the product will be standardised by comparison with a commercial product using different analytical methods.

2 General Materials and Methods

2.1 Materials

Initially, red and white grape seed, a waste product of wine production (Figure 2.2b and Figure 2.2c) was investigated separately. As the study progressed I made use of oil expressed grape seed (OEGS) (Figure 2.2d). The seed was collected from Mr Wilhelm Arnold, who is based in Paarl, in the Western Cape (Figure 2.1). Mr Arnold obtains both red and white seed varieties from wine producers. He then sun dries and separates the skin from the seed. By using suitable machinery, he presses oil out of the seed. What remains, as a waste product, is a compressed seed "cake". It is this cake that we made use of in our subsequent extraction procedure.

Figure 2.1: Mr Wilhelm Arnold as pictured in the South African Sunday Times (9 December 2001) in a photo entitled: "PIP PROFITS:Wilhelm Arnold turns grape pips into healthy oil - Picture: AMBROSE PETERS"

Technical grade ethanol, hexane, methanol, acetone, dichloromethane, butanol was obtained from Merck. Hydrochloric acid, vanillin, 2,2-Diphenyl-1-picraylhydrazyl (DPPH), Folin Ciocalteu reagent, ammonia ferric sulphate hydrate, catechin, gallic acid, ascorbic acid were obtained from Sigma-Aldrich.

The commercial products Procyanidin, Pro-oxidin and Immunochoice were obtained from Biomox (Pty) Ltd. Biomox also supplied grape seed extracts from Warren Chemicals and Afriplex.

Figure 2.2: (a) Grape seed before and after the skins are removed. b, c and d shows the white, red and oil expressed grape seed.

2.2 Preparation of grape seed

Red and white grape seed partially within their skins were dried under shade at room temperature. The seeds were separated from the skins using a sieve, carbohydrate material attached to the seed may not have been removed completely by this process. The seeds were then milled into a fine powder with a Junkel and Kunkel model A10 mill. The powder was stored at room temperature until required.

The oil expressed grape seed (OEGS) was obtained in the form of a hard crust, which was first ground with the help of a pestle and mortar and then ground into a fine powder using the mill. For large scale studies the material was ground into a fine powder using a Macsalab mill 200.

2.3 Initial extraction procedures

Initial extraction procedures involved both direct extraction and serial extraction. Serial extraction refers to the process of extracting a given quantity of grape seed with one solvent, collecting the extract and re-extracting the marc with another solvent. Direct extraction refers to the process of extracting a given quantity of grape seed with one solvent only

2.3.1 Serial extraction

Red and white grape seed powder (2 g) were separately weighed and mixed with 10 ml of hexane. The mixture was vigorously shaken for 10 min and centrifuged at 4000 rpm for a further 10 min. The supernatant was decanted and filtered, the marc re-extracted with 10 ml of dichloromethane, agitated for 10 min and centrifuged at 4000 rpm for a further 10 min. The process was repeated using ethanol, acetone, methanol and water respectively. The filtered supernatant from each extraction was poured into a separate pre-weighed glass vial and the solvent removed under a stream of cold air. The weight was determined and stock solutions of 10 mg/ml made up for chromatographic analysis.

2.3.2 Direct extraction

Red and white grape seed powder were split into four samples of 2g each. The powder was extracted with 10 ml of either acetone, ethanol, methanol or water for 10 min on an orbital shaking machine and centrifuged at 4000 rpm for an additional 10 min. The supernatant was decanted and the marc re-extracted with 10 ml of the same solvent used in the first extraction. Supernatant from the same solvent was combined, filtered and dried. The extracts were then reconstituted in the original extractant to make stock solutions of 10 mg/ml for TLC.

2.4 TLC analysis of extracts

The chemical constituents of the grape seed were analysed using silica gel Thin Layer Chromatography (TLC) plates (Merck, Kieselgel 60 F_{254}) and developed with one of four eluent systems:

- Ethyl acetate/methanol/water: 40/5.4/4 [EMW] (polar/neutral)
- Chloroform/ethyl acetate/formic acid: 5/4/1 [CEF] (medium polarity/acidic)
- Benzene/ethanol/ammonium hydroxide: 90/10/1 [BEA] (non polar/basic)
- Butan-2-ol/acetic acid/water: 14/1/5 [BAW] (polar/acidic)
- Water/Acetic acid/Formic acid/Ethyl acetate: 20/2/3/70 [WAFE] (polar/acidic).

Development of the chromatograms took place in a pre-saturated closed glass tank. Samples were quickly applied and run to minimise oxidative modification of the constituent compounds. The separated compounds were observed under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600).

Further detection of separated compounds by using vanillin sulphuric acid (0.1g of vanillin: 28 ml of methanol: 1ml sulphuric acid) spray reagent and chromatograms were heated at 100 ^{0}C for optimal colour development (Stahl, 1969).

In order to visualise antioxidant compounds on the TLC plates DPPH, a purple coloured free radical, was sprayed and antioxidant compounds were seen to be yellow bands against a purple background (Takao *et al*, 1994).

3 Selection of the best extractant

3.1 Use of individual solvents

3.1.1 Introduction

The starting point of this study was to investigate how solvents of different polarities, extracted compounds from red and white grape seed. In this regard, hexane, dichloromethane, acetone, ethanol, methanol and water were used for serial and direct extraction. This was a preliminary investigation of the character of compounds present in grape seed and therefore I also used unsafe solvents in the procedures.

3.1.2 Results and Discussion

3.1.2.1 Serial extraction

In the serial extraction process, hexane extracted the highest quantity (10 %) of light green coloured compounds, in both the red and white seeds. Hexane, being a non-polar solvent would extract non-polar compounds. Triglycerides, free fatty acids, phospholipids, pigments and unsaponifiable substances have been reportedly extracted from grape seed and as such, grape seed is used as a commercial source of culinary oil (Gomez *et al.* 1996). The lowest quantity extracted was with methanol (1 %) in the red seeds and acetone (1.3 %) in the white seed (Figure. 3.1). As the polarity increased the colour of the extract changed from light green in hexane and dichloromethane through to reddish brown in methanol and whitish in water, which indicated a degree of selectivity of the different extractants. The total quantity extracted was identical for both the red and white seed varieties.

Three solvent systems were initially used for TLC of these extracts (Figure 3.3). CEF and BEA, both relatively non-polar solvent systems, did not separate the polar antioxidant compounds. EMW, a polar solvent system, showed better separation of the compounds, although some components remained at the origin.

Hexane and dichloromethane extracts did not have high concentrations of antioxidant compounds as seen by the absence of yellow bands upon

spraying with DPPH. It therefore appears that non-polar compounds from grape seed do not possess antioxidant compounds. Ethanol and methanol had more bands that showed antioxidant activity, whilst water extracts had minimal activity (Figure 3.3). Based on the observation that compounds that stayed at the origin had the most activity, the conclusion drawn was that the antioxidant compounds were very polar. This is not surprising since antioxidant activity in grape seed has been attributed mainly to highly polar oligomeric proanthocyanidins (Prieur *et al*, 1994)

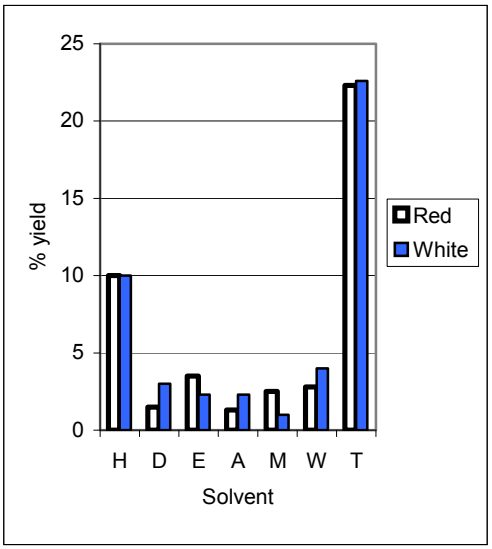


Figure. 3.1: Percentage of original mass extracted in serial extraction using hexane (H), dichloromethane (D), ethanol (E), acetone (A), methanol (M), water (W). T represents the total of all the solvents.

3.1.2.2 Direct extraction

In the direct extraction procedure acetone extracted the highest quantity in both the red (7 %) and white (12 %) seeds. This may be explained by the fact that, acetone, a medium polarity solvent, extracts both polar and non-polar compounds. The lowest quantity extracted was recorded in the water extract (2.5 %) for the red seed and ethanol (4 %) for the white seed (Figure 3.2).

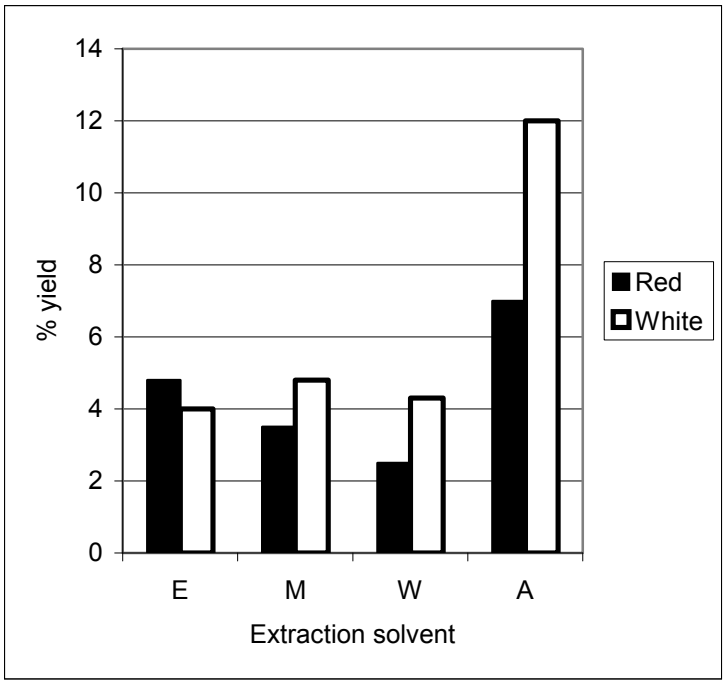


Figure 3.2: Percentage of original mass extracted in direct extraction using ethanol (E), methanol (M), water (W) hexane (H), dichloromethane (D), acetone (A),

Generally a lower mass was extracted with solvents in the serial extraction (section 2.3.1). This may be explained by the fact that solvents have an overlap of extraction ability, hence in the direct extraction they take up a wider range of compounds. In the serial extraction compounds that could have been extracted in one solvent might have gone into a preceding solvent.

The intensity of the antioxidant spots for ethanol, acetone and methanol in the direct extraction was lower than that of the serial extracts (Figure 3.4), possibly because of the presence of non-active non-polar compounds. It was observed in the direct extraction that the extracts could not dry off completely because of the presence of a green oily substance. This is in contrast to extracts from the serial extraction where extracts that came after the DCM extraction did not contain any oily substances. It was seen in section 2.3.1 that non-polar compounds did not have antioxidant compounds. Considering that for the purposes of TLC 10 mg/ml were spotted on the plates in each case, extracts from the direct extraction procedure that contained non-polar compounds would exhibit a lower intensity of antioxidant spots. However

extracts from the serial extraction in which the previous extraction with hexane and DCM acted as a de-fatting process would contain more antioxidant compounds and the spots would thus be more intense.

In both the serial and direct extraction procedures, it was observed that the red seed variety showed both higher extract colour intensity and brighter areas of antioxidant activity. Methanol being a relatively polar solvent extracted a smaller quantity of non-polar compounds and hence had more antioxidant compounds. The water extract showed minimal antioxidant activity, this is because water generally extracts sugars and salts.

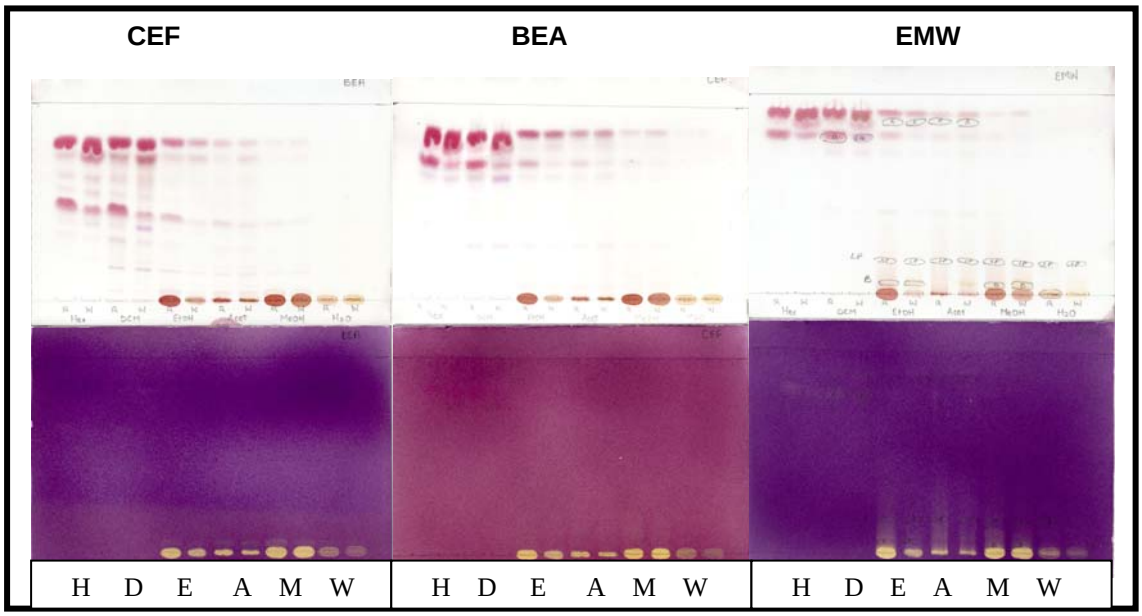


Figure 3.3: TLC profile of serially extracted grape seed, from left to right, using hexane (H), dichloromethane (D), ethanol (E) acetone (A), methanol (M) and water (W) as extractants on red (left of solvent symbol) and white (right of solvent symbol) seeds. CEF, BEA and EMW were used as the eluent system and the plates sprayed with acidified vanillin (top) and DPPH (bottom)

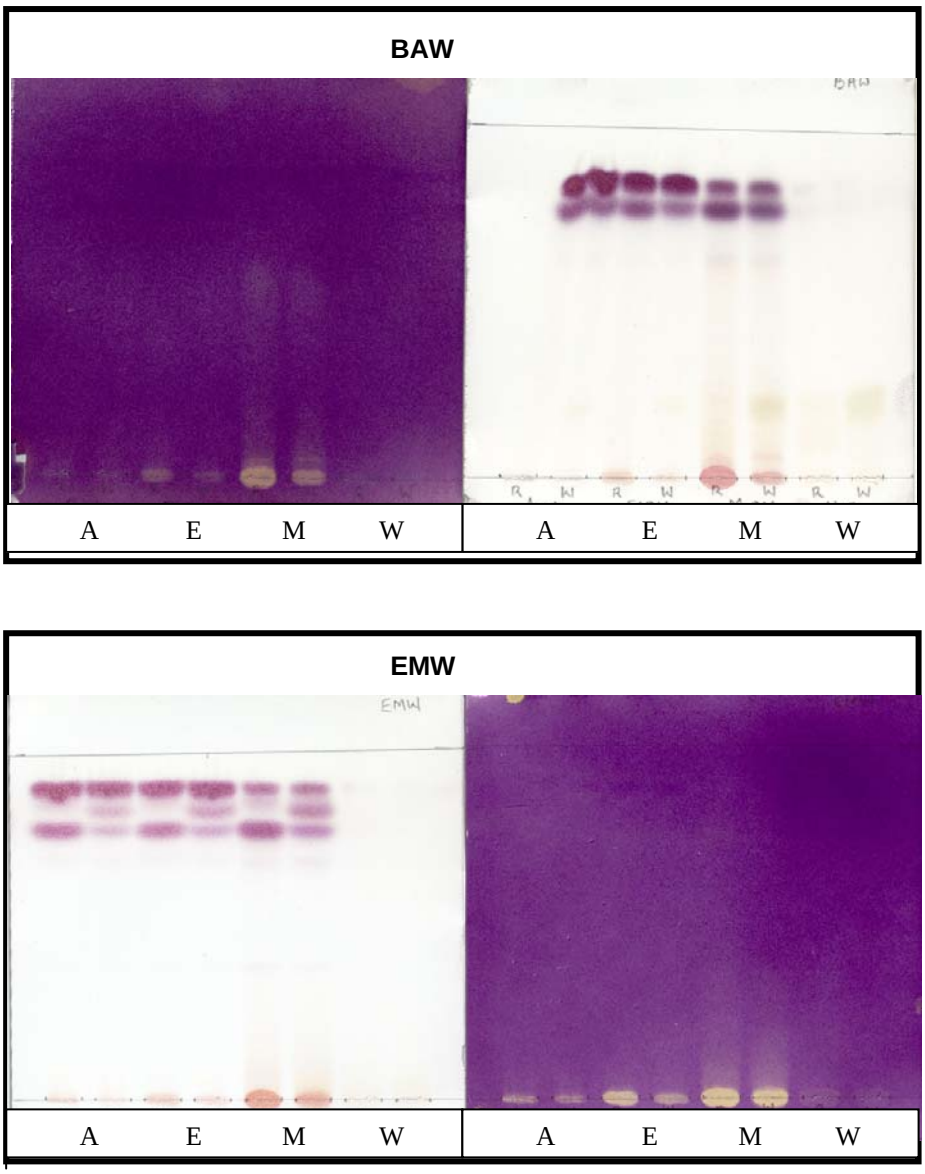


Figure 3.4: TLC profile of acetone (A), ethanol (E), methanol (M) and Water (W) extracts for red (left of solvent symbol) and white (right of solvent symbol) seed varieties using BAW (top) and EMW as the solvent system and spraying with vanillin and DPPH.

3.1.3 Conclusions

From the foregoing work it was concluded that non-polar compounds, extracted by non-polar solvents did not exhibit antioxidant activity. Furthermore water also did not extract a high concentration of compounds with antioxidant activity.

3.2 Extraction with ethanolic solutions

3.2.1 Introduction

Although solvents like acetone and hexane in section 2.3.1 and 2.3.2 extracted the highest yield, they exhibited the least antioxidant activity. Methanol effectively extracted antioxidant compounds, however we were not keen to make use of it. This was because of the possibility of residual traces appearing in an end product that would be ingested. Ethanol and water are safer solvents in extraction procedures that would result in ingestible products because they are safe, inexpensive and readily available and as such they have been used for patented effective extraction of grape seed (Nafisi-Movagher, 1999). However ethanol and water as individual solvents had low extraction yields (section 2.3.1 and 2.3.2). In work on extraction of proanthocyanidins from grape seed Pekic *et al.* (1998) concluded that the presence of water increases permeability of seed tissue, enabling a better mass transport by molecular diffusion. Based on the work of the above two groups, we decided to investigate the efficiencies of ethanol water mixtures as extraction solvents. TLC profiles of the extracts were compared to that of a Warren Chemical commercial product (WC) and a catechin standard. The following work was done on red grape seed, as it seemed to exhibit a higher antioxidant activity as seen in section 3.1.2.2.

3.2.2 Materials and methods

Five-gram samples of red grape seed powder were separately mixed with 50 ml of either distilled water, 20%, 40%, 60%, 80% ethanol in water or 100% ethanol. The mixtures were vigorously shaken for 10 minutes and centrifuged for a further 10 minutes. The yield extracted was determined and the extracts were analysed by TLC.

3.2.3 Results and Discussion

Different ratios of water and ethanol used to extract grape seed resulted in a larger quantity of material extracted (Figure 3.5), compared to the individual extraction abilities of ethanol and water (Figure 3.2). The quantity extracted gradually increased, reaching a maximum when 40 % ethanol solution was

used. The second best yield was obtained by 60 % ethanol solution, followed by the 80 % ethanol solution. It is surprising that 100 % ethanol extracted 8.5 % of the red grape seed, whereas it previously (3.1.2.2) extracted only 4.8 %. This may be ascribed to different solvent to powder ratios used, 5:1 in section 3.1.2.2 and 10:1 in this instance.

Since the TLC eluent systems used in section 2.3.1 and 2.3.2 did not separate the compounds well, another solvent system, WAFE, was used and it resulted in a better separation of the compounds (Figure 3.6). There was a higher occurrence of antioxidant compounds compared to the profile of the serial and direct extracts.

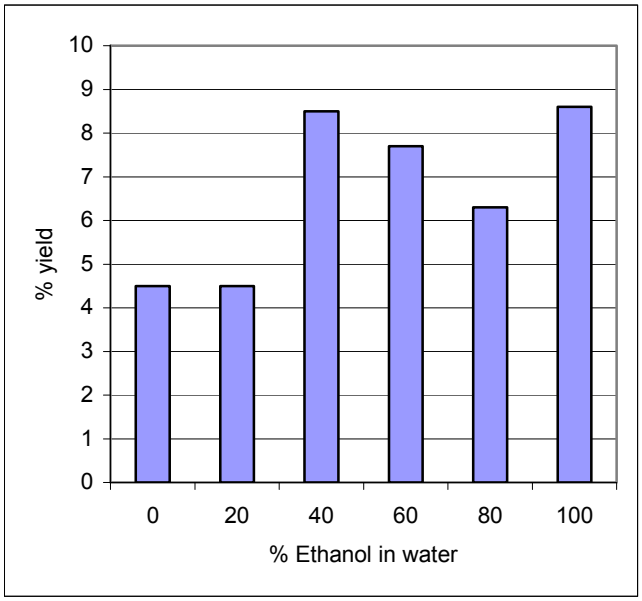


Figure 3.5: Extraction ability of different concentrations of ethanol in water

Generally the antioxidant TLC profile of the ethanolic extracts were similar, (Figure 3.6). Differences in antioxidant activity, if any, were therefore expected to be discerned by methods that quantify antioxidant activity. All extracts apparently contained catechin, and the profiles looked identical to that of the commercial product. At least three antioxidant compounds were visible in each case.

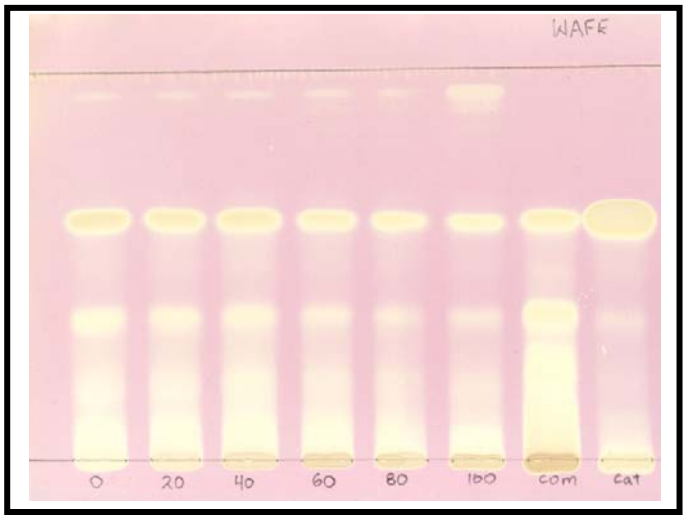


Figure 3.6: TLC of extracts obtained by different concentrations of ethanol in water using WAFE as the eluent, Catechin (cat) and the Warren Chemical Commercial product (com) were used as standards

3.2.4 Conclusions

Although water and ethanol extracted lower quantities from grape seed individually, their combination resulted in a greater quantity of material extracted with, 40 % ethanol in water having the highest yield. The quantity extracted was more than that extracted by acetone for the red seed (Figure 3.2). There was also an increased occurrence of antioxidant compounds as visualised on TLC plates. Subsequent studies used the ethanolic extractions to investigate more parameters that could be optimised.

4 Investigation of factors affecting extraction yield

4.1 Investigations on red seed

4.1.1 Introduction

The main thrust of this section was to evaluate the factors that might have an effect on extraction yield, with the aim of finding conditions that result in a high percentage yield. The issue of major concern is increasing the yield of antioxidant constituents whilst keeping the non-actives to a minimum.

Nafisi-Movagher *et al.*, (1999) in their patented procedure suggested that heating under reduced pressure resulted in effective extraction of proanthocyanidins. Franc *et al.* (1998), also describe a patented process in which temperatures of between 50 ^{0}C and 70 ^{0}C are used to extract proanthocyanidins.

Factors that were thought to affect extraction yield were, temperature of extraction, duration of extraction, ratio of grape seed to extraction solvent and the type of seed. These were investigated and described here. From work done in section 3.2 it was evident that 40 % ethanol in water resulted in the highest extraction yield and red seed (RGS) was superior over white. Further work as reported in this section was therefore done using 40 % ethanol solution to extract the red seed variety.

4.1.2 Materials and Methods

4.1.2.1 Influence of varying temperature

RGS powder (3 g) was extracted with 30 ml of 40% ethanol solution, at 20 ^{0}C, 40 ^{0}C, 60 ^{0}C, 80 ^{0}C and 100 ^{0}C. Although water:ethanol forms an azeotrope that boils at 80.5 ^{0}C, and experiments were done at high altitude, that also lowers the boiling point, the boiling 40 % was considered to boil at 100 ^{0}C for simplicity. Extraction at 20 ^{0}C was assisted with agitation whilst the bubbling effect of higher temperatures was considered enough agitation. Heating was done for thirty minutes under reflux after which the extracts were made up to the original volume to compensate for the loss of solvent during the extracting procedure. The extract from each temperature was separately centrifuged and filtered and dried.

4.1.2.2 Influence of duration of heating

To investigate the effect of duration of heating on the quantity extracted, RGS powder (40 g) was extracted with 400 ml of 40% ethanol solution by boiling under reflux for 180 min. 10 ml aliquots were removed from the mixture at 30 min intervals, centrifuged, filtered and dried.

4.1.2.3 Sample mass to solvent volume proportions

Different volumes of 40% ethanol solution, to grape seed powder ratios were extracted under reflux for thirty minutes at 100^{o}C. The percentage extracted at each given ratio was calculated.

4.1.3 Results and discussion

4.1.3.1 Influence of varying temperature

There was a linear relationship between temperature used during extraction and quantity extracted represented, by the equation y = 0.0428x + 5.096, having a 98.5 % fit when temperatures between 20 and 80 ^{0}C were used for extraction (Figure 4.1). The % yield increases gradually from *c* 6% at 20 ^{0}C to about 8.5 % at 80 ^{0}C. Boiling the ethanol: water mixture resulted in a % yield of 9 %. As the temperature increased, the kinetic energy of the solvent molecules increases, resulting in an increased interaction between the extractable material and the solvent and a consequent extraction of a higher quantity of material. An increased temperature may also result in the rupturing of cellular membrane, leading to the release of extractable compounds

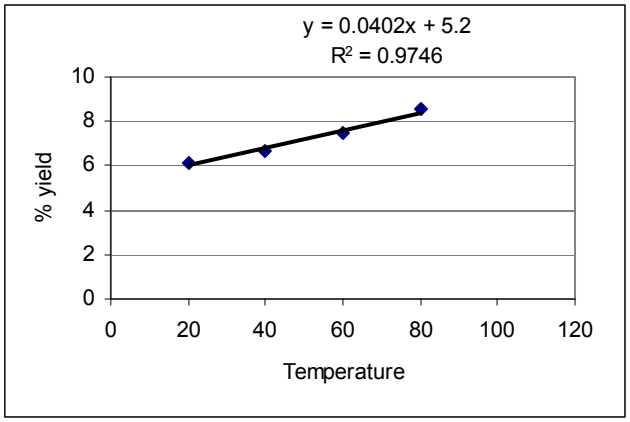


Figure 4.1: The relationship between temperature and % yield after 30 minutes of extraction

4.1.3.2 Influence of duration of heating

Working on the basis that extracting at 100^{o}C resulted in a large quantity of extractable material, the length of boiling time necessary to obtain the highest quantity was determined. There was a steep rise in % yield between 30 and 90 min , a slight increase in time would thus result in a significant increase in % yield. The % yield reached a maximum of *c* 15% at 180 minutes (Figure 4.2).

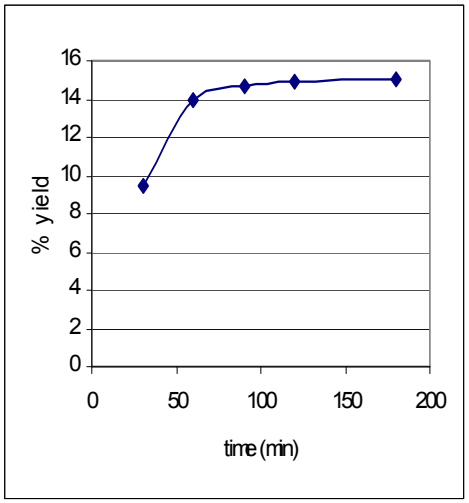


Figure 4.2: Effect of extraction at 100 ^{0}C for different heating time periods on extraction yield

The energy costs incurred on increasing the yield from 9.5 % to 15 % may however not compensate for the gain in quantity extracted, especially if some of the products may decompose upon increased exposure to high temperatures. Consequently I selected thirty minutes as the time of extraction, at least for the next series of experiments.

4.1.3.3 Sample mass to solvent volume proportions

As the amount of solvent increased the amount of material extracted increased (Table 4.1). Increasing the volume of solvent results in a steep diffusion gradient between the solid phase and the liquid phase resulting in a higher % yield. A smaller solvent volume reaches saturation point at a faster rate thereby diminishing the diffusion gradient. For the purposes of industrial application a large quantity of solvent would not be considered cost effective, thus a balance has to be found

between the volume of solvent used and the quantity extracted. It was decided that a ratio of 1:5, grape seed to solvent ratio could be optimal.

Table 4.1: Differences in quantities extracted at 100 ^{0}C under reflux, obtained with varying powder to solvent ratio

Powder:Solvent ratio	% Yield
1:3	4.45
1:4	4.47
1:5	5
1:6	6.66
1:10	8.83

4.1.4 Conclusions

An increase in temperature results in an increase in quantities extracted and the longer the extract is exposed to high temperatures, the higher the quantities extracted. Increasing the solvent ratio results in an increase in quantity of material extracted. A cost effective balance has to be maintained, when deciding on the duration of heating and the amount of solvent to sample ratio. It was thus decided that mixing in a ratio of 1:5 and extracting at 100^{o}C for 30 min could be optimal.

4.2 Investigations on oil expressed grape seed (OEGS)

4.2.1 Introduction

It was apparent from the serial extraction that non-polar compounds extracted with hexane did not have antioxidant activity and from the direct extraction that the presence of non-polar compounds decreased the antioxidant activity on a mass nase. Based on these two observations grape seed from which oil had been cold expressed (OEGS) was acquired: (a) as this was thought that this could result in a higher extraction yield since the non-polar content would be significantly reduced and (b) the OEGS was a low cost waste product. Although it was concluded in section 4.1.4 that 1:5 powder to solvent ratio was optimal, studies here were carried out with a 1:10 ratio to allow for comparison with previous investigations (section 3.2.3).

4.2.2 Materials and Methods

Five samples of seed powder (5 g) were extracted with 50 ml of either distilled water, 20%, 40%, 60%, 80% ethanol in water or 100% ethanol. The mixtures were vigorously agitated for 10 minutes and centrifuged for a further 10 minutes. The yield extracted was determined.

4.2.3 Results and Discussion

The quantities that were extracted with different ethanol in water ratios were higher compared to those extracted form the non-expressed red grape seed (Figure 4.3). This may be because, the absence of the viscous non-polar oil, results in a better interaction between the solid phase and the solvent. It was interesting to note that the same trend of % yield increasing and reaching a maximum at 40 % ethanol solution was repeated. Distilled water and 20 % ethanol solution had identical % yields, a repetition of the scenario observed in the red grape seed (Figure 4.3).

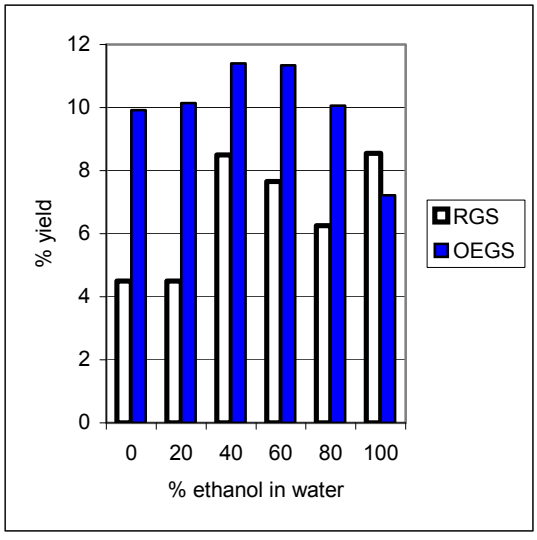


Figure 4.3: Extraction efficiency of different ethanol concentrations in water on OEGS obtained in the current investigations and on RGS obtained from Figure 3.5

4.2.4 Conclusion

The further use of OEGS was justified because of the increase in quantity extracted and the lack of oil that interfered with antioxidant activity. In further studies, OEGS was extracted in a ratio of 1:5 (powder to solvent), at 100^{o}C for thirty minutes. This resulted in a 12 % yield. Up to this stage the main focus was on the total quantity extracted. The quality of grape seed extract however depends on the antioxidant activity. The next chapter sought to quantify the antioxidant activity and content of polyphenols, present in the extract obtained under these conditions.

5 Quantitative and qualitative analysis

Optimal conditions generated in section 4 were used to OEGS using ethanol solutions described in section 3.2.2. Briefly, OEGS was extracted in a ratio of 1:5 with the extractant at 100^{o}C for thirty minutes. The extract was centrifuged, filtered and brought to complete dryness in a vacuum rotary evaporator at 95 ^{0}C resulting in a 12 % yield. Although the solvent was removed under vacuum leading to a lower temperature, the final product was taken to complete dryness. This would have led the extract being subjected to 95^{0}C for *c*. 45 min. These were the extracts analysed for their free radical scavenging activity and their polyphenolic constituents in this section.

5.1 Free radical scavenging activity

5.1.1 Introduction

The primary aim of this study is concerned with developing a product with high antioxidant activity. The mostly widely used methods for measuring antioxidant activity are those that involve the generation of a free radical species, which are then neutralised by antioxidant compounds (Arnao *et al*, 2001). The assays can be divided into two, the inhibition assays, where the extent of the scavenging by hydrogen or electron donation of a pre-formed free radical is the marker of antioxidant activity as well as assays involving the presence of antioxidant system during the generation of the radical (Re *et al*, 1999). In most cases, estimation of antioxidant activity is in aqueous systems and lipid soluble antioxidants would not be determined accurately.

This study made use of the method that involved reaction of free radicals with 1,1 diphenyl-2-picryl hydrazyl (DPPH), and following the reaction spectrophotometrically at 515 nm. 1,1 diphenyl-2-picryl hydrazyl (DPPH) (Figure 5.1) is a purple coloured stable free radical which does not dimerize and can hence be prepared in crystalline form (Tedder & Nechvatal, 1967).

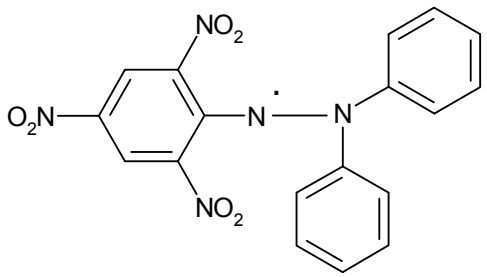


Figure 5.1: Chemical structure of 1,1 diphenyl-2-picryl hydrazyl (DPPH)

The DPPH method measures electron-donating activity of other compounds in the mixture and hence provides an evaluation of antioxidant activity due to free radical scavenging. DPPH is therefore reduced from a purple compound to a light yellow compound by electrons from oxidant compounds. Reaction of DPPH with hydroxyl groups involves a homolytic substitution of one of the phenyl rings of DPPH yielding 2-(4-hydroxyphenyl)-2-phenyl-1-picryl hydrazine as a major product whilst 2-(4 nitrophenyl)-2phenyl-1-picrylhydrazine is also formed via a series of secondary processes.
The concentration of DPPH at the end of a reaction will depend on the concentration and structure of the compound being scavenged.

5.1.2 Materials and methods

The effect of each antioxidant on DPPH free radical was determined according to an adaptation of the method described by Brand-Williams *et al.* (1995). A calibration curve was prepared by serially diluting a stock solution of 0.01 mg/ml (in methanol) and using methanol as a blank, the absorbances of the different concentrations were measured at 515 nm. Methanolic solutions (0.1 ml) of the different extracts were added to 3.9 ml of 0.025 mg/ml DPPH in methanol. Absorbances at 515 nm were measured at time zero and after 30 min for each solution. The absorbance of the initial DPPH solutions varied hence the exact initial DPPH concentration was calculated from a DPPH calibration curve. DPPH solution was prepared fresh on each day it was needed. Catechin, ascorbic acid and Warren Chemical grape seed extract product (WC) were used as standards.

5.1.3 Results and discussion

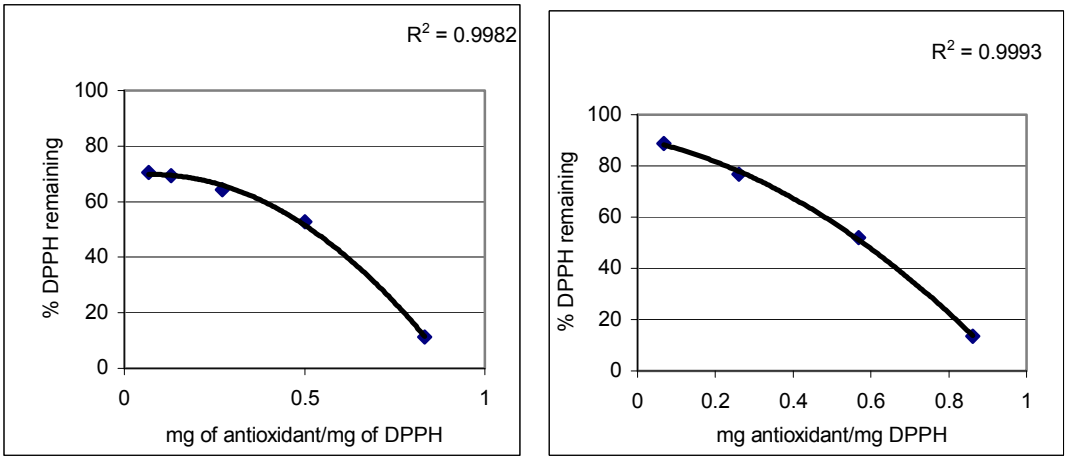
The exact initial DPPH concentration was calculated from the calibration curve that had the following equation.

$A_{515nm} = 26.3[DPPH] + 0.076 \quad r^2 = 0.9994$

For each antioxidant concentration tested the percentage of DPPH remaining after 30 minutes was calculated as follows

$\%\ DPPH_{remaining} = [DPPH]_{T=0\ min} / [DPPH]_{T=30\ min}$

The values obtained were plotted against mass of antioxidant/mass of DPPH, (Figure 5.2), from which the concentration required to decrease the initial concentration of DPPH by 50 %, EC_{50}, was obtained. The inverse of the EC_{50} values, the Antiradical efficiency (AE), was calculated (Figure 5.3). The higher the AE, the more potent the antioxidant.



a **b**

Figure 5.2: Relationship between mass of antioxidant per mass of DPPH with the percentage of DPPH remaining in the reaction medium for (a) 80 % and 40 % EtOH solution extract.

Antioxidant activity of the ethanol extracts gradually increased and reached a maximum when 80 % ethanol solution was used as the extracting solvent. 100 % ethanol has a 0.11 % lower activity than the 80 % ethanol extract and was 5.6 % less active than 60 % ethanol extract (Figure 5.3). From section 3.2.3, 40 % ethanol extract had the highest % mass yield; it however had an AE value less than that of the 60 % and 80 % ethanol solution extract. Differences in activity may have resulted from the extraction of different size

or type of compounds or the fact that inert compounds may have been extracted by 40 % ethanol. Phenolic antioxidant activity depends on different structural features such as the O-H bound dissociation energy and steric hinderance derived from bulky groups substituting oxygen in the aromatic ring (Shahidi & Naczk, 1995). The concentration of DPPH at the end of a reaction depends entirely on the concentration and structure of the phenolic compounds. Two theoretical termination processes exist:

$$DPPH^{\cdot} + DPPH^{\cdot} \longrightarrow DPPH{-}{-}DPPH \quad 1$$

$$PheO^{\cdot} + DPPH \longrightarrow DPPH{-}{-}PheO \quad 2$$

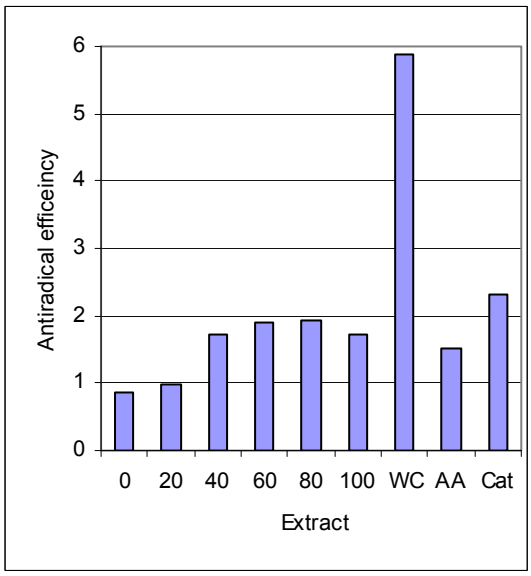


Figure 5.3: Antiradical efficiency values, determined by the DPPH method, for different extracts and standards, where, 0 to 100 are the different ethanolic extracts, WC-Warren Chemical commercial product, AA-ascorbic acid and Cat-catechin

The first equation may be inhibited by steric hinderance and equation 2 may be inhibited by aromatic ring substituent volumes (Sanchez-Moreno *et al*, 1998).

Equation (2) will compete with the reaction

$$PheO^{\cdot} + PheO^{\cdot} \longrightarrow (PheO)_2 \quad 3$$

Eighty percent ethanol could thus be extracting low molecular weight oligomers, which do not obstruct the interaction between DPPH and the hydroxyl groups. Forty percent ethanol, which extracted the highest % yield but had low antioxidant activity, could have been extracting either high molecular weight compounds that could not interact with the DPPH.

The commercial product imported by Warren Chemicals (WC) had an activity that was three times higher than the 80 % ethanol extract (Figure 5.3). The method used was either insufficiently selective for antioxidant compounds or it inhibited the activity of the compounds. High temperatures used in the extraction and drying processes may have been responsible for the latter. These temperatures were used in the extraction process based on the premise that an increase in temperature resulted in an increase in quantity extracted (section 4.1.3.1).

In addition the extract was taken to complete dryness in the rotary evaporator, which may have exposed it to 95 ^{0}C for *c.* 45 min that may have decomposed the antioxidant compounds. Unfortunately the process for preparing WC is an industrial secret, it may have been spray dried, a procedure that would not expose the antioxidant constituents to extreme temperatures.

5.1.4 Conclusion

Although 40 % ethanol in water extracted the greatest quantity as seen in section 3.2.3, 80 % ethanol in water had the highest antioxidant activity. The activity of the ethanol extracts compared to the commercial product was very low representing an activity that only 33 % of that of the commercial product.

5.2 Analysis of polyphenolic content

5.2.1 Introduction

The study of condensed tannins has been difficult because of the structural complexity of these compounds. According to Waterman & Mole (1994), commonly used quantification methods include reactions of the A-ring with an aromatic aldehyde (vanillin-HCl), oxidative depolymerization of

proanthocyanidins (Butanol-HCl test), method) and oxidation-reduction reactions (Folin Ciocalteu method).

5.2.1.1 Vanillin HCl method for proanthocyanidins

The Vanillin-HCL method was first developed by Burns in 1971 to measure specifically the presence of flavanols (Price *et al*, 1978). The original method was modified by Price *et al.* (1978), who noted that it did not describe how the conditions were selected and which parameters must be closely controlled to ensure reproducibility.

Vanillin, 3-methoxy, 4-hydroxy benzaldehyde, (Figure 5.4a) in acid solution captures a proton and gives an electrophilic radical (Figure 5.4b), which is able to condense with the aromatic with activated sites on the benzene ring (Figure 5.4c) to produce a coloured product (Figure 5.4d) (Ribereau-Gayon, 1972). An activated benzene ring site is described as one that contains an excess of electrons. This happens when there are electron donating groups like the hydroxyl group which are attached to the benzene ring. An increased electron density enhances the ability of the ring to undergo substitution reactions.

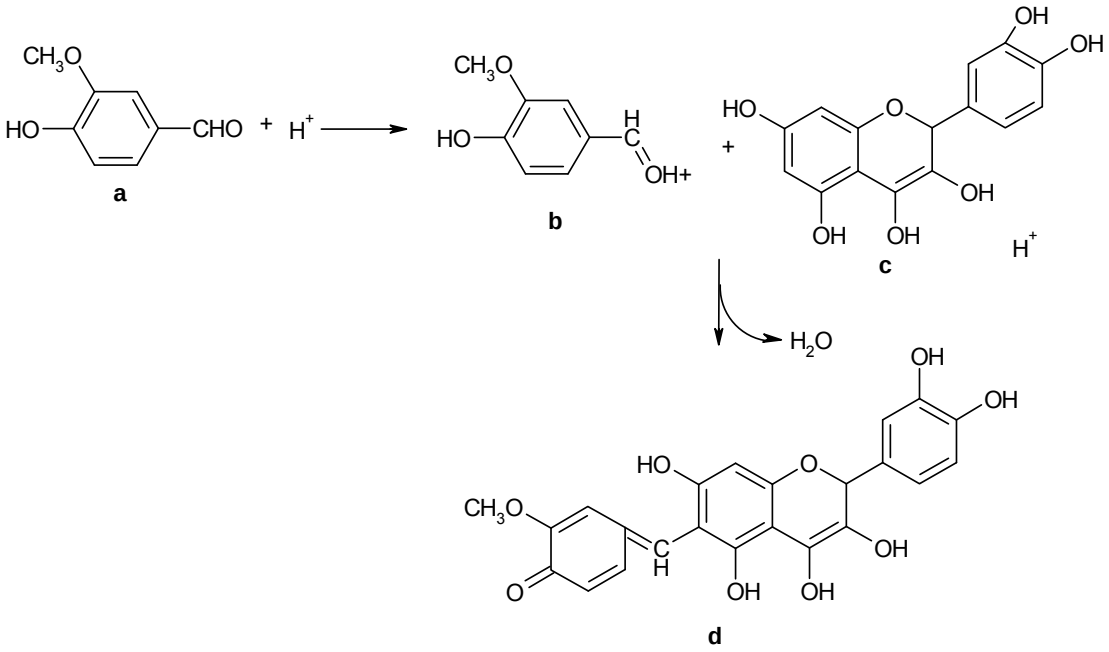


Figure 5.4: Reactivity of acidified vanillin with catechin (Gayon-Ribereau, 1972).

Other groups like the carbonyl group have an electron withdrawing effect and consequently decrease the reactivity of the benzene ring. Catechins have alternating electron donating hydroxyl groups on the A-ring, this makes position six and eight very active. This activity is enhanced by the absence of electron withdrawing groups.

5.2.1.2 Folin-Ciocalteu test

The Folin-Ciocalteu method determines the total free phenolic groups and is therefore a method to determine total soluble phenolics. The reaction is based on the reduction of phosphomolybdic acid by phenolics in aqueous alkali. The chromophore produced is a blue phosphotungstic-phosphomolybdic acid, which according to Schofield et al (2001), is of undefined structure. The chemistry of this method is not well understood (Schofield *et al*, 2001).

5.2.1.3 Butanol-HCl test

This is a colorimetric reaction which is specific for the polyflavan structure, using an acid catalysed oxidative depolymerization of condensed tannins to yields red anthocyanidins (Figure 5.5) (Schofield *et al*, 2001). The presence of transition metal ions in the assay is an important factor for colour development (Hagerman *et al*, 1998). Ferric ions are considered the most efficient transition metal ions in catalysing the colour formation of the butanol-HCl reaction (Porter *et al*, 1985).

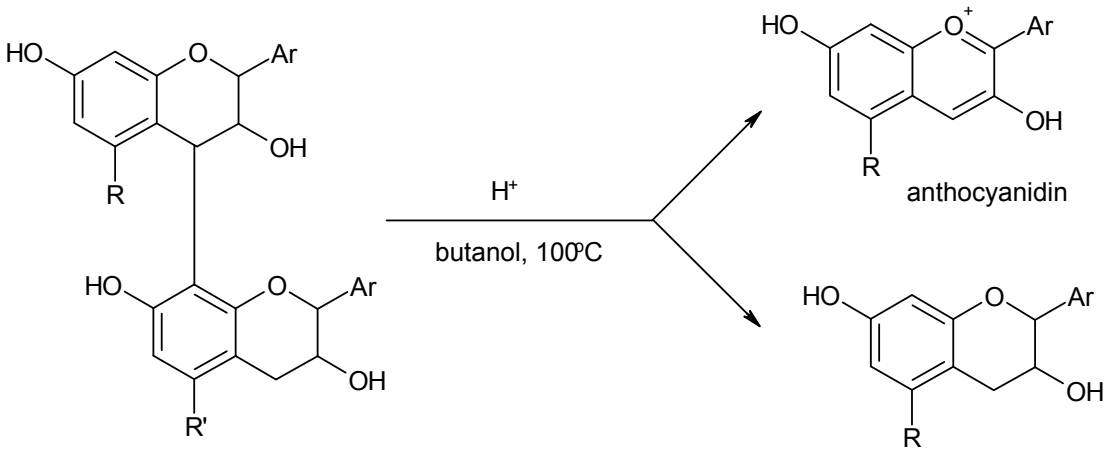


Figure 5.5: Chemistry of the butanol-HCl reaction. The reaction involves oxidation and the terminal unit does not give a colored anthocyanidin product structure

5.2.2 Materials and Methods

All experimental determinations were done in triplicate and the mean of the values determined.

5.2.2.1 Vanillin- HCl method for proanthocyanidins

Acidified vanillin was prepared by mixing equal volumes of 8 % conc. HCl in methanol and 1 % vanillin in methanol. The solution was used soon after preparation as standing for long periods resulted in colour development. The following procedure was carried out in a 30 ^{o}C water bath: 1 ml of a 0.5 mg/ml methanolic extract was mixed by vortex (Whirlmax vortex-Cenco Instruments) with 5 ml of the acidified vanillin, and left to stand in the water bath for twenty minutes. The absorbance of the solutions were then read at 500 nm, using the methanolic extract as a blank.

A catechin standard was prepared by preparing a 2 mg/ml stock solution of catechin in methanol. The stock solution was diluted to 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml, which were treated as the methanolic extracts.

5.2.2.2 Folin-Ciocalteu method for total polyphenols

The test solution, 0.05 ml of 1 mg/ml, was added to 3 ml of distilled water, swirled and followed by an addition of 0.25 ml Folin-Ciocalteu phenol reagent. After *c*. 2 min 0.75 ml of 20 % sodium carbonate was added and the whole mixture was made up to 5 ml with distilled water and vortexed. Two hours after the sodium carbonate was added, the absorbance was measured at 760 nm. The same mixture without the test solution was used as a blank.
To prepare the standard, gallic acid, 5 mg, was dissolved in 5 ml of 40 % EtOH in water and the mixture thoroughly shaken after which 0.05 ml was transferred to a test tube and treated as the test sample.

5.2.2.3 Butanol-HCl method

Reagents were prepared as follows
$NH_4Fe(SO_4)_2 12H_2O$ solution: 2 g of $NH_4Fe(SO_4)_2 12H_2O$ were weighed and mixed with 100 ml of 2N HCL.

n-Butanol HCL solution: 95 ml of n-butanol were mixed with 5 ml of conc HCl. The test solution was made up to 0.1 mg/ml in methanol. Blank and sample solutions were made in borosilicate tubes as follows:

	Sample (ml)	Blank (ml)
$NH_4Fe(SO_4)_2 12H_2O$ solution	0.2	0.2
n-Butanol HCL solution	6.0	6.0
Methanol		1.0
Product solution	1.0	

The tubes were stoppered, thoroughly mixed, and heated in boiling water for 40 min after which the vessels were cooled in a water bath. The absorbance of the sample solution was measured against the prepared blank solution at 546 nm.

5.2.3 Results and Discussion

5.2.3.1 Vanillin-HCl method

The catechin equivalent value shows a gradual increase with an increase in ethanol. 80 % ethanol showed the highest value. 100 % and 60 % EtOH had similar catecin equivalent values (Figure 5.6).

This method of proanthocyanidin estimation has the disadvantage of using catechin as a reference. This is because the reactivity of vanillin with catechin is different from that of vanillin with proanthocyanidins (Scalbert, 1992). In addition, Subodh *et al.* (1976) investigated the specificity of the vanillin-HCL method and they concluded that the vanillin reagent is not completely specific for flavanols. Catechin gave the greatest colour intensity but this was matched by phloretin a dihydrochalcone. It is however important to note that dihydrochalcones have not been reported in grape seeds. WC had a catechin equivalent content two and a half times that of the best performing ethanolic extract i.e 80 %.

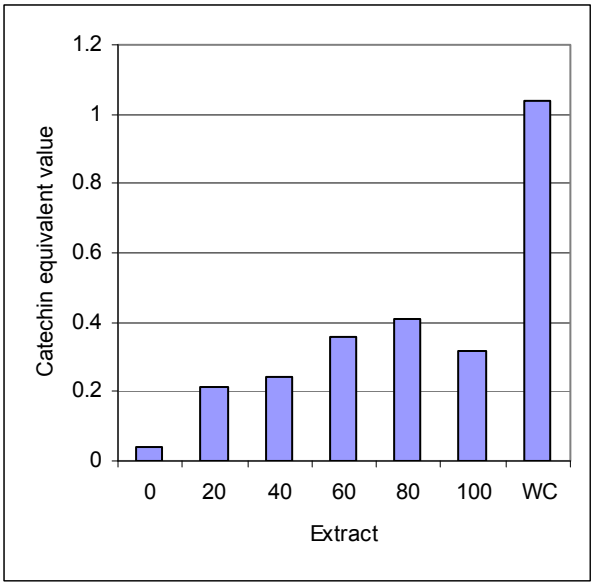


Figure 5.6: Catechin equivalent values for the ethanol in water extract and the Warren Chemical (WC) commercial product.

5.2.3.2 Folin Ciocalteu method for total polyphenols

The quantity of polyphenols as detected by this method followed a similar pattern as shown by the DPPH and the vanillin assay with the values increasing gradually and reaching a maximum with grape seed extracted with 80 % ethanol solution (Figure 5.7). The Warren Chemical extract had three times the amount of polyphenols compared to the 80 % extract.

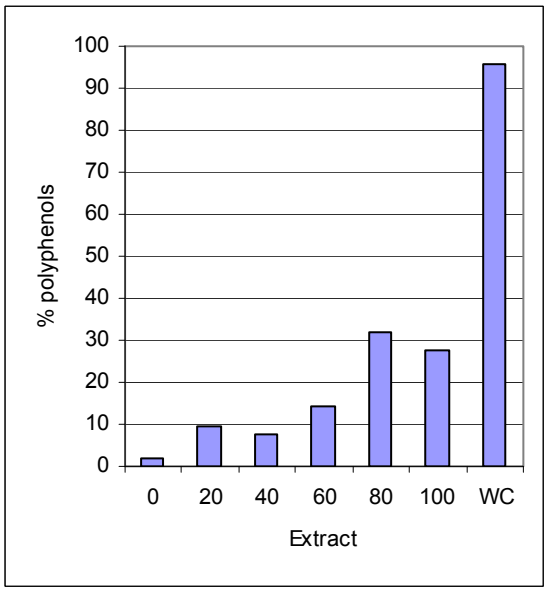


Figure 5.7: Percent polyphenols as detected by the Folin-Ciocalteu method

The problem with this method is that polyphenolic constituents of the grape seed extracts contain varying numbers of hydroxyl groups per molecule, which produce different results relative to gallic acid. Two grape seed extracts with the same quantity of polyphenols but different compositions can produce two different results. In addition this method does not differentiate between tannins and many phenolics that are not tannins. Interfering compounds such as ascorbic acid, tyrosine and possibly glucose are also measured (Waterman & Mole, 1994).

5.2.3.3 Butanol-HCl method

Results obtained from this method were not reproducible (Figure 5.8). Not only did the percentage proathocyanidin value differ from one test to another but also the source of the highest percentage of proathocyanidin extracted differed with each new test. The inconsistencies in the values may be because of side reactions that occur during the transformation of proanthocyanidin into anthocyanidins, resulting in red-brown polymers that absorb around 450 nm (Scalbert, 1992). The Butanol-HCl reaction is commonly used to quantify proanthocyanidins, it is however recognized that some proanthocyanidins are insoluble in common solvents and therefore proanthocyanidins in plant materials may be underestimated (Schofield *et al*, 2001). However the mean values showed the proanthocyanidin content of the extracts increasing from when water was used as a solvent, reached a peak with the use of 40 % ethanolic solution and gradually decreasing to when absolute alcohol was used. Schofield *et al.* (2001), states that the test should be used with caution as a quantitative assay, the assay's greatest strength lies in the confirmation of the presence of a polymeric interflavan structure. Given these drawbacks WC had consistently higher values than the ethanolic extracts.

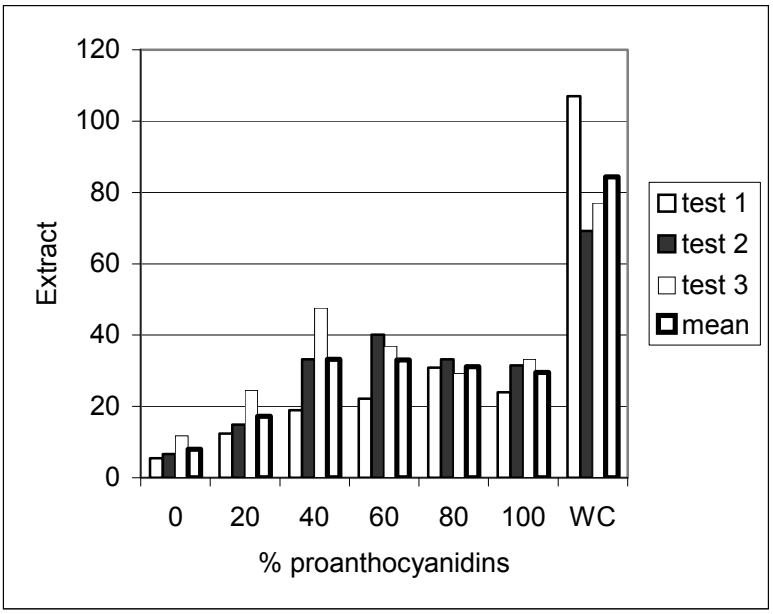


Figure 5.8: % proanthocyanidin of different concentrations of ethanol extracts and a commercial product (com) as determined by the Butanol-HCl method.

5.2.4 Conclusions

Comparison of the ethanolic extracts shows that 80 % ethanol solution yielded the best results in all of the determinations except with the Butanol-HCl method. It was thus used as the solvent of choice in the studies that followed. Although methods used to quantify polyphenols have their limitations, they all overwhelmingly show inferiority of our ethanolic extracts comparing to the commercial product (Table 5.1). This prompted troubleshooting and re-evaluation of our efforts. The first port of call was the investigation of the effect of temperature on the antioxidant activity of the extract. The extract obtained by extracting with 80 % ethanol solution in a ratio of 1:5 at 100^{o}C for thirty minutes and dried in an oven at 95 ^{0}C will be henceforth referred to as Laboratory Product 1 (LP1).

The best performing extract, 80 % ethanol, was 33 %, 39 %, 33 %, and 37 % as active as the commercial product using the DPPH, Vanillin-HCl, Folin-Ciocalteu and Butanol-HCl test respectively.

Table 5.1: A comparison of activities obtained from ethanolic extracts and the commercial extract (WC) using the DPPH method (antiradical efficiency), the Vanillin-HCl method (catechin equvalence), the Folin-Ciocalteu method (% polyphenols) and the Butanol-HCl method (% proanthocyanidins)

Extracts	Antiradical Efficiency	Catechin equivalence	% polyphenols	% proanthocyanidins
0	0.86	0.04	2	8
20	0.99	0.22	9.5	17.3
40	1.72	0.24	7.6	33.2
60	1.89	0.36	14.2	33.1
80	1.92	0.41	32	31.1
100	1.72	0.32	27.4	29.6
WC	5.88	1.04	96	84.4

6 Effect of temperature on antioxidant activity

From the results of section 5.1 and 5.2 it was evident that the laboratory extract had a low antioxidant activity compared to the commercial product. High temperatures were used in the extraction and drying processes. Although high temperatures may result in high yields, it may be a major factor limiting the activity of the extract. The effect of temperature on antioxidant activity of the product was thus investigated. To allow for rapid screening of the antioxidant activity, a new parameter was defined, the [DPPH] $t_{1/2}$, the time it takes to reduce the initial DPPH concentration by half. The lower the [DPPH]$t_{1/2}$ the higher the antioxidant activity of the given extract. This parameter differs from the method used in section 5.1, in that the process of working with different extract concentrations to plot a graph from which the antiradical efficiency will be calculated is avoided. In this instance one concentration is used (10 mg/ml of extract and 25 µg/ml DPPH) and the result is obtained much more rapidly. Eighty percent ethanol was used as the extracting solvent based on the results obtained from section 5.1 and 5.2. This section investigated the effect of temperature in the extraction procedure and the drying process separately.

6.1.1 Materials and methods

6.1.2 Investigating the effect of heat on antioxidant activity

Two OEGS powder samples were separately weighed, and separately mixed with 80 % ethanol in water in a ratio of 1:5. One sample was agitated for a period of 60 min, and the other was boiled under reflux for a period of 30 minutes. After the extraction period the samples were separately centrifuged, filtered and the solvent removed under reduced pressure in a vacuum rotary evaporator at 95^0C. The DPPH $t_{1/2}$ of the two extracts was determined. The two extract were analysed by TLC using EMW as the eluant and acidified vanillin as the spray reagent.

6.1.3 Rapid screening free radical scavenging method

DPPH preparation and use of the spectrophotometer was as in section 5.1.2. 10 µl of a 10 mg/ml solution of the extract was added to the DPPH reagent. The change in absorbance as the reaction progressed was noted at 30 sec intervals and recording stopped after half of the initial absorbance was reached. The calibration curve from section 5.1.2 was used to calculate the concentration of DPPH and this was plotted against time. The time it took to reduce DPPH absorbance by 50% was used as a measure of antioxidant potency, the smaller the value, the more potent the antioxidant.

6.1.4 Results and Discussion

Although heat extraction resulted in an increase in the quantity of material extracted, there was a consequent reduction in the antioxidant activity when compared to the same quantity of cold extracted material. The cold extraction procedure gave an average [DPPH] $t_{1/2}$ of 97.4 ± 0.3 sec whilst the heat extraction procedure gave an average [DPPH] $t_{1/2}$ of 105 ± 5.2 sec. Possible explanations of the differences are that heat breaks down thermo labile compounds resulting in modified compounds which either exhibit a low antioxidant activity or compounds that totally lose activity. Heat may also result in loss of volatile antioxidant active compounds. It is also possible that a larger quantity of non-antioxidant compounds were extracted. A given quantity of extract would thus contain a large quantity of inactive compounds, resulting in low activity on a mass basis. TLC was used to investigate differences, if any, between compounds from the heat and cold extraction procedure (Figure 6.1). Some compounds were visible in the heat extract and not the cold extract. This implies that either different compounds were extracted with the heat or the heat resulted in breakdown of some compounds.
Differences might also exist within the non-mobile compounds. There is a high possibility that the heat resulted in the extraction of high molecular weight, polar non-mobile polysaccharides and polyphenols that do not exhibit antioxidant activity.

The commercial product had an antioxidant activity of 35.7 ± 0.3 sec. The cold extracted product was thus 34 % as active as WC whilst the heat extracted

product was 37 % as active as WC. These activities were similar to those obtained in section 5, where the antioxidant activity of 80 % ethanolic extract was 33 % as active as WC. The elimination of excess temperature in the extraction procedure thus did not have a profound effect on the activity of the extract.

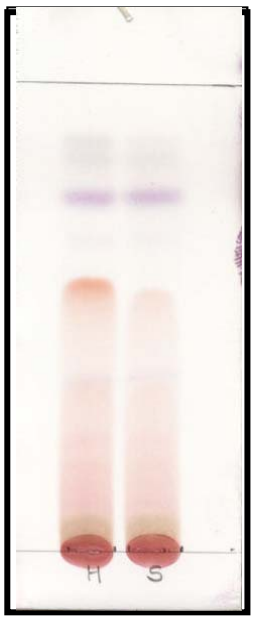


Figure 6.1: TLC profile of heat (H) and cold (S) extracted grape seed extract using EMW as eluent and acidified vanillin as a spray reagent.

6.2 Effect of drying temperature on the antioxidant activity

A 100 g sample of grape seed powder was mixed with 80 % ethanol, agitated for thirty minutes, centrifuged and filtered. The extract was split into four equal volumes of 25 ml. The four samples were dried under varying temperatures; under a cold stream of air, and at either 40 ^{0}C, 60 ^{0}C and 100 ^{0}C until dried. The DPPH $t_{1/2}$ of the different extracts were determined.

6.2.1 Results and discussion

Drying using a cold stream of air and at 40 ^{o}C and 60 ^{o}C did not show any significant differences in the antioxidant activity (Table 6.1). However drying at 100 ^{o}C modified compounds resulting in a partially insoluble and inactive extract. The insolubility of the compounds may be used explain the high [DPPH] $t_{1/2}$ of 286.8 ± 2. The decomposition process may have been

aggravated by the fact that the compounds were already extracted and were thus more susceptible to heat degradation. It has been reported that phenolic antioxidants exhibit significant decomposition at high temperatures, giving rise to a number of breakdown products that in turn can be decomposed (Hamama & Nawar, 1991).

Table 6.1: Effect of drying temperature on antioxidant activity (DPPH $t_{1/2}$), of cold extracted grape seed as shown by the mean of two determinations (a and b) for each temperature tested

Drying temperature	**a**	**b**	**mean**
Cold air stream	68.3	62.1	65.2 ± 4.4
40 ^{o}C	64	69	66.5 ± 3.5
60 ^{o}C	65.1	67	66 ± 1.3
100 ^{o}C	285.4	288.2	286.8 ± 2.0

This observation agrees with the findings of Larrauri *et al.* (1997) who compared antioxidant activity of freeze dried grape pomace peels with material dried at 60 ^{o}C and observed insignificant differences. Extractable polyphenols decreased from 4.3 % in the freeze dried material to 4.1 % in the material dried at 60 ^{o}C whilst condensed tannins decreased from 27.0 % to 26.2 %. However, comparing to freeze-dried samples a reduction of 18.6 % and 32.6% of the extractable polyphenol content was obtained with drying temperatures 100 ^{o}C and 140 ^{o}C, whilst condensed tannins decreased by 11.1 % and 16.6% respectively. Such an effect would consequently result in low catechin equivalent values, low total polyphenols and low percentage of proanthocyanidins.

6.3 Conclusion

The elimination of excess heat in the extraction and drying procedure resulted in improved activity of the extract. Drying the extract using temperatures of up to 60 ^{0}C had no significant effect on the activity of the extract. After these findings, the method of extraction was that of extracting OEGS by shaking for

thirty minutes with 80 % ethanol solution, mixed in a ratio of 1:5, and dried at temperatures under 60 ^{o}C. The product obtained in this way was referred to as LP2. The elimination of high temperatures in the extraction and drying procedures resulted in LP2 being 55 % as active as WC using the DPPH $t_{1/2}$. (Compare with 33 % of LP1 in section 5.1.3). The highest contribution towards increased activity would have come from reduction of high temperature in the drying process, as it was evident that removal of excess temperature in the extraction procedure resulted in minor differences in activity (section 6.1.4). The activity of 55 % obtained with LP2 although an improvement from LP1 showed that changing the drying temperature did not resolve the problem of much lower activity of LP2. As a next step the chemical composition of the different extracts were determined.

7 Comparison of NMR spectra

7.1 Introduction

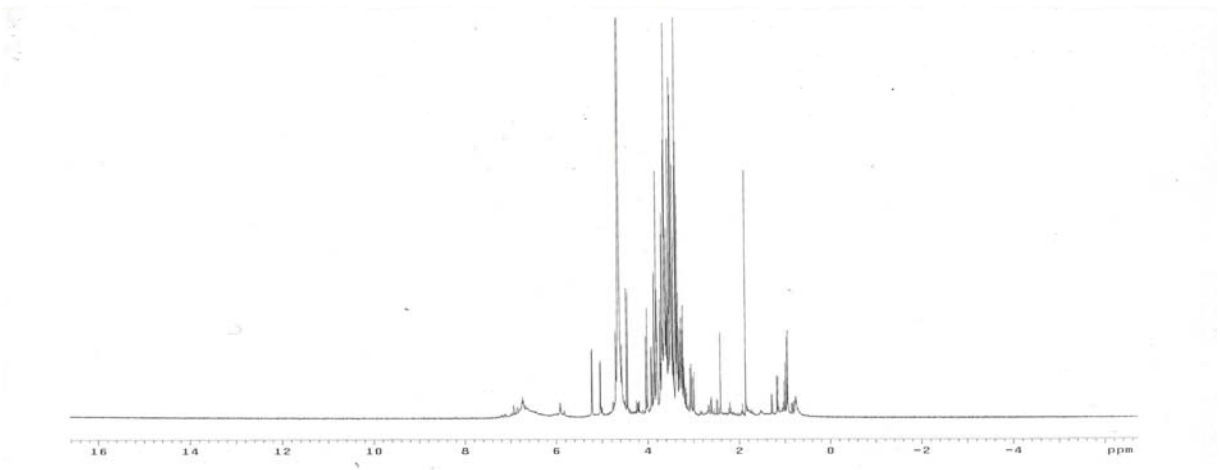
Nuclear Magnetic Resonance (NMR) spectroscopy was used to help determine the type of constituent compounds in LP2 and WC. NMR makes the direct observation of atoms possible by determining the structure of an organic compound by measuring the magnetic moments of its hydrogen or carbon atoms when placed between the poles of a powerful magnet (Harborne, 1973). The chemical environment of gyromagnetic nuclei leads to a small shift in the resonance frequency, the chemical shift, that is measured in either delta or parts per million (ppm). The chemical shift is measured relative to an inert standard, usually trimethylsilane (TMS). Functional groups find their expression in the chemical shift resulting in an intensity/frequency-recording, the NMR spectrum (Holzgrabe *et al*, 1998). The area of an NMR signal is directly proportional to the molar amount of the detected isotope. In ^{1}H NMR spectroscopy, each H-atom leads to at least one signal and since most molecules of phytochemical interest contain more than one H-atom, the spectra are complex. ^{1}H NMR of complex mixtures cannot result in detection of single components but the sum of the functional groups in the mixture can be determined (Holzgrabe *et al*, 1998). In this study NMR was initially used as tool to give a gross analysis of LP2 and WC. This enabled me to get an idea of the possible constituents of the two preparations and pick up crude phytochemical differences, if any. I stress crude because the NMR of tannins is specialized and is better elucidated by magic angle spinning because of their size and polyhydroxylated nature.

7.2 Materials and Methods

Both LP2 and WC were dissolved in d-DMSO and d-water. ^{1}H NMR spectra were obtained on a 300MHz Varian (Oxford Instruments) NMR machine by Freddy Mahubela at MEDUNSA.

7.3 Results and discussion

LP2 was not completely soluble in water but spectra were obtained and major differences between WC and LP2 were apparent (Figure 7.1a). The LP2 spectrum was crowded between the 3 ppm and 4 ppm regions (Figure 7.1a). A great majority of proton resonances of sugars appear in a very small spectral width of 3.0 – 4.2 ppm (Agrawal, 1992), with a lot of overlap which derives from non-anomeric sugar methane and methyline protons, which have similar chemical shifts in different monosaccharide residues. This implies that there are high concentrations of sugars present in LP2



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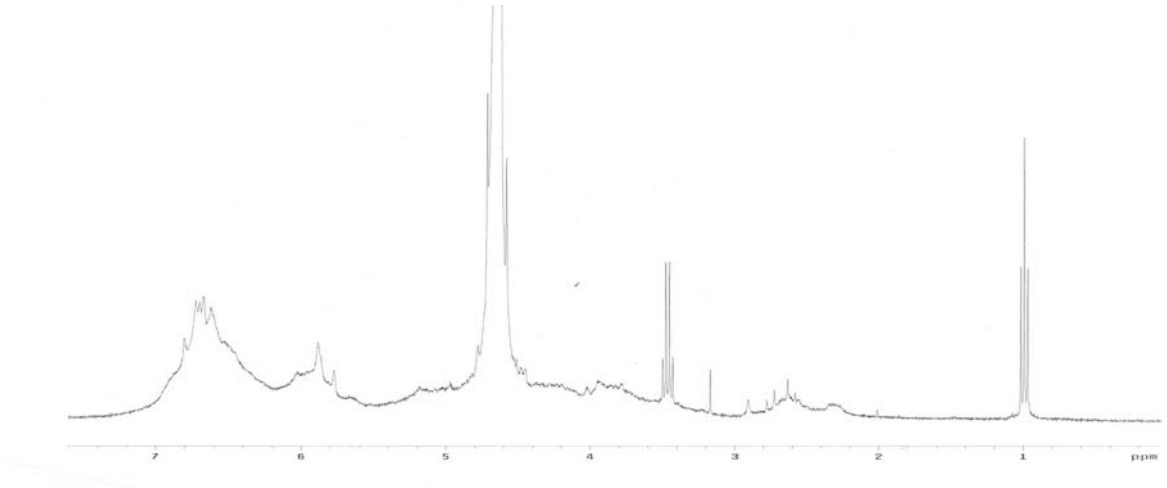

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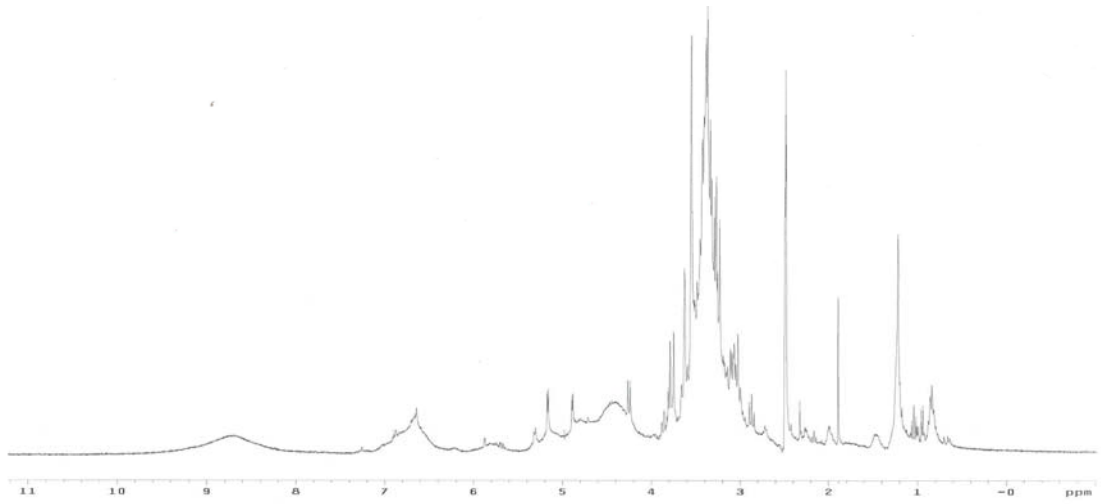
Figure 7.1: NMR spectra of LP2 (a) and WC (b) dissolved in d-water

WC did not show the occurrence of sugars (Figure 7.1b). Both WC and LP2 completely dissolved in DMSO, and LP2 was again crowded between 3 ppm and 4 ppm (Figure 7.2a). The main difference between the water and the DMSO dissolved spectra for LP2 was that in DMSO, there were broad peaks between the regions 6 ppm and 9 ppm. They were also present in the commercial product, (Figure 7.2b), being more pronounced than in the water dissolved commercial product. It is assumed that the signals in this region are due to phenolic compounds and they occur as broad peaks due to their polyhydroxylated nature.



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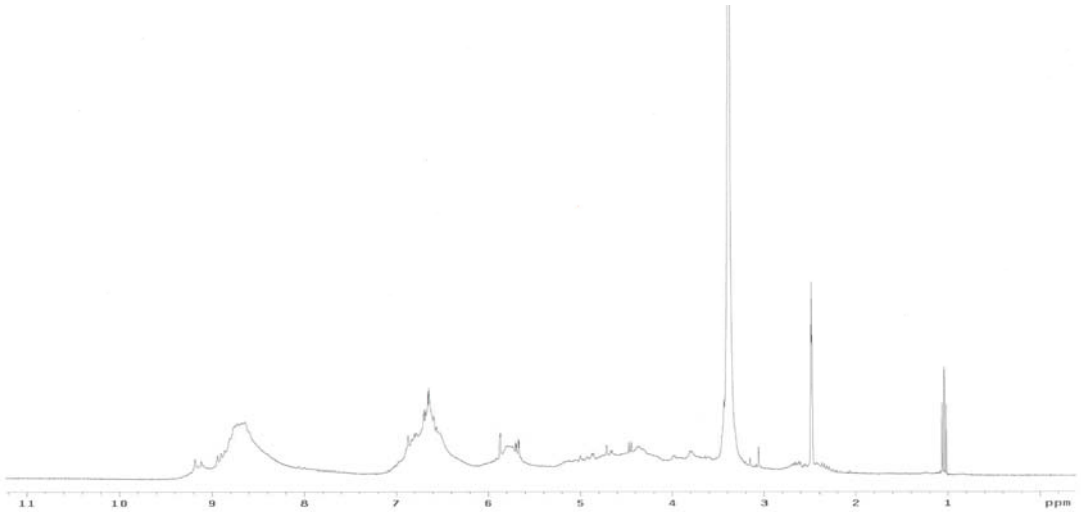

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Figure 7.2: NMR spectra of LP2 and WC dissolved in d-DMSO

Another marked difference between the spectra of LP2 and WC as seen in (Figure 7.2) was the presence of peaks in the region between 0 and 3 ppm. This region is normally associated with aliphatic compound. This implies that LP2, thus has more non-polar compounds than WC. From section 3.1 these non-polar compounds are inactive and only serve to reduce the potency of the product.

7.4 Conclusion

LP2 appears to contain several sugars compared to WC. These sugars would result in low antioxidant activity per given mass of extract and their exclusion would thus result in improved antioxidant activity (Siddhuraju *et al*, 2002). The second conclusion drawn was that since the sugars dissolved in water and the hydroxyl containing groups between 6 ppm and 9 ppm had a limited dissolution in water, the sugars could possibly be selectively removed by extracting the extract with water. Incidentally in the wine fermentation industry, hot water is used to recover all the remaining sugars from grape seeds and the wash water is normally fermented and distilled to obtain alcohols (Gomez *et al*, 1996). The fact that water takes up a limited amount of antioxidant compounds is evident from section 3.1 where the water extract had the least antioxidant activity and the least amount of proanthocyanidins and polyphenols.

LP1 also contained a significant amount of non-polar compounds that would have a negative effect on antioxidant activity, and it was envisaged that their removal would improve the activity of the extract.

NMR indicated that the obvious differences between LP2 and WC was that the former contained sugars and non-polar compounds which would have a confounding effect on the potency of the product. Further work was therefore targeted at removing these constituents.

8 Removal of sugars

8.1 Introduction

The presence of considerable quantities of sugars such as glucose, fructose, reducing disaccharides, and sucrose has been envisaged to have a deactivating effect on the antioxidant potential of compounds present in a given extract (Siddhuraju *et al*, 2002). This is because sugars such as pentoses, hexoses reducing disaccharides have been reported to be strong prooxidants in aqueous emulsion systems (Yamaguchi et al, 1984). It was thus thought that removal of sugars present in LP2 as visualised by NMR spectroscopy (section 7.3) would result in enhanced antioxidant activity. Initially LP2 was extracted with distilled water. Considerations of the cost of extracting the grape seed with the ethanol solution, drying the extract, extracting with water and drying again led to the thought of extracting grape seed powder with water before extracting with the 80 % ethanol solution. The concept of total antioxidant activity is introduced in this section, what it seeks is to relate the antioxidant activity, represented in this instance by the $DPPH_{t1/2}$, to the quantity of material extracted. It is thus obtained by dividing the mass extracted by the $DPPH_{t1/2}$ (Eloff, 2001 – personal communication).

8.2 Materials and methods

8.2.1 Extracting grape seed extract with water

LP2 (5 g) was extracted with 20 ml of distilled water on a shaking machine for 5 min, centrifuged at 4000 rpm for 10 min, filtered and the supernatant and *c.* 10 mg of the residue was stored for further analysis. The remaining residue was re-extracted with 5 ml of distilled water and the supernatant and a sample of the residue were again stored for further analysis. The whole process was repeated resulting in a total of four supernatants and four residue samples. The DPPH $t_{1/2}$ of the supernatants and the residues were determined. The residues and supernatants were also analysed by NMR spectroscopy.

8.2.2 Extracting grape seed powder with water

Three, 30 g OEGS powder samples were prepared. The first sample was extracted once with water in a mass to volume ratio of 1:10, the second sample twice and the third sample three times. The final water extract was collected and dried in each case and the atomic composition detected by NMR spectroscopy. The residue from each of the three samples was cold extracted with 80 % ethanol solution. The DPPH $t_{1/2}$ of the three different ethanol extracts was determined and the extracts were analysed by NMR spectroscopy.

8.3 Results and discussion

The first water extract of LP2 removed the highest quantity of material, 58.4 %, relative to the other four water fractions (Table 8.1). This also represents 89.5 % of the total water extractable compounds.

Table 8.1: The total antioxidant activity (aa) of residual samples (R_1 – R_4) of grape seed extract (LP2), that remained after consecutive extractions with water (W_1- W_4) on an original grape seed mass of 2.5 g.

Sample	% of original mass	DPPH $_{t1/2}$	Total aa
R_1	41.6	33.5	31
R_2	37.6	27.8	33.8
R_3	35.2	43.5	20.2
R_4	34.6	53.5	16.2
W_1	58.4	81	18
W_2	4.0	43.8	2.3
W_3	2.3	52.8	1.1
W_4	0.58	28.9	0.0145

The residue after the second water extraction had the lowest DPPH $_{t1/2}$ of 27.8 sec and the highest total antioxidant activity of 33.8 (Table 8.1 and Figure 8.1). The commercial product tested under the same conditions gave a DPPH $_{t1/2}$ of 22.3 sec. Comparison of this value with the DPPH $_{t1/2}$ of sample R_2 (27.8 sec) shows that R_2 was 80 % as active as the commercial product. This

represented a vast improvement from product LP2, which was 55 % as active as the commercial product (section 6.3).

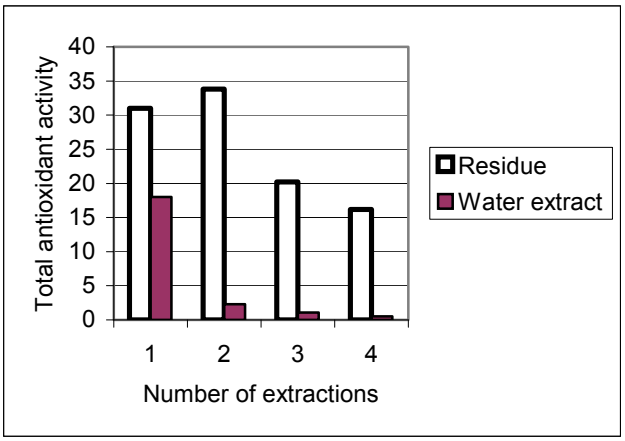


Figure 8.1: Total antioxidant activity of different fractions of residual and water extracts, during washing of grape seed extract with water.

The first water extract, although having a relatively high DPPH $_{t1/2}$ value of 81 sec, had a significantly high total antioxidant value of 18. NMR spectra of the above extracts in DMSO showed the first water extract (Figure 8.3a) as having a large quantity of sugars compared to the second water extract (Figure 8.3b). The first residue from the water extraction was less crowded than the extract extracted with water in the region corresponding to the sugars with the regions that supposedly correspond to the phenolic groups being more pronounced (Figure 8.3c). Subsequent residues did not show any marked spectral differences from the first residue. Washing of the extract with water therefore selectively removed sugars that compromised the antioxidant activity of the extract.

8.3.1 Extracting with water before ethanolic extraction

Whilst the above procedure resulted in a product with increased activity, the process of extracting the grape seed with the ethanol water solution, drying, extracting with water and drying again would be costly. This consideration prompted the thought of first washing the grape seed powder with water and then extracting with the ethanol solution, this way the drying procedure would be performed once. The water extract was white in colour and it could be decanted after the mixture was left to stand.

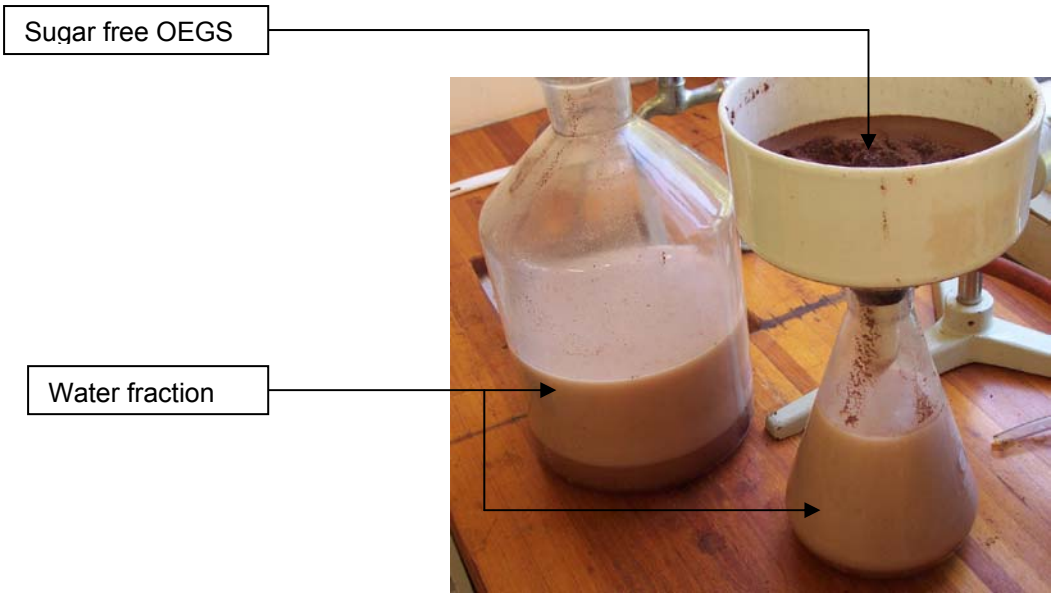
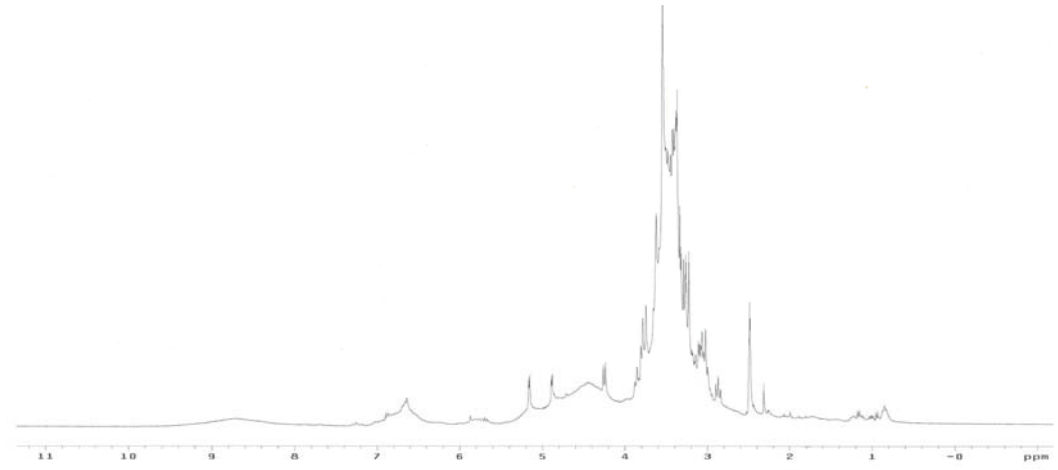


Figure 8.2: Large-scale extraction of OEGS with water, showing the white water fraction that will be discarded, and the sugar free OEGS.

The results obtained are shown in Table 8.2. The extract extracted twice with water (C2) thus had an activity higher than the commercial product, which had a DPPH $_{t1/2}$ of 30.7. The complete procedure extracted 2.1 % of the original grape seed material. The first water wash extracted 12 % of the original material. Antioxidant activity of the water extracts was low, after 30 minutes of reaction time they reduced DPPH by 85.4 %, 66.3 %, 86.3 % for the first, second and third water extracts respectively. Water therefore selectively removed non-active compounds.

Table 8.2: Antioxidant activity of water washed seed powder, C-cold extracted, WC- Warren Chemical product, W-water extracts

Sample	% extracted	DPPH $_{t1/2}$	Total aa
C1	4.2	42.9	14.5
C2	2.1	27.2	11.3
C3	3.5	45.5	11.5
WC		30.7	
W1	12		
W2	3.1		
W3	1.1		



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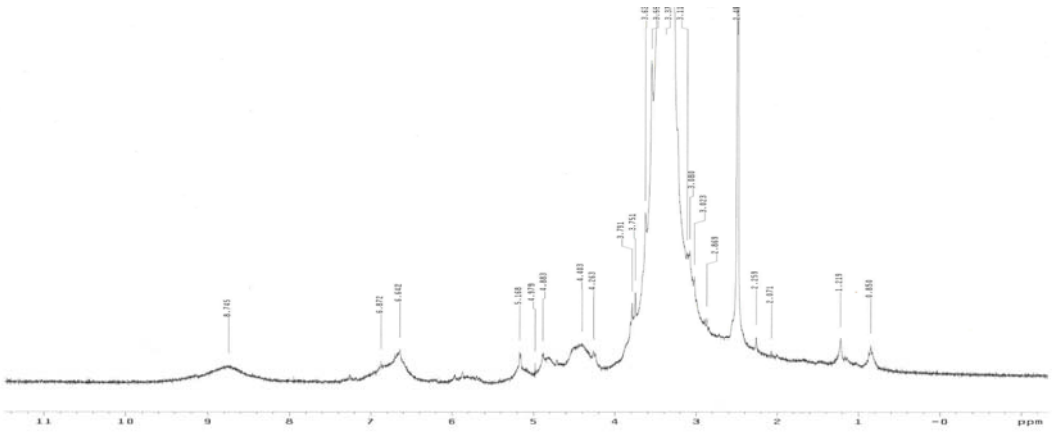

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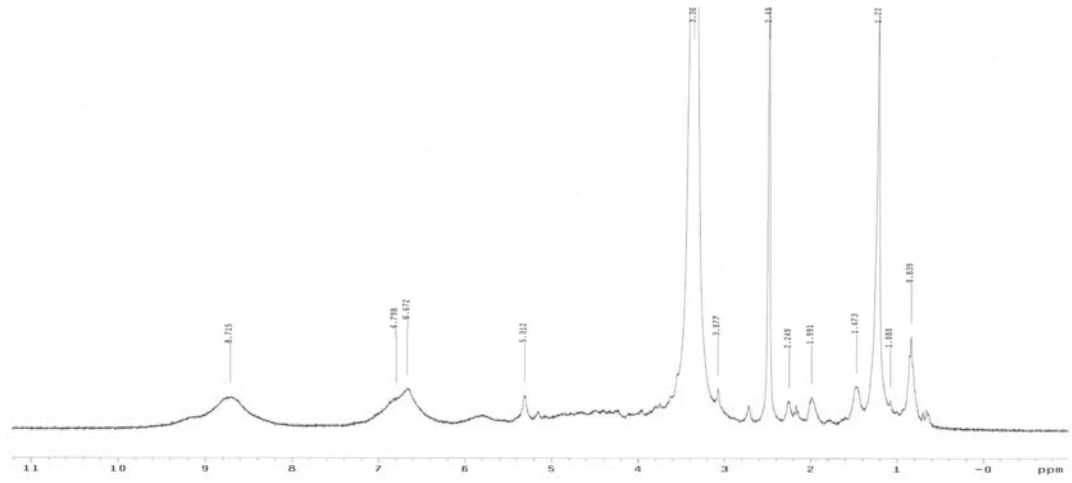


c

Figure 8.3: NMR spectra of water fraction after first (a) and second (b) wash with water and (c) is the remaining residue after washing with water once

The water extracts therefore selectively removed compounds that did not have antioxidant activity.

Grape seed was thus extracted twice with distilled water in a ratio of 1:10, the marc re-extracted on a shaking machine with 80 % ethanol solution in a ratio of 1:5 for thirty minutes. The product obtained via this process was referred to as LP3. LP3 was compared to WC using the full DPPH method (section 5.1.2), the Vanillin-HCl method (section 5.2.2.1) and the Butanol-HCl method (section 5.2.2.3).

Further tests done on the grape seed first extracted with water and then with 80 % ethanol solution are shown in Table 8.3.

Table 8.3: Comparison of LP3 with a commercial product using the complete DPPH test (AE), the Vanillin-HCl test (catechin equivalent) and the Butanol-HCl test (% proanthocyanidins).

	AE	**Catechin equivalent**	**% Proanthocyanidins**
LP3	6.7	1.54	85
Commercial	10	2.32	98

8.4 Conclusion

Water selectively removed sugars from the extract, and was effective even when the water extraction procedure came first. Due to the absence of these sugars the activity of the extract vastly improved relative to WC. Before improvements, the laboratory extract was about 33 % as active as WC in the tests done except the Vanillin-HCl test where it had an activity of 40 %. After improvements, the laboratory extract was 67 % as active in the DPPH and the catechin equivalent test and 83 % as active in the Butanol-HCl test. As stated in section 7.4, the presence of non-polar compounds would also negatively affect the antioxidant activity negatively, hence work in section 8 sought to remove these compounds.

9 Removal of non-polar compounds

9.1 Use of DCM

9.1.1 Introduction

Work from section 3.1 showed that the presence of non-polar compounds resulted in a decrease in the antioxidant activity of the extract. Moreover they also resulted in a lower yield of extractable mass (Figure 4.3). Comparison of NMR spectra of LP2 And WC (Fig.. 7.2) showed the presence of numerous non-polar compounds LP2 compared to WC. The removal of non-polar compounds was thus expected to further increase the antioxidant activity of LP3.

9.1.2 Materials and methods

9.1.2.1 Extracting LP3 with DCM

LP3 (5 g) was mixed with 25 ml of DCM and vigorously shaken for 10 min. The extract was filtered and the concentration of the DCM extract determined. The remaining marc was referred to as LP4.

9.1.2.2 Comparison of LP4 with commercial products

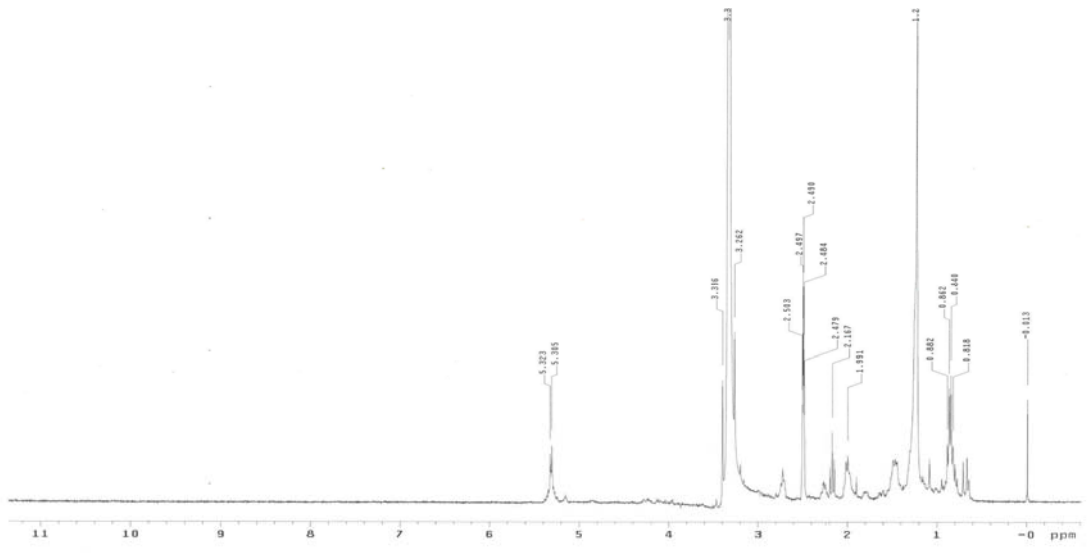
The activity of commercial products from other sources was compared to LP4, using the Vanillin-HCl, the DPPH, Folin-Ciocalteu and Butanol-HCl test.

9.1.3 Results and Discussion

9.1.3.1 Extracting LP3 with DCM

Initially DCM was used to remove the non-polar compounds from LP3, and the end product was compared to commercial products from different sources. However consideration of the toxic effects of DCM if found in considerably high residual quantities led to the investigation of the possible use of the resin Diaion HP-20. Dichloromethane is a non-polar solvent that can be used in extraction processes that produce ingestible products. The FAO/WHO expert committee on food additives met in Geneva in 1970 over the issue of using DCM. They decided that the use of this solvent should be restricted to that determined by good manufacturing practice, which would be expected to result in minimal residues unlikely to have any significant toxicological effect.

DCM extracted green coloured material that represented 1.62 % of the extract and NMR spectra reflected effective selective removal of non-polar compounds (Figure 9.1).



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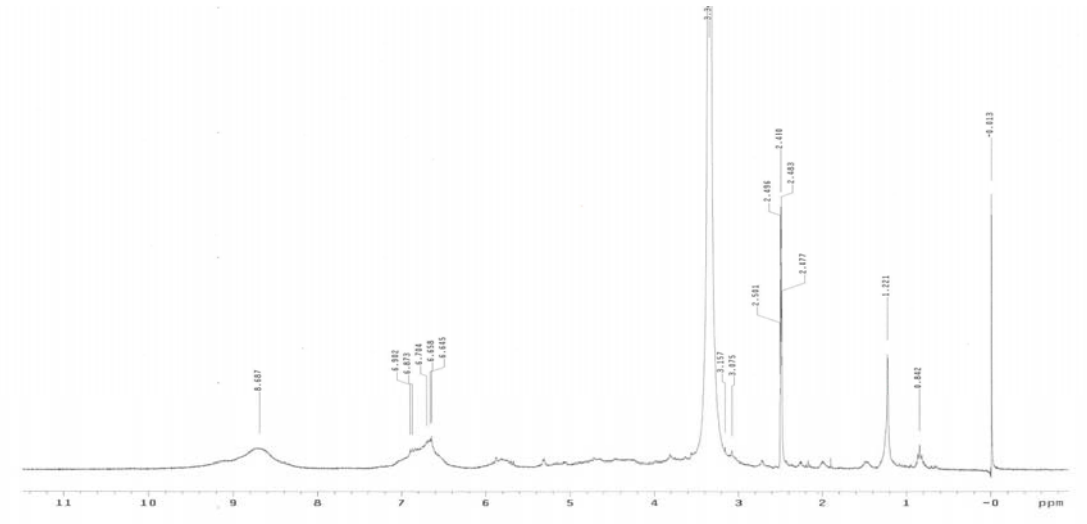


b

Figure 9.1: NMR spectra of compounds removed with DCM (a) and the remaining LP4 (b)

It can be noted from Figure 9.1 that the region above 6 ppm had no signals, which meant that polar compounds that I associate with most of the activity did not partition into DCM. The product that remained after extracting with DCM, LP4, showed a minimal amount of aliphatic compounds compared to LP2 (Figure 7.2).

9.1.3.2 Comparison of LP4 with commercial products

The final product DCM extracted product was compared with ten commercial products using the DPPH test, the Folin-Ciocalteu, Vanillin-HCl, and the Butanol-HCl methods. The products and abbreviations used to refer to them are shown below, other products had additional constituents which are shown in brackets:

Extract after DCM extraction	LP4
Product before DCM extraction	LP3
Warren Chemical extract	WC
Pro-oxidin (selenium, vitamin C, vitamin E)	O
Procydin (ascorbate, bioflavonoids, vitamin E)	C
Immunochoice (plant sterols and sterolins, digestive enzymes)	I
Petrow extract	R
Afriplex samples	M, P, B and A

The Butanol-HCl test showed that the LP4 had a proanthocyanidin content of 101.63 %, a value that was second to the Afriplex product M content of 116.93 % (Figure 9.2a). LP4's EC_{50} of 0.090 mg of antioxidant/mg of DPPH was second best to that of WC that had a value of 0.070 mg of antioxidant/mg of DPPH (Figure 9.2b). The total polyphenols as shown by the Folin-Ciocalteu method were ranked sixth, with a content of 29.95 % compared with the highest value of 54.1 % of WC (Figure 9.2c). In the Vanillin-HCl test LP4 was the fourth best product with a catechin equivalent value of 3.9 mg. Afriplex product M had the highest value of 5.89 mg (Figure 9.2d). It should however be noted that the presence of other constituents would impact on the antioxidant activity of the commercial products – the presence of vitamin C, E and selenium is expected to have an additive effect on the antioxidant activity whilst the presence of inert substance in the product formulations is expected to result in the lowering of the activity.

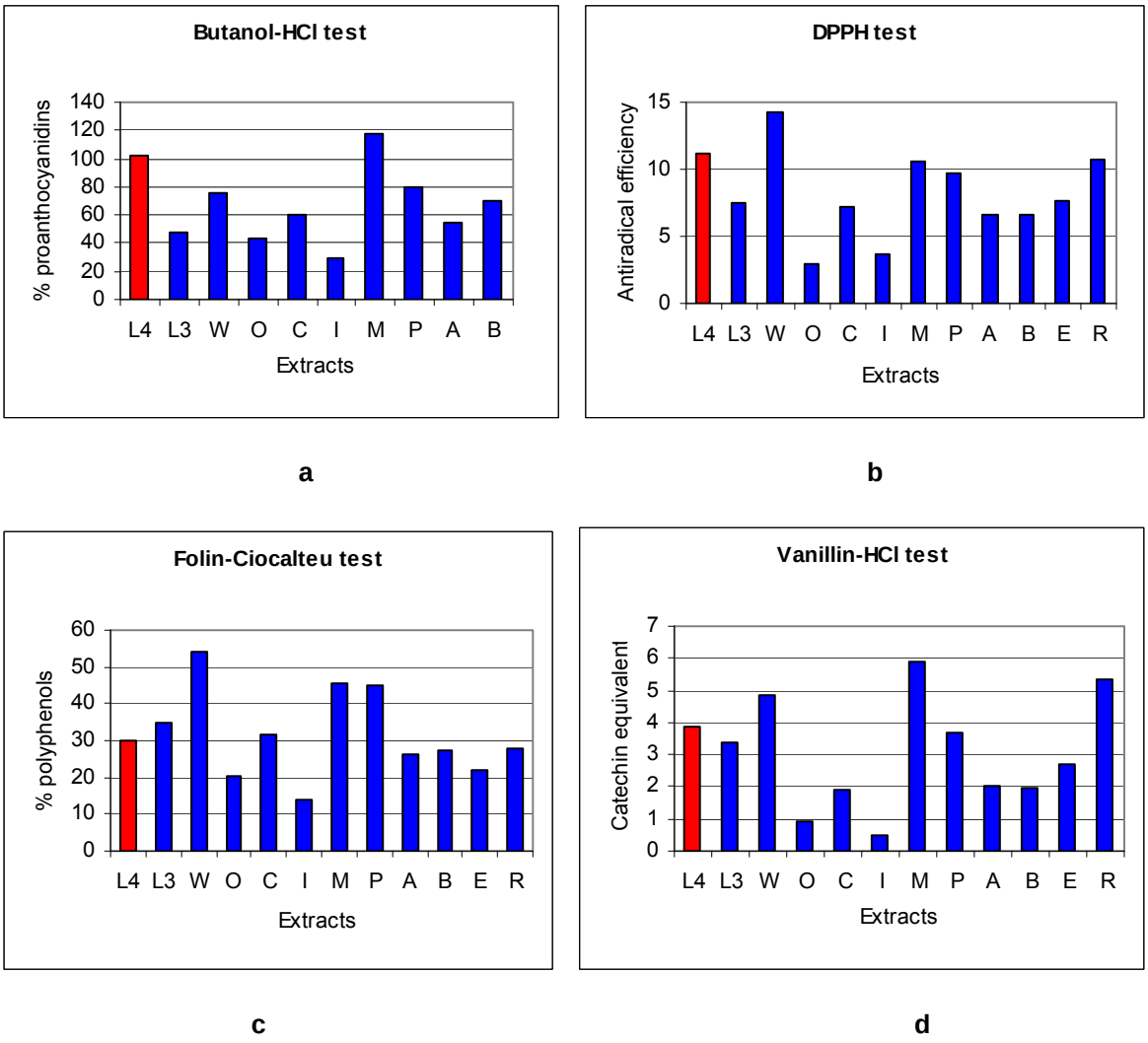


Figure 9.2: Comparison of LP4 (L4) and LP3 (L3) with different commercial extracts including WC (W), using the Butanol-HCl (% proanthocyanidin), DPPH (antiradical efficiency), Folin-Ciocalteu (% polyphenols) and Vanillin-HCl test (catechin equivalent)

9.1.4 Conclusion

Dichloromethane effectively removed non-polar compounds and this led to a further improvement in the activity of the extract, LP4.

The proanthocyanidin content of LP4 was 133 % relative to that of WC (Figure 9.2a), which was an improvement from the activity of LP1 that was 37 % as active as WC (Table 9.1). The antioxidant activity, as represented by the antiradical efficiency, of LP4 was much higher (78 %) than that of LP1 (33 %) (Table 9.1). The catechin

equivalent value and % polyphenols also showed improvement although not as high in the latter.

Table 9.1: Percentage activities of LP1 and LP4 in comparison with the commercial product, using different analytical methods

Parameter	% activity of WC	
	LP1	LP4
% proanthocyanidins	37	133
Catechin equivalent (mg)	39	80
Antiradical efficiency	33	78
% polyphenols	33	55

The use of DCM was however not attractive as there was a possibility of high quantities of residual solvent being found in the final product. This drawback led to the investigation of the possible usefulness of Diaion-HP20 in removing non-polar compounds as reported in section 9.2.

9.2 Use of Diaion-HP20

9.2.1 Introduction

Diaion^{R} HP20 is a synthetic adsorbent that possesses a hydrophobic styrene–divinylbenzene polymeric structure (Kabay *et al*, 2003). The characteristics of the resin are shown in Figure 9.1.

Table 9.2: Characteristics of Diaion HP20

Characteristic	Value
Moisture content %	56.5
Swelling (ml/g)	3.45
Pore volume (ml/g)	1.18
Specific gravity	1.01

The resin is widely used in a broad range of applications, including, the purification of antibiotics amino acids and sugars. Diaion HP20, if effective, would not result in the

presence on undesirable residues. The capability of Diaion to selectively bind to non-polar compounds was first investigated followed by the investigation of its carrying capacity and the ability to recycle it.

9.2.2 Materials and Methods

9.2.2.1 Use of Diaion to remove non-polar compounds

Diaion (10 g) was pre-washed and left to stand in ethanol for 10 minutes and packed into a column of diameter 25 mm and a column bed of height 800 mm. Grape seed extract, 600 ml of a 5 mg/ml solution, previously prepared by washing grape seed powder twice with water and then extracting with 80 % ethanol in water was passed through the Diaion. The Diaion was then washed with 150 ml of absolute ethanol to remove remaining extract. Subsequently acetone (150 ml) was passed through the column to remove bound material, and regenerate the column.

9.2.2.2 Determination of Diaion saturation point

Ten grams of Diaion were prepared as in section 9.2.2.1 and packed into a column. The extract, 3000 ml of a 5 mg/ml, was passed through the column. Samples (30 ml) were collected, dried and reconstituted in 20 ml of water; shaking the tubes in a hot water bath assisted dissolution. Hexane (30 ml) was added into the tubes and the water-hexane mixture was and left to stand after which 20 ml of the hexane fraction was pipetted out and dried. The dried hexane fraction was reconstituted in 1 ml of hexane, which was used for spectrophotometric quantification.

9.2.2.2.1 Spectrophotometric quantification

Green coloured non-polar material previously extracted from grape seed by DCM was used to draw a standard concentration versus absorbance curve. The straight line drawn was represented by: $y = 0.2121x + 0.0085$ and had a 99.9 % fit.
The absorbance of the fractions from the above work was measured and the quantity of non-polar material calculated.

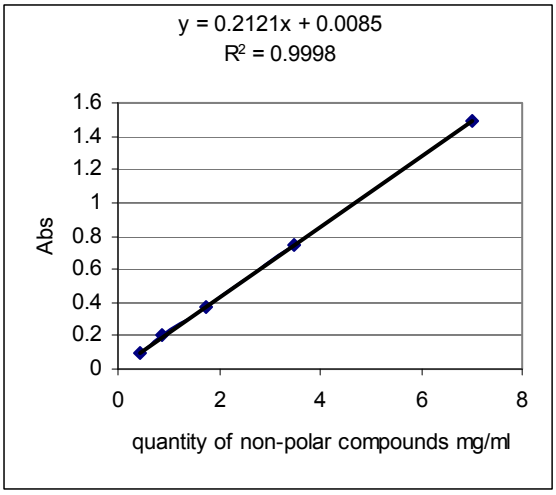


Figure 9.3: Standard curve showing the relationship between quantity of non-polar compounds previously extracted from grape seed and optical density

9.2.2.3 Effect of acetone and water regeneration on Diaion efficiency

10 g of Diaion was pre-washed in ethanol and packed into a column. 600 ml of a 5 mg/ml extract was passed through the Diaion. After each 600 ml run, the column was washed with 200 ml of ethanol, acetone and water respectively, after which another 600 ml of extract was added. 30 ml samples were collected on the first second and fifth elution. The weight of the acetone fractions was determined in each case.

9.2.3 Results and Discussion

9.2.3.1 Use of Diaion to remove non-polar compounds

Passing the extract through the Diaion resulted in green colouration of the Diaion. Elution with clean ethanol removed a red extract but not the green colour. However subsequent elution with acetone removed the green coloured compounds from the Diaion. The colour was the same as that of the grape seed expressed culinary oil and of the DCM extract obtained in section 9.1.2. The conclusion derived from this study was that Diaion selectively bound the non-polar compounds in the grape seed extract.

9.2.3.2 Determination of Diaion saturation point

If Diaion could be use as indicated in section 9.2.3.1, it was important to find out how much of the green coloured non-polar compounds could be carried by a given

quantity of Diaion. Attempts to determine the saturation point of Diaion produced results shown in (Figure 9.4).

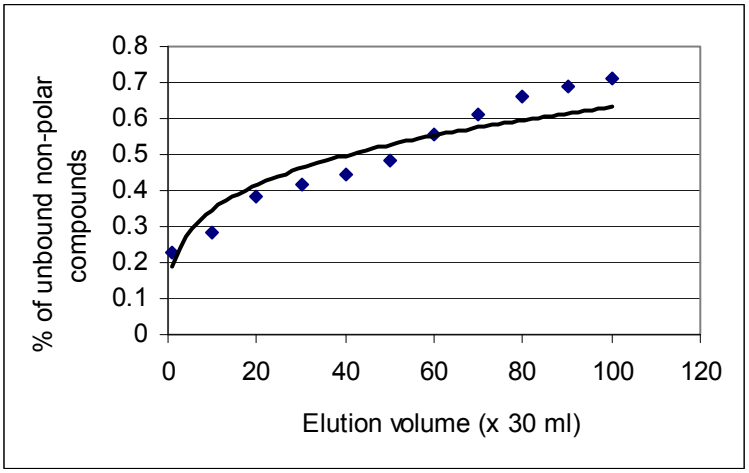
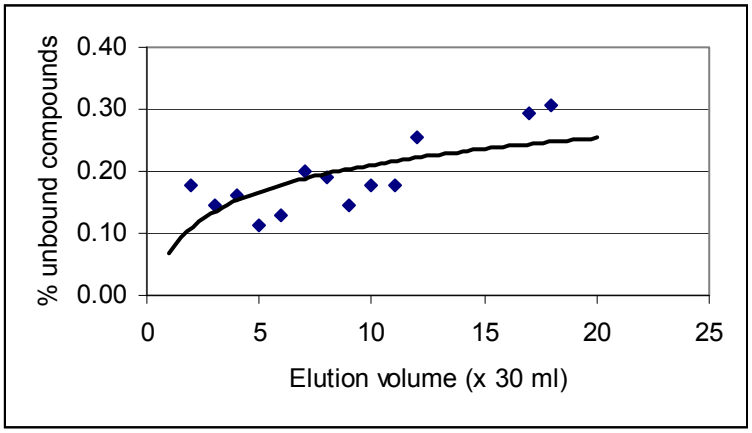


Figure 9.4: Percentage of non-polar compounds that did not bind to the Diaion.

An increase in the volume of extract passed through the Diaion, resulted in a greater incidence of unbound non-polar compounds (Figure 9.4). The amount of non-polar compounds passing through the Diaion increased logarithmically as the volume of the extract increased. Initially 0.2 % of the non-polar compounds passed through and after 3000 ml of the extract were eluted, *c* 0.6 % of non-polar compounds were not bound to the Diaion. It was determined that the amount of unbound non-polar compounds became significant after 1200 ml of the extract was eluted. Ten grams of Diaion could therefore efficiently purify 7,5 g of grape seed extract.

9.2.3.3 Effect of acetone and water on Diaion efficiency

Re-use of Diaion was the next major concern; this had implication on the cost of the whole procedure. Washing of the Diaion with acetone and water could have possibly had an effect on the carrying capacity of Diaion. The effect of re-use on the binding capacity of Diaion was thus determined. After the first elution of 600 ml of the extract, 247 mg of non-polar compounds were removed by acetone. The second elution of acetone removed 248 mg whilst the fifth elution removed 248 mg of non-polar material.



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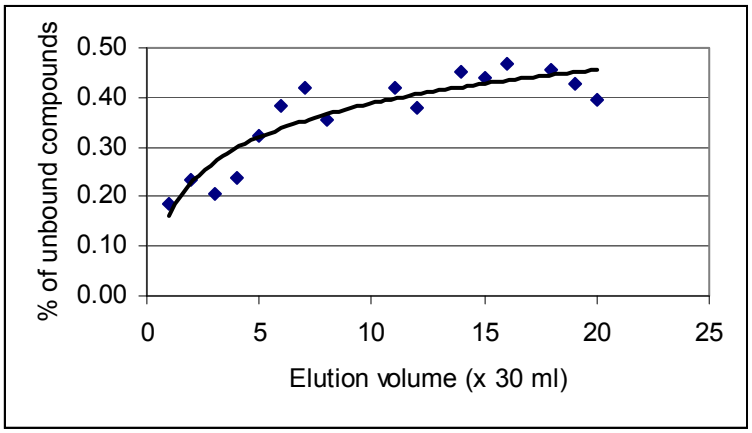

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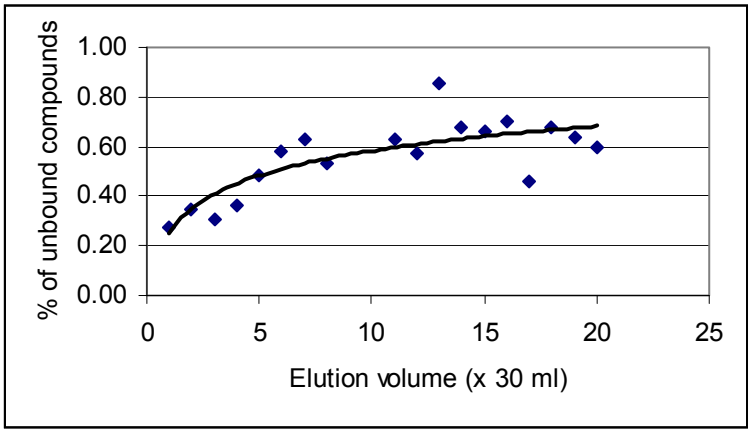

c

Figure 9.5: % unbound non-polar compounds in consecutive use and washing of Diaion, where with ‘a’ is the first elution, ‘b’ the second elution and ‘c’ the fifth elution

The quantity of non-polar compounds that were not bound by the Diaion during consecutive use and washing of the Diaion did not vary significantly between the first and the fifth elution of the grape seed extract. As the Diaion was repeatedly eluted with the extract and later with acetone, the quantity of non-polar material that went through gradually increased with each run. After 600 ml of the first and second elution were passed through *c.* 0.25 % of the unbound material was found in the extract. In the fifth round of elution and removal of non-polar compounds *c* 0.7 % of non-polar compounds were found in the extract (Figure 9.5).

9.2.4 Conclusion

Diaion HP20 can be used to replace the use of DCM in the removal of non-polar compounds from grape seed. Diaion has the advantage that it can be re-used without affecting its carrying capacity and no toxic substances will be found as residues in the final product. The next section sought to compare the antioxidant activity of grape seed extract whose non-polar compounds were removed by Diaion (LP5). In this instance antioxidant activity was compared using the Trolox equivalent antioxidant capacity.

10 Comparison of LP5 and commercial products

10.1 Introduction

The successful removal of non-polar compounds using Diaion resulted in the final laboratory product (LP5). The antioxidant activity of LP5 compared to commercial products was quantified using the trolox equivalent antioxidant capacity assay (TEAC) and the chemical constituents were analysed by NMR.

The TEAC is a decolourisation assay which measures antioxidant activity in relation to trolox, a water soluble vitamin E analogue (Re *et al*., 1999). The method involves prior generation of the radical monocation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{+}$). The blue/green chromophore $ABTS^{\cdot +}$ is produced through the reaction between ABTS and potassium sulphate (Re *et al*., 1999). The addition of antioxidants to the free radical reduces it to a colourless ABTS, a reaction that depends on the concentration of the antioxidant and the duration of the reaction. The extent of decolourisation as percentage inhibition of the free radical is then calculated relative to the reactivity of Trolox under the same conditions (Re *et al*., 1999). One difficulty in assessing antioxidant activity is the option of which method to use. Different methods seem to give different values and methods have their advantages and disadvantages. The TEAC has the major advantage that it is applicable to both aqueous and lipophilic systems (Re *et al,* 1999).

10.2 Materials and Methods

10.2.1 Preparation of ABTS

Mixing 192 mg of ABTS with 50 ml of water made a 7 mM stock solution of ABTS. The ABTS free radical was produced by reacting ABTS stock solution with 33 mg (2.45 mM) of potassium sulphate (final concentration). The solution was prepared 12 - 16 hrs before use and stored at 4 ^{0}C, until needed.

10.2.2 Experimental procedure

Different concentrations of the laboratory products, commercial extracts or Trolox were prepared by serially diluting 1 mg/ml of each sample.

The prepared $ABTS^{+}$ solution was diluted with ethanol to an absorbency of 0.7 ± 0.02 at 734 nm (ethanol used as blank) after which 1.0 ml was added to 10µL of the 1 mg/ml solution of Trolox. The absorbance reading was taken after 6 min of reaction time. This was repeated for the remaining concentrations of Trolox and all the other extracts. All determinations were carried out in triplicate.

A Trolox standard line was prepared by plotting percentage inhibition of the $ABTS^{+}$ radical against concentration of Trolox. The curve had a gradient of 176.3 and a percentage fit of 99.9 % (Figure 10.1).

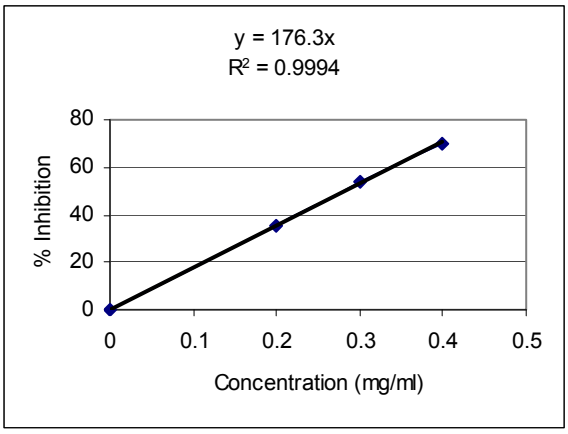

Figure 10.1: Standard curve of % inhibition of $ABTS^{+}$ against concentration of trolox after 6 minutes of reaction time.

Data from the other extracts was analysed in a similar manner and the gradient obtained for a particular extract was divided by the gradient of Trolox resulting in a Trolox equivalent value

10.3 Results and Discussion

LP5 had the best antioxidant activity as reflected by the highest TEAC value of 1.72 (Figure 10.2), a value that was 72 % higher than that of WC (1.00). Diaion therefore selectively removed non-polar compounds without compromising the activity of the extract. It was interesting to note that the activity of LP4 (1.44) which had non-polar compounds removed by DCM was lower than that of LP5.

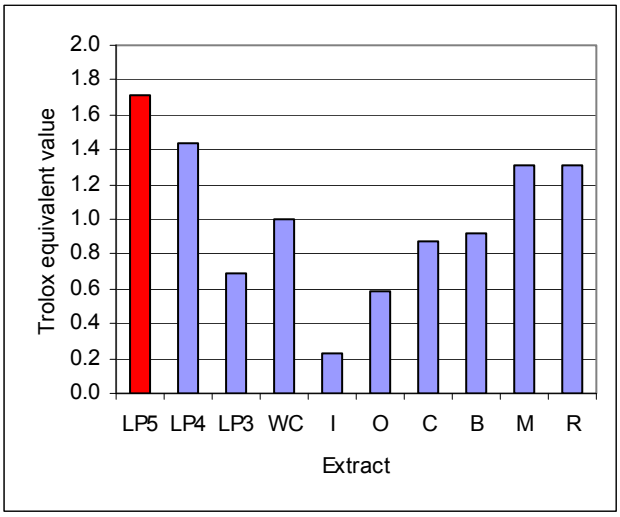

Figure 10.2: Trolox equivalent values of Laboratory products (LP) and commercial products

Also of interest is the fact that using the DPPH method in section 9.1.3.2 showed LP3 to be less potent than WC, however in the present case LP3 appears to be more potent than WC. The question that arises is; what method should one use? From work I have done, the TEAC is more favourable than the DPPH method in that the free radical formed is much more stable and the method is much more rapid.

NMR spectra, comparing LP5 and WC were obtained as reported in section 6. The spectrum showed that LP5 and WC were identical for practical purposes (Figure 10.3). Broad peaks, normally associated with phenolic compounds that would result in the desired antioxidant activity were present between the regions 6 ppm and 9 ppm in both products. Sugars and non–polar compounds were previously identified as compounds that would result in the lowering of antioxidant activity (section 7.3). Both products did not show the occurrence of sugars between the regions 3.0 – 4.2 ppm. Non-polar compounds were also not present in the region normally associated with aliphatic compounds (0 – 3

ppm). The method used to obtain LP5 therefore effectively removed sugars and non-polar compounds.

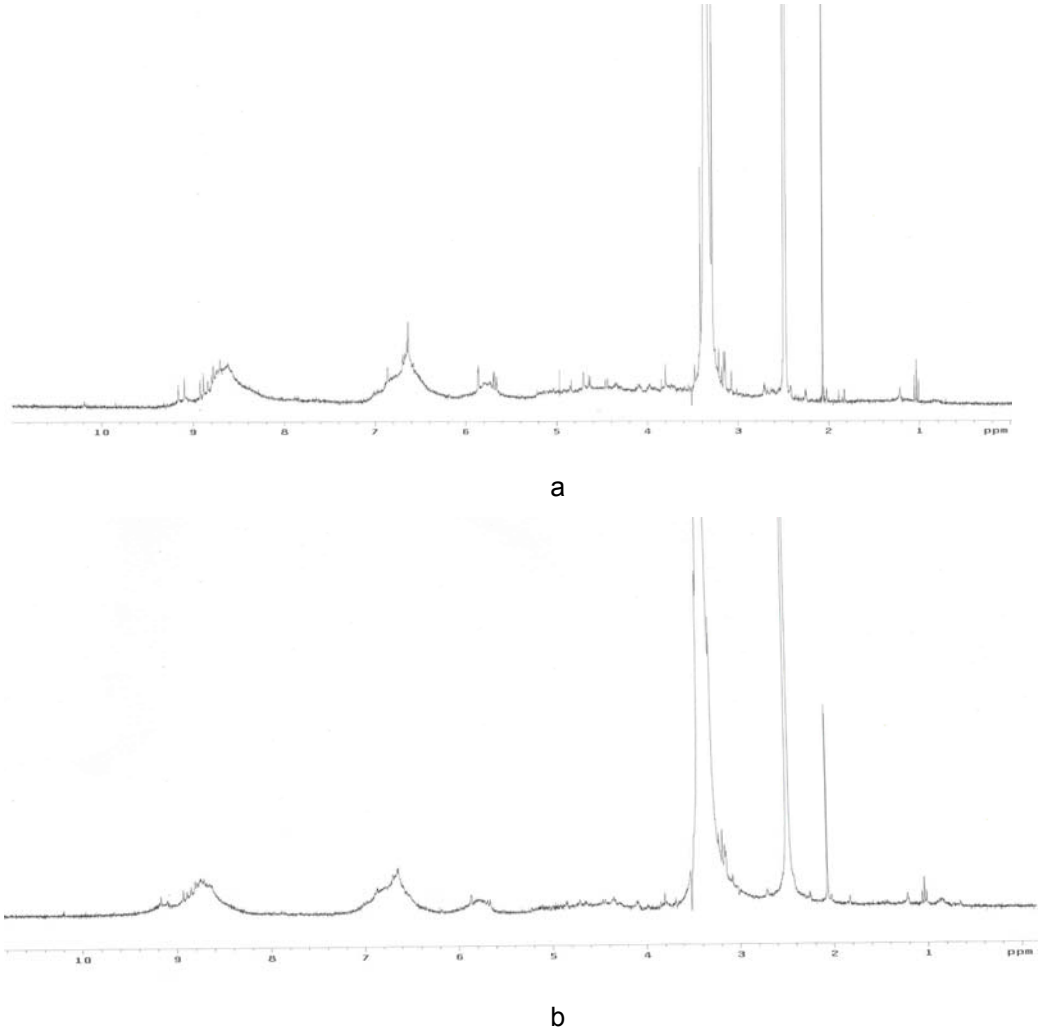

Figure 10.3: Comparison of NMR spectra of LP5 (a) and WC (b)

10.4 Conclusion

The laboratory product LP5 was substantially more active than the commercial product using the TEAC assay. The NMR spectra of the two products were identical. The overall method used was simple and rapid, this therefore meant that the original aim of using grape seed obtained from a local source to make a low cost and effective product, which will be able to compete with products already in the market was met.

11 General Conclusion

Initially compounds extracted by non-polar solvents did not exhibit antioxidant activity and their presence resulted in lower antioxidant activity as visualized by the TLC based DPPH spraying method. TLC was however not effective in separating compounds from grape seed, mainly because of the extremely polar nature of the proanthocyanidins. A possible solution to this problem would be to use reverse phase chromatographic plates or HPLC.

Investigation on extractable yield revealed that acetone extracted the highest quantity of material, however consideration of safety led to the use of a combination of ethanol and water as extraction solvents. Although water and ethanol extracted low quantities from grape seed individually, their combination resulted in a greater quantity of material extracted with 40 % ethanol in water having the highest yield. There was also an increased occurrence of antioxidant compounds as visualised on TLC plates.

There was a linear relationship between extracting temperature and quantity extracted; in addition the longer the extract was exposed to high temperatures the higher the quantities extracted. Increasing the volume of solvent ratio resulted in an increase in the quantity of material extracted. A cost effective balance however has to be maintained, when deciding on the duration of heating and the amount of solvent to sample ratio. Extracting in a ratio of 1:5 at 100^{o}C for 30 minutes was selected for further studies.

Although 40 % ethanol in water extracted the highest quantity of material, using the above conditions, 80 % ethanol solution extract had the highest antioxidant activity. This initial product was referred to as LP1 and had an activity that was 33 % of a commercial product, WC, in all of the determinations except the Butanol-HCl method.

In an effort to improve the quality of the product the effect of temperature on the antioxidant activity of the extract using the quantitative DPPH method was investigated. The elimination of high temperatures in the extraction and drying processes resulted in a product, LP2 that was 50 % as active as WC. Drying temperatures of up to 60^{o}C did not compromise the activity of the extract.

I used ^{1}H NMR to compare the composition of LP2 with the commercial product. By using NMR, it appeared LP2 contained high concentrations of sugars compared to WC. These sugars would result in low antioxidant activity per given mass of extract and sugar removal would thus result in improved antioxidant activity. Water was then used to selectively remove these sugars. The resultant product, LP3 was 67 % as active as WC in the DPPH test and the catechin equivalent test and 83 % as active in the Butanol-HCl test.

NMR also indicated that LP3 had a higher incidence of non-polar compounds compared to WC. It was already established at the beginning of the study that the presence of non-polar compounds resulted in a decrease of antioxidant activity. Work was therefore done on LP3 to remove the non-polar compounds.

DCM was next used to extract the non-polar compounds leading to product LP4. The use of DCM was however not attractive as there was a possibility of substantial quantities of residual solvent being found in the final product. It is also a valuable marketing tool if it can be stated that no toxic extractants were used in the procedure. This led to the investigation of the possible use of Diaion-HP20 in removing non-polar compounds.

Diaion effectively removed non-polar compounds from the extract and it could be used repeatedly without significantly compromising its effectiveness. The potency of the extract obtained with the use of Diaion (LP5) was measured by the Trolox equivalence antioxidant capacity assay and compared to commercial products. It was found that LP5 had an activity that was 72 % higher than that of WC. The progress followed in attaining the

proanthocyanidin enriched grape seed extract is shown in Table 11.1 and Figure 11.1 shows the final process which results in LP5

The percentage yield of the process was 5.5 % of the original OEGS mass. This value is much higher than yields reported in patented methods e.g. Frangi *et al* (1996) reported a yield of less than 1.5 % by weight of flavanloic monomers. Franc *et al* (1998) in their patented method obtained yields of 1.5 to 2.5%, which according to them, was 3 to 4 times higher than the yields obtained by other methods.

The aim of the study was to make a low cost and effective product, which will be able to compete with products already in the market. A local company, Afriplex (Pty) Ltd an affiliate company of Weir en Vig was impressed with the result of this study and a licensing agreement was reached.

Table 11.1: Progress followed in attaining the proanthocyanidin enriched grape seed extract, where " % activity" represents antioxidant activity of the products relative to WC using the DPPH method (LP1 – LP4) and TEAC assay (LP5).

Product	Extraction method	% activity	% yield
LP1	• Mix OEGS with 80% ethanol in a ratio of 1:5 • Extract for thirty minutes at 95 ^{0}C • Filter and dry extract at 95 ^{0}C	33.0	12.0
LP2	• Mix OEGS with 80 % ethanol in a ratio of 1:5 • Shake vigorously for thirty minutes • Filter and dry extract at below 60 ^{0}C	55.0	10.1
LP3	• Mix OEGS with distilled water in a ratio of 1:10 • Extract for thirty minutes, decant water extract and re-extract marc with distilled water • Decant water extract and extract marc with 80 % ethanol in a ratio of 1:5 • Filter and dry extract at below 60 ^{0}C	67.0	6.0
LP4	• Extract LP3 with DCM in a ratio of 1:10	78.0	5.9
LP5	• Mix OEGS with distilled water in a ratio of 1:10 • Extract for thirty minutes, decant water extract and re-extract marc with distilled water • Decant water extract and extract marc with 80 % ethanol in a ratio of 1:5 • Filter ethanol extract and pass it through Diaion HP20 in a ratio of 1g Diaion to 750 mg grape seed extract • Dry extract at below 60 ^{0}C	172.0	5.5

Figure 11.1: Flow chart showing the final procedure of extracting OEGS to produce a proanthoyanidin rich extract (LP5)**.**

12 Proposals for future work

Further studies that can emanate from this work are:

1. Investigate and troubleshoot any problems that may arise in the process of up scaling the procedure. This also involves fine tuning the process to make it as effective as possible.
2. The characterisation of the grape seed extract- an important step towards quality control. This will involve use of HPLC, and NMR and will seek to define the type of compounds present in the extract and the quantities in which they are present.
3. Testing the toxicity of the extract. The extract could be administered to a target animal, most probably rats or mice, and monitoring for any toxic effects.
4. Test the efficacy of the product as an immune system booster in animals challenged with infections

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