

**Potential health benefits of antioxidant
effects of wine on lipids**

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Faculty of Health Sciences
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ABBREVIATIONS AND SYMBOLS

| | |
|-------------|--|
| AAPH | = 2,2'-azo-bis, (2-amidinopropane) dihydrochloride |
| ABAP | = 2,2'-azo-bis (amidinopropane hydrochloride) |
| ABTS | = 2,2'-azinobis-3-ethyl benzothiazoline-6-sulphonate |
| Acetyl- CoA | = acetyl coenzyme A |
| ADH | = alcohol dehydrogenase |
| AMP | = adenosine 5'-monophosphate |
| ANOVA | = analysis of variance |
| ATP | = adenosine triphosphate |
| AUC | = area under the curve |
| BHT | = butylated hydroxytoluene |
| BHP | = butyl hydroperoxide |
| BP | = blood pressure |
| CAD | = coronary artery disease |
| cAMP | = cyclic AMP |
| CD | = conjugated dienes |
| CDP | = cytidine-5'-diphosphate |
| CHD | = coronary heart disease |
| cm | = centimetre |
| CoA | = coenzyme A |
| CRP | = C-reactive protein |
| CV | = coefficient of variation |
| CVD | = cardiovascular disease |

| | |
|-------------------|--|
| DMACA | = p-dimethyl amino cinnamaldehyde |
| DNA | = deoxyribonucleic acid |
| E | = extinction coefficient |
| EDTA | = ethylene diamine tetraacetic acid |
| EGF | = epidermal growth factor |
| eNOS | = endothelial nitric oxide synthase |
| FAD | = flavin adenine dinucleotide |
| FADH ₂ | = reduced FAD |
| FAS | = fetal alcohol syndrome |
| Fluoresein | = (3',6'-dihydroxyspiro[isobenzofuran-1[3H], 9'[9h]-xanthen]-3-one)(disodium) |
| FMD | = flow-mediated dilatation |
| FRAP | = ferric reducing ability of plasma |
| fTBARS | = free TBARS |
| g | = gram |
| GC | = gas chromatography |
| GSH | = glutathione |
| h | = hour |
| HDL | = high density lipoprotein |
| Hg | = mercury |
| HPLC | = high performance liquid chromatography |
| Hz | = herz |
| IQ | = intelligence quotient |
| IUPAC | = International Union of Pure and Applied Chemistry |
| kg | = kilogram |
| kJ | = kilojoule |

| | |
|------------------|---|
| L | = litre |
| LDL | = low density lipoprotein |
| LOOH | = lipid hydroperoxides |
| Lp(a) | = lipoprotein (a) |
| m | = metre |
| M | = molar |
| MDA | = malondialdehyde |
| milli (prefix) | = milli (10^{-3} x) |
| min | = minute |
| mol | = mole |
| MUFA | = monounsaturated fatty acids |
| MW | = molecular weight |
| NAD ⁺ | = nicotinamide adenine dinucleotide |
| NADH | = reduced NAD |
| NADP | = nicotinamide adenine dinucleotide phosphate |
| NADPH | = reduced NADP |
| nano (prefix) | = nano (10^{-9} x) |
| NO | = nitric oxide |
| NOS | = nitric oxide synthase |
| OFTT | = oral fat tolerance test |
| ORAC | = oxygen radical absorbance capacity |
| PBS | = phosphate buffered saline |
| pH | = negative logarithm of the hydrogen ion concentration |
| Pi | = inorganic phosphate |
| PL | = phospholipid |
| Ppi | = inorganic pyrophosphate |

| | |
|------------|---|
| PUFA | = polyunsaturated fatty acids |
| ROS | = reactive oxygen species |
| rpm | = revolutions per minute |
| SD | = standard deviation |
| SDS | = sodium dodecyl sulphate |
| SEM | = standard error of the mean |
| SFA | = saturated fatty acids |
| SOD | = superoxide dismutase |
| TAS | = total antioxidant status |
| TBA | = thiobarbituric acid |
| TBARS | = thiobarbituric acid reactive substances |
| TCA cycle | = tricarboxylic acid cycle (Krebs cycle, citric acid cycle) |
| T.Chol | = total cholesterol |
| TE | = Trolox equivalents |
| TEAC | = Trolox equivalent antioxidant capacity |
| TG | = triacylglycerol (triglyceride) |
| TRAP | = total peroxy radical-trapping antioxidant parameter |
| Trolox | = 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid |
| tTBARS | = total TBARS |
| μ (prefix) | = micro (10^{-6} x) |
| UFA | = unsaturated fatty acids |
| VLDL | = very low density lipoprotein |

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All photography for this thesis was by the author except where otherwise indicated.

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University of Cape Town

Abstract

Potential health benefits of antioxidant effects of wine on lipids

D M Blackhurst

The consumption of wine dates back to the Stone Age, but its potential health benefits only really began to make an impact after the French Paradox was postulated by Renaud and de Lorgeril in the 1990s. Their observations indicated that moderate consumption of red wine may have health benefits. Research since then has shown that wine, in particular red wine, is a source of a large number of different polyphenolic compounds that have antioxidant activity.

The finding that the consumption of red wine might have a beneficial health effect was very appealing, resulting in a large number of epidemiological and experimental studies. To date, controversy still surrounds wine and its health effects. Several pertinent questions are still to be answered: What *in vitro* methods can be used to determine the antioxidant effects of wine? What are some of the *in vivo* effects of wine? Can wine be used directly as an antioxidant in cooking of the food that would ordinarily be exposed to conditions that may induce peroxidation of lipids? What effects does wine have at the cellular level?

Four areas of research were undertaken in response to these questions. The first study examined the antioxidant effects of red wine on thermally-stressed polyunsaturated oil. The lipid peroxidation products present in various monounsaturated and polyunsaturated oils were examined: total and free thiobarbituric acid reactive substances being the most varied, while lipid hydroperoxides and conjugated dienes showed less variation. Thermally-stressed polyunsaturated fatty acid oil showed an increase above baseline of approximately 450% for conjugated dienes and lipid hydroperoxides, while there was a 34% and a 1080% increase of total thiobarbituric acid reactive substances and free thiobarbituric acid reactive substances respectively, over 75 hours. Macro and micro oil boiling assays were set up to determine the antioxidant capability of 9 different red wines, revealing similar results for all. The wines had a powerful antioxidant capability relative to copper, a pro-oxidant, diminishing by 80–100% the peroxidation products tested.

There was a trend for red wine to protect the unsaturated lipids in red meat against conjugated dienes during moderate cooking. Marinating changed the conjugated dienes in meat after cooking from the raw state by -23.3%, the lipid hydroperoxides by -21.6%, and the thiobarbituric acid reactive substances by 37.1%. Loss of fatty acids from the meat into the red wine marinade was minimal as established by gas chromatography, which also determined that the fatty acid profile was not significantly altered during the cooking.

The possible antioxidant effect of the consumption of red wine on postprandial lipoproteins (chylomicrons) was investigated. There was no significant difference between the peroxidation products in isolated chylomicrons generated with and without wine, nor did they differ in their resistance to copper-induced lipid peroxidation.

There was a significant increase in the diameter of the brachial artery, relative to the baseline, over 2 hours after moderate consumption of red wine, although flow-mediated dilatation of the artery did not increase significantly. Wine consumption also significantly impaired the vasoconstriction with ice immersion after 60 minutes, but did not alter the vasodilatation after trinitroglycerine.

Wine is a complex beverage which may have beneficial health effects. These studies show that wine has strong antioxidant effects that can be simply described with nutritionally relevant polyunsaturated fatty acids in a boiling experiment, that there is a potential for wine to limit lipid peroxidation during food preparation and that its immediate effects on flow-mediated dilatation need further investigation. Ultimately, only a prospective, controlled interventional study evaluating cardiovascular outcomes can conclusively prove the health benefits of wine consumption.

CHAPTER 1. WINE, ITS HISTORY AND CULTURE, AND THE CHEMISTRY OF ITS EFFECTS ON LIPIDS AND HUMAN HEALTH

1.1. The history and culture of winemaking

1.1.1. History and culture

The origins of wine can be traced back as far as the Stone Age due to the discovery in Northern Iran in 1996 of a pot of fermented grape juice, probably the earliest form of wine. The pot was dated back to 5400–5000 BC (1). Literature includes a number of gods and goddesses of wine: Liber the Roman god, Siduri the Sumerian goddess, Sekhmet the Egyptian goddess, Sura the Indian goddess, Tepoztecatl the Aztec god and Ssu–ma Hsiang–ju the Chinese god.

Ancient Egyptian pottery shows that wine played an important role in their civilisation. Passages from the Bible refer to wine as “the blood of grapes”. In the Koran, Paradise is described as a place where there are “rivers of wine, an exquisite delight for drinkers” (2). Ancient Chinese medicine refers to the medicinal use of fermented drinks and medical writing from the 6th century B.C. from India describes the health benefits of alcoholic drinks.

The growing of vines and winemaking spread through Egypt to the west and south and also became entrenched in the Greek civilisation. The ancient Greeks travelled and traded overseas and took with them vines and the knowledge of wine production. Their trading with the Romans at about the first century A.D. marks the beginning of vine cultivation in Europe. Grape vines that are grown now are related to these vines. They belong to the family Vitaceae, genus *Vitis*. *Vitis* is divided into two subgenera: *Euvinis*, the true grapes, and the *Muscadinia*. The wine industry of the world is built upon one of the 60 *Vitis* species, called *Vitis vinifera*, which is often called the European grape (3).

By the end of the 5th century A.D. the Romans had established some of the world’s best-known vineyard areas for example Burgundy, Bordeaux and the Rhine valley of Germany. During the collapse of the Roman Empire and the ensuing Dark Ages, the Church became the focus of winemaking. By the 12th century the powerful Benedictine and Cistercian monasteries owned most of the French and German vineyards. Wars, the

power of the Church and royal marriages between the English, French and German nobility were some of the factors that controlled the wine markets well into the Middle Ages.

Initially the wine served and sold was very young and acidic. At first it was stored in flasks called amphorae, until the Romans developed glass blowing (4). This was followed shortly afterwards by the practice of sealing the bottles with stoppers. It was then possible to store wine for longer periods of time. The Bordeaux and Burgundy regions started producing wines specifically for ageing, called “reserve” wines.

Different types of wine were introduced, for example Champagne, a “sparkling” wine with bubbles of carbon dioxide generated by a second fermentation. It became very popular in the Café societies of the 1720s. Fortified wines were introduced, at first by adding grape brandy to wine to enable the wine to be transported by ship without the wine becoming vinegar. In 1730 the sweet fortified red wine, Port, was introduced in England.

During the French Revolution of 1789 the vineyards were taken from the Church and nobility and auctioned back to the citizens. By the 19th century the wine market was doing very well. The vine spread to other continents. In South Africa, Jan van Riebeeck planted the first vines in the Cape in the 1650s from French stock. In 1685 the Constantia estate was established. The first vines introduced into southeastern Australia were via the Cape. The gold rush of the mid-1800s resulted in the introduction of vines to California. A very popular sweet wine at that time was Constantia from South Africa.

In the 1860s, the French vines were decimated by an aphid-like insect known as phylloxera that attacked the roots of the vines (5). The disease spread across Europe and further down to the Cape, destroying most of the vines there by the 1880s (6). The disease was a disaster at that stage but the outcome was that only the most resistant vines survived and formed the basis of modern viticulture.

1.1.2. Winemaking, with special reference to South Africa

There have been huge advances in modern viticulture and winemaking science and technology during the last 35 years. Results from many studies have suggested that certain wine polyphenols play a positive role in human health, and for this reason

oenologists have attempted different strategies to produce wines that are rich in bioactive phenolic compounds, for example catechins and anthocyanins (7).

1.1.2.1. Vineyards

Vineyards around the world, including those in South Africa, fall mainly between 30–50° latitude (the temperate zones) in each hemisphere. The weather there is mostly ideal for growing and ripening the grapes: cold winters, long and warm summers with occasional rain followed by dry autumns for harvesting the grapes. Too much rain results in diluted juice with reduced flavour (2). Changing exposure of grapes to sunlight has been found to alter phenolic profiles in wine, (in particular quercetin in red wine) (8,9).

Choice of vineyard site and its associated climate and soil conditions often dictate which grapes are grown. Highly fertile soils are generally undesirable due to the excessive vigorous foliage growth these soils induce, as opposed to infertile soils, which induce vines to spend their energy on grape growth. The chemical composition of the soil is important and it is usually correctable if there are excesses or deficiencies. Two problems in winemaking are due to inadequate soil:

- 1) incomplete yeast fermentation, which is due to insufficient nitrogen in the soil and
- 2) wine with too high a pH, which is due to excess potassium in the soil (the usual pH for white wine is 3.0–3.3 and for red table wine is 3.2–3.4: (3)).



Figure 1.1. Steenberg vineyards in Cape Town.



Figure 1.2. Red grapes from Stellenbosch, Western Cape.

1.1.2.2. Harvesting the grapes

Picking the grapes by hand is still carried out fairly extensively in South Africa, but harvesting by machine is also used. This method is less costly and less time-consuming, but the grapes are often more damaged. White grapes are used in the making of white wine. Their skins range in colour from green to yellow to pink, but their juice is “white”. The juice of red grapes is also pale and the skins, being dark in colour, are used to colour the red wines.



Figure 1.3. Red grapes recently harvested.



Figure 1.4. Mechanical sampling of the grape load.



Figure 1.5. Container with mechanical screw for collection of grapes at off-loading.

1.1.2.3. Winemaking in general

Different winemaking techniques, including using different ageing temperatures and times, and oxygen exposure, are found to influence the class composition and concentrations of phenols in wine. For example during ageing, concentrations of anthocyanins (pigments that significantly contribute to red wine colour) (Figure 1.24)

decrease due to their progressive reaction with other phenols, especially the flavanols (10)

1.1.2.3.1. White wine making

After arriving at the winery, the grapes are either pressed immediately to separate the juice from the skins and seeds, or crushed lightly and left for approximately a day before pressing. Allowing the skins and juice to react together for this short time results in a more flavourful wine as well as increasing the concentration of polyphenols, which has been found to improve the antioxidant capacity of some white wines (11). The juice is then transferred into fermentation vats, which may be old-fashioned concrete (Figure 1.6), oak or stainless steel (Figure 1.9).



Figure 1.6. Concrete vats at the Vina Cousiña Macul winery in Santiago, Chile, South America.

1.1.2.3.2. Red wine making

In contrast to white wines, the juice as well as the skins and seeds are put into stainless steel vats or oak barrels for the fermentation process. During fermentation, anthocyanins are able to condense with tannins, from either the grapes or oak, in the presence of acetaldehyde, leading to the production of pigments which in solution are more resistant to pH change and bleaching by sulphur dioxide and are generally more stable than

solutions of anthocyanins alone. This is important for the retention of red wine colour (12,13). The presence of acetaldehyde is due to the slow uptake of small amounts of oxygen (micro-oxygenation) through oak barrels or by small measured additions to the stainless steel vats, and in the presence of a transition metal (iron, copper or manganese) the oxygen becomes activated. This activated oxygen, represented by hydrogen peroxide, is able to oxidise alcohol to acetaldehyde, and this stabilises the anthocyanin-tannin reaction (personal communication with Michael Buccholz, the winemaker at Distell Winery).

The majority of South African red wines are fermented in stainless steel vats, some with oak staves or oak chips added to the vat during the process. Concrete vats are still in use in South Africa. Fermentation temperatures are controlled at approximately 25–28°C for many South African red wines.



Figure 1.7. Red grape juice, seeds and skin are transferred into a vat.



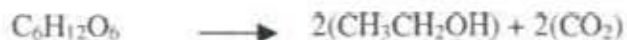
Figure 1.8. Red wine fermentation in a stainless steel vat.

During fermentation the colour from the anthocyanins as well as tannins from the skins leaches into the juice (must). This process is called maceration. Dark red wines (for example Merlot and Shiraz) require that the skins and juices react together for longer (for example two weeks) than the lighter-coloured red wines, which may have only a few days to react, before the juice is transferred to vats to continue with the fermentation process. Examples of red wines are Cabernet Sauvignon, Shiraz and Pinotage (from South Africa's own grape hybrid of Pinot Noir and Hermitage). A recent comparison of different cultivars of red wines from different regions worldwide showed wide ranges in catechin and epicatechin concentrations (14); those from South Africa were amongst the lowest studied. Table 1.2 shows compositional data of red and white wines. Among the potential reasons given for these differences were different climates, soils and in particular different winemaking techniques.

1.1.2.4. Fermentation

1.1.2.4.1. Fermentation in general

Fermentation is the biochemical process whereby sugar (predominantly glucose and fructose) in the juice of the grapes is converted into ethanol and carbon dioxide by the metabolism of yeast:



Glucose and fructose are metabolised by yeast to pyruvate via glycolysis. Pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol by yeast alcohol dehydrogenase (ADH).

Glycerol is the major fermentation product after ethanol and carbon dioxide (15). Apiculate and oxidative yeasts for example *Candida*, are the predominant yeasts on the grape, but *Saccharomyces cerevisiae* is the species mostly responsible for alcoholic fermentation as it is tolerant to ethanol (16). The optimal growth temperature for *S. cerevisiae* is 25°C (17). It is a complex process that may be affected by a number of factors that ultimately affect the quality of the wine, such as composition of the grape juice, the temperature of fermentation, concentration of sulphur dioxide and strains of yeast (18,19). Owing to insufficient yeast cells on the surface of the grapes, natural or cultivated yeasts are added after the grapes have been crushed. During fermentation, the heat generated by the process may be suppressed by controlling the temperature, for example in the stainless steel vats.



Figure 1.9. Stainless steel vats for fermentation.

Fermentation ceases when all the sugar has been converted to ethanol or when alcohol inhibits the process. Fermentation stops at an alcohol content of 8–10% (20). The

sediment that is formed from dead yeast cells at the bottom of a vat is known as “lees”. There are many volatile compounds produced as a result of fermentation, including isobutanol, ethyl hexanoate, acetic acid and valeric acid, and these are responsible for wine aroma (21).

The presence of sulphur dioxide in wine is due to deliberate addition to either must or wine, or also the production by yeast during fermentation. This results in growth inhibition or death of undesirable bacteria and yeast and prevention of brown pigment development. These bacteria include acetic acid bacteria (genera *Gluconobacter* and *Acetobacter*), which are present on the surface of grapes and have been found to be present during alcoholic fermentation and subsequent ageing of the wine, despite requiring aerobic conditions. They produce acetic acid from ethanol and so may contribute towards bacterial spoilage of the wine. Sulphur dioxide may prevent this (22). Sulphites in wine have been found to have antioxidant properties (23). Wine that contains sulphur dioxide must have this declared by labelling (in South Africa, Australia and the United States of America) because individuals lacking the sulphite oxidase enzyme are highly sensitive to the compound.

1.1.2.4.2. Malolactic fermentation

This secondary fermentation is an option for white wine but is one that most red wines undergo. It is a natural process that takes place after the main fermentation and before ageing and bottling. It involves the conversion of “tart” malic acid (which, with tartaric acid, are the dominant organic acids in wine: (24)) to lactic acid and carbon dioxide (Figure 1.10) by lactic acid bacteria, which renders a white wine “creamy” and a red wine “soft and buttery”.

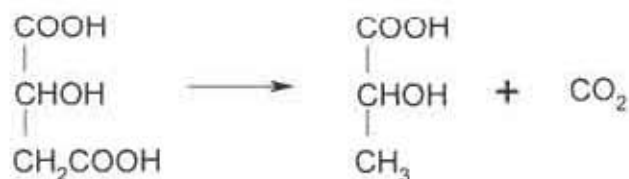


Figure 1.10. Conversion of malic acid to lactic acid.

1.1.2.5. Ageing

Most white wines are made for drinking young, for example Sauvignon Blanc and Chenin Blanc, whereas a few are best aged in oak, for example Chardonnay. Most white wines undergo minimal, if any, deliberate bottle-ageing.

Most red wines are aged, first in oak (for 6–24 months) and then in bottles (for up to 20 years). In red wines the length of ageing is dependent upon the quality of the grape, the cultivar and the winemaking process itself. After ageing, mature red wines have a red–brown colour and are less astringent than their younger counterparts, which are red–blue in colour with a fairly astringent taste. This is due to the reaction of anthocyanins in mature wines with the flavanols (10).

As ageing progresses, the aroma and flavour of the wines change, leading to the development of softer, richer wines. Maturation of red wine in oak results in complex interactions between compounds in wood and wine, involving oxidation, hydrolysis and polymerisation. This chemistry is not well-understood. The antioxidant activity of wine during the fermentation stage has been found to be significantly lower than that of aged wine, although their total phenol concentrations were similar. This suggests that the larger polyphenol polymers that appear during ageing play an important role in the total antioxidant activity of red wines (25). During the ageing process, the ellagitannins (Figure 1.15), owing to their rapid and easy oxidation, prevent oxidation of other wine components. These oxidised tannins then slowly release hydroperoxides into the wine (21). Generally, the younger the oak used and the smaller the barrel, the more distinct the flavour that is imparted to the wine from the harsh wood tannins. Older oak imparts a mellow character.



Figure 1.11. Ageing in wood.

1.2. HEALTH ASPECTS OF ALCOHOLIC BEVERAGES, WITH FOCUS ON WINE

This section provides information on alcoholic beverages, particularly wine, and describes the potential health benefits of consuming alcoholic beverages.

1.2.1. Background

There is little doubt that heavy consumption of alcohol is harmful to health, but whether moderate consumption has beneficial effects has caused much controversy. Hippocrates, the Greek physician and father of Western medicine (26) suggested nearly 2400 years ago that “food be your medicine and medicine your food”. He used wine for many ailments including diarrhoea and lethargy. Paracelsus (1493–1541), the Swiss physician, suggested that food can be a drug or a poison, and that the difference is a matter of dosage, and then approximately 400 years after that, Pasteur (1822–1895), the French chemist, described wine as the healthiest and most hygienic beverage. During the 19th century, the toxic effects of alcohol became evident and doctors were loathe to promote its consumption. Evidence of health benefits of consumption of alcoholic beverages only really began accumulating in the 20th century, with a large number of

studies taking place in the 1990s. To date this accumulated evidence shows paradoxical results: heavy drinkers suffer predominantly harmful results (27) while abstainers may fare less well than regular moderate drinkers in the risk for coronary heart disease (CHD), strokes and total mortality. Moderate drinking is now generally accepted as 10–35g alcohol/day (28,29). The risk of all-cause mortality in moderate drinkers is 20–30% lower than in abstainers (30). There are several problems associated with proving whether wine or any other alcoholic beverage has health benefits. To be effective, epidemiological studies should ideally be carried out over a number of years, because diseases against which alcoholic beverages may confer protection, e.g. cardiovascular disease (CVD), develop slowly. Similarly, harmful effects may only be evident in the long term. The changes in the body or health outcomes that are being studied may require large sample numbers to demonstrate an effect. Many studies rely on self-reported drinking data, and alcohol consumption is often under-reported (29,31). Patterns of drinking are also often very variable and therefore potential benefits and risks are accumulated only over long periods of time. There is also the problem that factors such as economy, gender, age, diet, smoking, social class, ethnicity and education play a role in population health and may therefore act as confounders in studies of effects of alcoholic beverages on health. The Atherosclerosis Risk in Communities Study (31) found contrasting associations between alcohol consumption and incidence of coronary heart disease (CHD) in White and Black subjects with an inverse association for White men and a positive association for Black men for 13g/day ethanol consumed as wine, beer and spirits. This might raise the question as to whether alcohol really has a cardioprotective effect or if the effect is confounded by lifestyle characteristics. Correlations between wine consumption and other health-promoting dietary factors have been reported (32).

1.2.2. Effects of alcohol

The effects of alcohol may appear as soon as 10 minutes after consumption, and peak within 40–60 minutes. Absorption of alcohol depends to a large extent on the amount and type of food in the stomach, for example high fat and carbohydrate foods reduce absorption rates and carbonated alcoholic drinks promote a faster absorption rate. The bulk of the absorbed alcohol remains in the blood until it is metabolised by the liver. If

the consumption of alcohol occurs at a faster rate than it can be metabolised by the liver, the blood alcohol concentration increases.

South African drinkers consume amongst the highest volumes of alcoholic beverages in the world, at approximately 50mL ethanol per drinker per day, making alcohol abuse one of South Africa's top ten health and social problems (33). The South African legal blood alcohol limit is 50mg /100mL (34) and the corresponding breath alcohol limit is 0.24mg/L. If a pregnant woman abuses alcohol, alcohol can cross the placenta to the fetus, and this can cause fetal alcohol syndrome (FAS). The fetus then develops congenital defects and growth retardation. Facial abnormalities, reduced mental functioning, central nervous system dysfunction and delayed development are characteristic of this syndrome. The highest prevalence of FAS in the world occurs in the Western Cape (35).

The Widmark formula is an estimation of the amount of alcohol consumed from a single measurement of a person's blood alcohol concentration (36). Saliva alcohol analysis correlates significantly with blood alcohol analysis and can therefore be used as an independent test for sobriety in place of blood or breath testing. The advantage of saliva is that it is obtained in a non-invasive manner (37,38).

The pharmacokinetics of alcohol metabolism have been studied extensively. Alcohol dehydrogenase reversibly oxidises primary and secondary alcohols to their corresponding aldehydes and ketones, much of which takes place in the liver. Aldehyde dehydrogenase (AcDH) oxidises the potentially reactive aldehydes to acetic acid. The reactions are summarised in Figure 1.12. ADH is found in animal retinas and liver and oxidation by liver ADH contributes to clearance of blood ethanol.

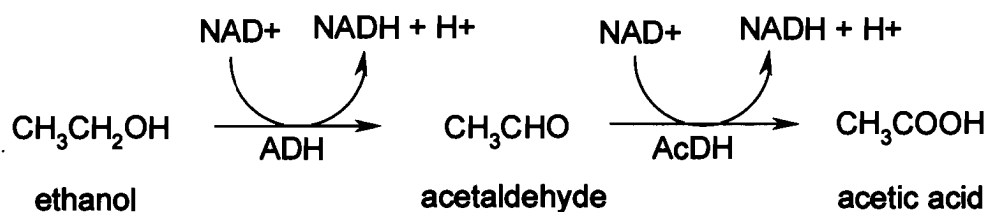


Figure 1.12. The metabolism of ethanol by ADH and AcDH.

There are 3 types of liver ADH: ADH1, 2 and 3. ADH 2 and ADH3 have polymorphisms that result in isoenzymes with particular kinetic properties. Epidemiological studies have shown an association between homozygosity of the fast-metabolising ADH3(1) allele, and alcohol-related diseases such as alcoholism and oropharyngeal cancer (39). Moderate drinkers showing homozygosity at the ADH3 locus for the allele associated with a slow rate of ethanol oxidation (ADH3(2)), have been found to have a significantly decreased risk of myocardial infarction (40), and higher high density lipoprotein (HDL) levels compared with non-drinkers who have the same genotype (41).

1.2.3. The controversial issues of alcohol consumption and health

Although the balance of epidemiological evidence is in favour of moderate consumption of alcoholic beverages being conducive to good health, the central questions are still largely unanswered. It will be difficult to resolve the question of whether wine has additional health benefits (for example cardioprotective effects) compared with beer or spirits and if so, whether red wine is better than white wine. It will also be difficult to resolve the different contributions of alcohol and phenols to health and the specific effects of the different polyphenols in wine as well as their net effect, without resorting to interventional studies or experimental evidence.

1.2.3.1. Does wine have more health benefits than for example beer or spirits and if so, is red wine better than white wine?

There is still dispute over whether any particular one of the alcoholic beverages offers more protection than the others. Many reports support the proposal that wine has especially-beneficial effects on health, relative to the other alcoholic beverages (28,30,42-45). Wine and beer are complex mixes of compounds. Beer, like wine, is produced by fermentation. Both beverages comprise water, ethanol, acids, esters, aldehydes, ketones and phenolic compounds, although many of their phenolic compounds are different. Beer generally comprises 3-6% ethanol and wine 10-14%, whereas spirits, usually prepared by distillation, contain approximately 40% ethanol. Sherry and port are "fortified" by additional alcohol to approximately 20%. Wine is also reported to be less harmful than beer and spirits in that it contains far lower concentrations of nitrosamines, a

group of N-nitroso compounds such as nitrosodimethylamine that are broad-acting and potent carcinogens (46,47). Vliegenthart *et al* (48) showed the individual relative risk of CAD after consumption of wine to be lower than that after consumption of beer or spirits, in asymptomatic subjects. However, some studies have found benefits with all the alcoholic beverages on the risk of CHD and mortality (49-52) while others have reported a greater beneficial effect from spirits than wine and beer (53). Because different diets are often associated with consumption of different alcoholic beverages, it may be difficult to attribute the benefit to the beverage. It has been suggested that perhaps wine and beer have similar physiological effects, but that differences in the risk factor patterns amongst those who drink these beverages could create the differences (54), for example wine consumption is associated with a higher intake of healthy food such as fruit, vegetables, fish and olive oil (32,55). Additionally, in communities with higher alcohol consumption, alcohol-related diseases may be the reason for abstention. Wine drinkers have been found to have higher IQs, to smoke less, exercise more, to be better educated and have a higher socio-economic status than beer and spirit drinkers (55-57). Red wine is often reported as having greater health benefits than white wine, and this is usually attributed to the higher total polyphenol content of red wines (58). Some studies have shown that red wines have greater antioxidant activity than white wines (59,60), whereas other studies have found white wine phenols have a greater antioxidant effect than those from red wines (61). However, some white wines that have an increased extraction of grape skin polyphenols, have similar antioxidant characteristics to red wines (11). Studies by Glidewell *et al* (62) suggest that if superoxide-scavenging ability determines health benefits, then white wine is at least as effective as red wine, as measured by spin-trapping electron paramagnetic resonance spectroscopy. The interpretation of reported differences in health benefits of the different alcoholic beverages should be viewed with caution until confounding effects have been clearly ruled out or mechanistic differences are evident.

1.2.3.2. *Is it the alcohol or the polyphenols in the wine that is more beneficial?*

The influence of the ethanol in alcoholic beverages has not been fully established and reports are contradictory. Ethanol influences metabolism in a number of ways, by acting

as a fuel source, altering fluid balance, increasing HDL concentration (63,64) and reducing platelet aggregation (65). Ethanol has also been found to increase elimination of polyphenols from the plasma compartment (63). Doll (66) reported that it is the ethanol in alcoholic beverages that is responsible for the beneficial health effects rather than the other characteristics of the beverage. Likewise, a review by Rimm *et al* (49) concluded that most of the benefits of alcoholic beverages derive from the alcohol rather than the other components of each beverage. This is refuted by a number of studies which demonstrate that the beneficial effects of wine in particular are derived from the non-alcoholic fraction, for example Wallerath *et al* found that an increase in expression of endothelial nitric oxide synthase (eNOS) by red wine was not associated with ethanol (67), (although a study by Abou-agag *et al* has subsequently proved that ethanol can increase eNOS (68)). eNOS is an enzyme responsible for the production of nitric oxide (NO), which enhances vasodilatation. Endothelium-dependent vasodilatation was found to increase after consumption of 250 mL dealcoholised red wine (69) and the ingestion of 113 mL of dealcoholised red wine increased plasma antioxidant capacity (70). Sato *et al* (71) also demonstrated the cardioprotective effects of ethanol-free red wine extracts, in their ability to improve post-ischaemic ventricular function. Short-term (7 days) treatment of red wine polyphenols was shown to decrease blood pressure and reduce infarct size and oxidative stress in ischaemia-reperfusion in rats (72). Consumption of alcohol-free red wine extract, equivalent to 375 mL red wine, inhibited *ex vivo* copper-induced LDL oxidation (73). Sato *et al* found that both the polyphenols and the alcohol in red wine reduce the incidence of CHD, but by different mechanisms. Polyphenols such as resveratrol and proanthocyanidins acted as *in vivo* antioxidants, while the alcohol adapted hearts to oxidative stress. Both then triggered a reduction of proapoptotic transcription factors, resulting in reduced cardiomyocyte death (74).

1.2.3.3. What are the specific effects of the different polyphenols in wine and what is their net effect?

Since the 1990s, potential benefits of specific non-alcoholic components of alcoholic beverages, in particular red wine, have received attention. Although many studies show

an added advantage from red wine with its high concentration of polyphenols relative to white wine and spirits, there is as yet no epidemiological consensus.

The action of polyphenols and flavonoids

At the cellular and molecular level, polyphenolic compounds have a broad range of biological activities that depend on the individual structural characteristics:

- Their antioxidant action may prevent activation of carcinogens (75) and inhibit cell and tissue damage due to free radicals (76), leading to reduction of development of atherosclerosis and inflammatory diseases.
- They have been shown to inhibit growth and induce apoptosis of several cancer cell lines, including colorectal cancer cells, at nM and μ M concentrations (77-79), and human breast and lung cancer cell lines (80,81). These concentrations are potentially achievable *in vivo*.
- Flavonoids are lipid-soluble and can be incorporated into cell-membranes, sometimes interfering with normal membrane functioning.
- They may interact with a number of enzymes for example receptor kinases and protein kinase C (82).
- In many organisms, calorie restriction leads to a reduction of the ageing process and a subsequent increase in maximum lifespan. It has been postulated that a number of the effects of polyphenols (in particular resveratrol) might be due to their action of mimicking the calorie-restriction response mediated by sirtuins. Sirtuins are a family of NAD⁺-dependent protein deacetylases. SIRT1 is one such human deacetylase that has been found to negatively regulate the p53 tumour suppressor (by deacetylating lysine 382 of p53), leading to increased cell survival (83).

The polyphenols quercetin and resveratrol in wine have both been found to be cardioprotective (84,85). Polymeric proanthocyanidins in red wine have also been found to protect against myocardial complications subsequent to ischaemia and reperfusion (86). As described in Section 1.3, there is a wide range of different polyphenols in wine, and there is also large variation amongst these polyphenols, especially the flavonols, in the different wines (87). One of the most important of these is the antioxidant

hydroxystilbene, resveratrol (Figure 1.19). It is found mostly in red wines, but to a lesser extent in white and rosé wines (88), and recently, for the first time, permission was given by the United States government to label bottles of two different pinot noir wines, which have high concentrations of resveratrol, with their antioxidant information (89). Resveratrol, along with its hydroxylated analogues, has been found to have a number of health benefits, mainly in the field of cancer research (90). It may act as a phytoestrogen by binding to oestrogen receptors and thus blocking the mitogenic response of oestrogens (31,91). Reports in 1997 and 2005 summarised the anticancer activities of this compound (92,93): it has cytostatic properties via inhibition of initiation, specifically inhibition of free radical formation, promotion and progression of carcinogenesis; it reduced the incidence of carcinogen-induced cancer development in a mouse mammary gland culture and in a mouse skin cancer model; it induced differentiation and apoptosis in a number of different tumour cell lines, such as human breast cancer (94), human leukaemia (95) and oesophageal cells. It has been shown that resveratrol may be converted to piceatannol, a known stilbene antileukaemic compound, by the cytochrome P450 enzyme CYB1B1 found in human tumours (96). In 2000 it was reported for the first time that resveratrol had a direct antimicrobial effect when it was found to inhibit the growth (*in vitro*) of *Helicobacter pylori* (*H.pylori*), a bacterium associated with gastric cancer (97). Resveratrol is able to inhibit platelet aggregation (84,98,99). Resveratrol also has antioxidant properties: it inhibits peroxidation of lipids in cell membranes mainly by scavenging peroxy radicals in the membrane (100). It is also able to reduce iron-induced lipid peroxidation of rat liver microsomes (101). That resveratrol inhibits oxidant-induced peroxidation of LDL was first reported in 1993 (102) and corroborated by a study in 2002 (103). Resveratrol and its analogues, for example astringinin (104), protect against ischaemia-reperfusion injuries in rat hearts (105,106) by preventing superoxide-dependent inflammatory responses (such as endothelial barrier disruption) brought about by reperfusion. Resveratrol however, is usually found in low quantities in the diet, and might be inefficient at normal nutritional intake concentrations. Resveratrol may itself be oxidised to a phenoxyl radical which forms quinone, a highly reactive prooxidant which exerts cytotoxicity (107). The half-life of resveratrol is short (human plasma half-life is 9.2 ± 0.6 hours), and much may be lost before absorption (108,109).

Studies have shown that catechins induce regression in tumours, both *in vitro* and *in vivo*, by several mechanisms, only some of which have been characterised. Doss *et al* (110) showed that catechins are scavengers of the growth factors that promote carcinogenesis.

1.2.3.4. What constitutes moderate drinking?

The definitions of moderate and heavy drinking are arbitrary, as is the definition of a 'glass' or a 'drink'. A moderate intake of alcohol has been reported as much as 2–5 glasses per day (111), as little as 1 glass (112) or more usually ≤ 2 drinks daily for men and ≤ 1 drink daily for women (113). "Anstie's Limit" is still sometimes quoted, setting the upper safe limit of sensible drinking to approximately 45mL ethanol per day (114). Studies examining the impact of alcoholic beverages on health, report the intake variably as units or grams of alcohol, representing almost pure ethanol, per day. A unit of alcohol is defined as approximately 10g ethanol, which is equivalent to 1 drink (100mL wine, 200mL beer, 50mL fortified wine and 25mL spirits (48). The fermentation process that yields wine and beer is limited by the sugar content and the alcohol concentration, since the latter will eventually inhibit fermentation. Red wines average 13% and white wines 12% alcohol. Spirits have higher alcohol concentration due to distillation. The "proof" is a measure of the alcohol (ethanol) concentration of alcoholic beverages, being twice the percentage of alcohol by volume. Sherry and port are "fortified" by additional alcohol. Data from many studies summarised in the J-shaped curve (Figure 1.13) suggest that an increase in all-cause mortality, all-stroke and CHD relative risks, may be evident from approximately 35g alcohol per day, and that "moderate drinking" could be defined as approximately 10–35g alcohol (115).

1.2.4. Studies of health benefits of alcoholic beverages

Evidence for the beneficial effects of the consumption of alcoholic beverages on health may be derived from epidemiological or experimental research. In the absence of a controlled interventional study, there should be strong evidence from both avenues of investigation, and the benefits should exceed the harm before public health

recommendations can be made to include the consumption of alcoholic beverages in daily living.

To date there have been numerous epidemiological as well as experimental studies showing the compelling evidence of health benefits of alcoholic beverages, in particular wine, and their various components. The limitations of epidemiological studies include the lack of validated biomarkers, lack of long-term controlled clinical studies, and lack of sufficient knowledge of bioavailability for relevant control in *in vitro* and *in vivo* studies. Many of the conclusions from epidemiological studies are based on results from meta-analyses, a term coined in 1976, where associations are often tenuous at best. According to Shapiro (116), meta-analyses are based on studies that are often incomplete and disputed. He suggested that meta-analysis of all observational studies be stopped. Greenland however, suggested that analysis of these studies acts as a means to compare the studies and should be used to identify patterns amongst results (117), while Taubes (118) suggested that results from epidemiological studies should be treated with a measure of scepticism and an awareness of inappropriate associations.

1.2.4.1. Epidemiological studies

In 1926, the first finding of a U-shaped association between all-cause mortality and consumption of alcoholic beverages was reported by Raymond Pearl in a book called *Alcohol and Mortality* (119,120). Since then, a large number of epidemiological studies have shown a U-or J-shaped association between alcohol consumption and all-cause, particularly cardiovascular, mortality (Figure 1.13). This relationship may be influenced by factors such as drinking patterns, age, gender and genetics. The shape indicates that moderate daily alcohol consumption results in a significant reduction in mortality compared with individuals who abstain or drink alcohol to excess.

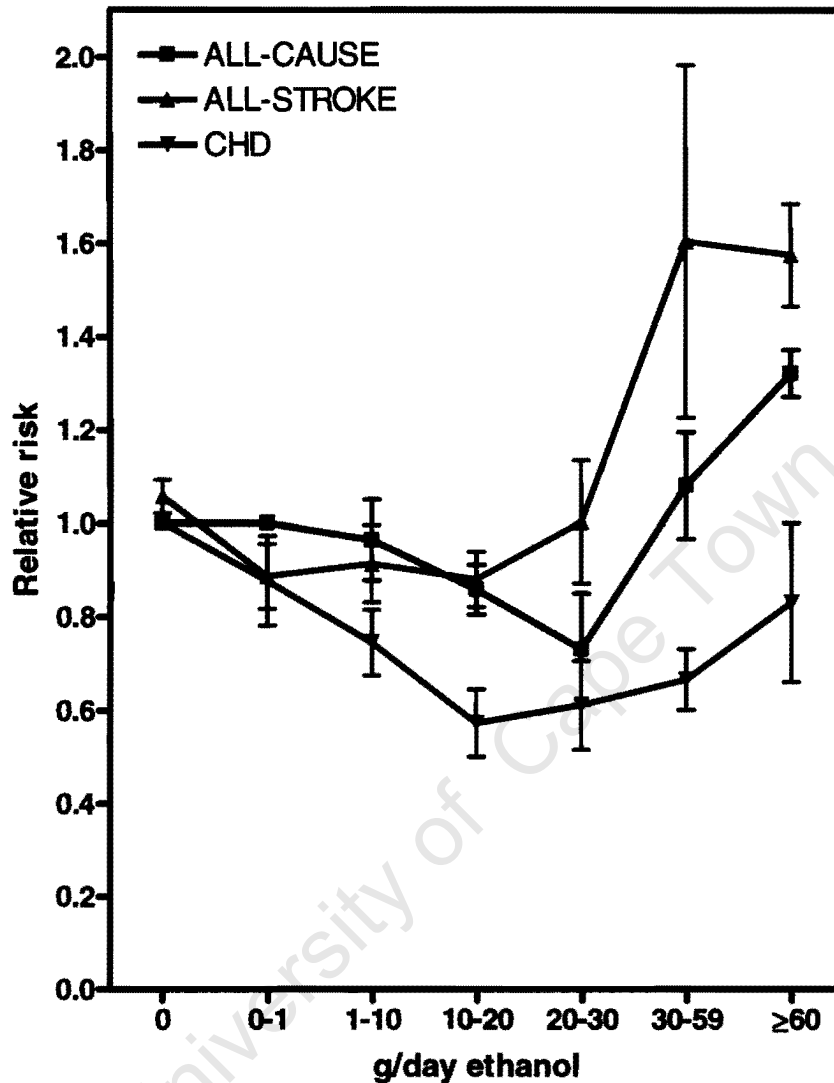


Figure 1.13. All-cause mortality, all-stroke and CHD risk in relation to alcohol consumption. Data reflect the means and SEM from 7 all-cause, 11 all-stroke and 11 CHD studies respectively.

Results from worldwide epidemiological studies show that the beneficial effects of moderate consumption of wine may be divided into several areas of health:

- *Protection against CHD and stroke:*

Worldwide, CVD is the leading cause of death, responsible for approximately 30% of all deaths globally. Arteriosclerosis is the most significant factor contributing to the pathogenesis of CHD. People with CHD typically become symptomatic after age 40

years, but it is thought that CHD begins much earlier. In one study of cardiac transplant donors, 17% of those aged <20, 39% of those aged 20–29, and 85% of those aged >50years, had demonstrable lesions of atherosclerosis (121). Moderate consumption of alcoholic beverages, especially red wine, is associated with a decreased risk of CHD of up to 50%, relative to abstainers (30,122), but the causal mechanisms are not fully understood. It has been suggested that, because inflammation plays a role in the initiation and progression of atherosclerosis, the protective role of red wine may be due to a reduction in inflammation. Some studies have shown only a moderate reduction in certain markers of inflammation, for example serum fibrinogen (a protein that plays a key role in thrombosis, also appears to reflect an inflammatory state (123), and which is associated with CHD in older adults (124,125)) and C-reactive protein (CRP) (also associated with CHD (125,126)), whereas other studies found lower levels of CRP (127,128) and fibrinogen (126,128) among healthy moderate drinkers.

Epidemiological studies over the last three decades have established a strong inverse relationship between alcohol intake and fatal or non-fatal CHD (30,122,129–134). The vast majority of research results concerning the health benefits of alcohol consumption, in particular wine, concern the benefits to vascular disease, in particular CHD. In 1979, St. Leger *et al* showed the strong inverse relation between alcohol, especially wine, consumption in developed countries particularly France, and ischaemic heart disease mortality (135). The ‘French Paradox’, a sometimes controversial phenomenon reported in 1991 in an American television programme, and described by Renaud and de Lorgeril in 1992 (136) describes the lower rate of mortality from CHD among the French compared with other developed countries, despite similar dietary intakes in these countries. The explanation has been that their relatively high consumption of red wine protects the French from heart disease, and that the resveratrol in the wine was specifically responsible (102,137). However, it has been suggested that the paradox may partly be due to incorrect statistics and recording of mortality data (at that time France was known to under-report ischaemic heart disease deaths (135)) and partly because of the influence of past, rather than present, fat intake, leading to a “time lag” in influencing the risk for heart disease (138). This time lag theory generated much discussion amongst clinicians and scientists, who suggested that the rates of CHD are in fact the same in

France as in other countries in Southern Europe but these rates are all lower than in Northern Europe; that the effect of high plasma cholesterol concentrations on CV risk in populations from Southern Europe is not the same as that from Central or Northern Europe or the United States, and that after allowance has been made for effects of past animal fat intake, there is a significant association between wine consumption and mortality from heart disease (139-142). There was after this, however, an increase in the number of studies seeking to provide information on the health benefits of wine consumption. A study by Wannamethee *et al* (143), however, has since found that non-drinkers, with or without CHD, should not be encouraged to take up regular drinking of alcoholic beverages, due to the increased risk of non-cardiovascular mortality with no reduction in CHD. Moderate alcohol consumption may reduce the risk of fatal myocardial infarction in people with ischaemic left ventricular systolic dysfunction (144). In addition, moderate alcohol consumption reduced the risk of cardiovascular complications, such as CHD recurrence, unstable angina, cerebral stroke and peripheral embolisms, after recent myocardial infarctions (145).

A number of studies in the 1990s reported a reduced risk of myocardial infarction after alcohol consumption and a reduction in atherosclerotic mortality (45,49,52,135,146). Various other studies have reported a reduction in total and ischaemic stroke (147) and a reduction in vascular deaths (coronary thrombosis and ischaemic stroke) of between 30–50% (148,149). Alcohol consumption was found to provide possible benefit to those with pre-existing cerebrovascular disease (150). However these are merely associations, and do not resolve the question as to whether it is the alcohol consumption itself or other factors that are responsible for the benefit of decreased mortality. There have been a number of studies showing a positive correlation between alcohol consumption and stroke, but most have been after heavy and few after moderate consumption of alcohol (133). Also, approximately 80% of strokes are ischaemic in nature (151,152) and consumption of alcohol is reported to affect the 2 types differently. Several large epidemiological studies have reported an increased risk of haemorrhagic stroke but a lower risk of ischaemic stroke (133,153,154). The protection towards ischaemic stroke may be due to the effect of alcohol on HDL-cholesterol (43). Strokes are also more likely to occur in life-long abstainers than current non-drinkers (155).

- *Prevention of cancers:*

Data for effects of moderate alcohol consumption on cancer are inconclusive. Alcohol consumption, in particular heavy drinking, especially of beer and spirits, has long been associated with an increased risk of cancers of the upper digestive tract (156-158), although it has been reported that cancers of the head and neck areas, and of the oesophagus, might be related to confounding factors in that heavy drinkers are invariably smokers (43,159), and oesophageal cancer is unusually sensitive to environmental factors and diet (160). There is less certainty about the association of alcohol consumption with other cancers. Moderate consumption of red wine has been found to protect against lung cancer (44). A review by Longnecker *et al* (161) of a number of epidemiological studies concluded that alcohol consumption may be associated with mouth, pharynx, larynx, oesophagus and liver cancers. The association was unclear with breast and large intestine cancer but appeared not to increase the risk of melanoma or cancers of the stomach, lung, bladder, prostate, ovary or endometrium. A meta-analysis of data from 38 studies on alcohol and breast cancer showed that even though the results varied widely, the relative risk, compared with nondrinkers, increased linearly by 7% (162) and 11% (163) for daily consumption of 1 alcoholic drink. Pooled analyses from 6 prospective studies also showed a linear increase in incidence for women consuming approximately 1.5–60g alcohol per day (164). However, in the Framingham Study, with a follow-up of more than 40 years, consumption of alcohol of up to 15g per day was not associated with an increase of breast cancer in women (165) nor in premenopausal women consuming up to 11g alcohol (166).

- *Risk of hypertension:*

Epidemiological studies have shown the association between alcohol drinking and increased blood pressure (BP). Regular heavy consumption of alcohol is associated with epidemiological evidence of raised BP and seems to be independent of the type of alcoholic beverage (167). There seems to be a threshold dose of 20–30g ethanol per day, above which BP unequivocally increases.

- *Reduction in the risk of dementia:*

Compared with abstention, moderate consumption of alcohol, particularly wine, is

associated with better cognitive function and memory in the aged (approximately 65 years and older), while excessive drinking increases the risk (168-170).

- *Type 2 diabetes mellitus (DM) and the risk for CVD:*

Diabetics are a group at high risk for CVD. Results from the Physician's Health Study showed a decreased risk for type 2 DM in healthy men who consumed moderate amounts of alcoholic beverages (171,172) and a similar risk for CHD among diabetic and nondiabetic volunteers after moderate alcohol consumption (173). Solomon *et al* found moderate alcohol consumption was associated with reduced CHD in women with type 2 diabetes (174). Two large studies found a positive association between moderate to high alcohol consumption and the incidence of DM in Japanese men (175,176). Wannamethee *et al* found a U-shaped association between alcohol consumption and risk of DM, with moderate drinkers showing the lowest risk (177). Various other epidemiological studies have found a lower prevalence of DM amongst moderate drinkers compared with abstainers (178-181). However a systematic review by Howard *et al* (182) found moderate alcohol consumption to be associated with a decreased incidence of diabetes and a decreased incidence of heart disease amongst diabetics.

- *Decrease in bone mineral density:*

Bone density was found to be significantly higher in postmenopausal women who consume moderate amounts of alcohol compared with non-drinkers (183,184) but results showed that heavy drinking was associated with low bone density and a subsequent high risk of fracture.

1.2.4.2. Experimental studies

Experimental studies with alcoholic beverages have shown a variety of potentially favourable effects:

- *Ethanol preconditioning:*

Chronic low dose consumption of ethanol was found to maintain the heart in a protected state in a rat heart model, so that when ischaemia occurred, damage as measured by infarct size, was reduced (185).

- *Reduction in oxidation of low density lipoprotein (LDL):*

That oxidative modification of LDL is implicated in atherogenesis was first published in 1989 by Steinberg *et al* (186). Frankel *et al* were the first to show the protective effect from red wine on oxidation of LDL (187-190).

- *Vasoprotective effect:*

After red wine consumption the resting brachial artery diameter and resting blood flow increased significantly but were unchanged after dealcoholised red wine which led to the significant increase of flow mediated dilatation (FMD) of the artery (69). Red wine and red wine polyphenols, as well as white wine (191), have been found to improve endothelial function in human volunteers (192-195) by increasing the expression of eNOS (196). Martin *et al* studied the mechanisms whereby red wine polyphenols (including anthocyanins, catechins and flavonols) effect an increase in NO and reported that this was via Ca²⁺-dependent pathways and activation of the tyrosine kinase and phospholipase C pathways (197). French red wines were found to be more active than German red wines in upregulating the eNOS gene in human endothelial cells (67). However, this study highlights one of the shortcomings of experimental studies and that is their relevance. Incubation of red wine with human cells is not clinically relevant and does not reveal what really happens when humans consume the wine. This is because plasma and tissues are exposed to polyphenols *in vivo* only after they have undergone extensive modification during first-pass metabolism (Section 1.2.5). At this stage the polyphenols are very seldom aglycones, although deconjugation to produce aglycones may occur, but only at certain sites. A second factor that highlights the shortcomings of experimental studies is that the concentration of wine or polyphenols used is often not physiologically relevant. It should be of the same order as the maximum plasma concentrations found after a polyphenol-rich meal (approximately 0.1–10 μmol/L (198).

- *Effects on lipids and lipoproteins:*

A high serum HDL concentration is associated with a decreased risk of CVD, and HDL concentrations are significantly and consistently increased in humans and other animals after consumption of wine (199,200), and alcoholic beverages (52,136,199,201). It is well-documented that ethanol causes elevated concentrations of plasma HDL (202,203) and to date, no other diet or lifestyle factor is able to do this so consistently. The response of the major HDL subfractions to alcohol consumption is not clear, and variable

responses have been reported (202, 205). Consumption of alcoholic beverages raises plasma triacylglycerol (204-206), raises total cholesterol levels but decreases lipoprotein (a) (Lp(a)) (199,207-209). Atherosclerotic lesions may be accelerated to advanced plaques by the formation of new blood vessels, which supply the neighbouring cells with oxygen and nutrients. Red wine polyphenols are able to prevent this neovascularisation by inhibiting activation of matrix metalloproteinase 2, which is expressed abundantly in atherosclerotic lesions (210).

- *Risk of hypertension:*

Experimental studies on humans have corroborated the results from epidemiological studies of increase of BP from alcohol consumption and the subsequent decrease of BP upon alcohol withdrawal (211). The direct pressor effect of alcohol has been proposed as the basis of the association of alcohol consumption with increased BP (212). In a randomised, controlled intervention study, consumption of approximately 40g red wine and beer elevated the BP (213). However, consumption of 2.5–10g alcohol was found to be associated with a decrease in risk, relative to abstainers (214).

- *Inhibition of platelet adhesion and aggregation:*

Platelet aggregation influences the development of atherosclerosis by the formation of blood clots in arteries and there are a number of studies demonstrating the anti-aggregating effect on platelets of alcohol (51,136,215-217); red wine (208,218,219); dealcoholised red wine (218,220), where polyphenols interfere with arachidonic acid metabolism, and resveratrol and quercetin in a dose-dependent fashion (84).

- *Effect on plasma homocysteine:*

A raised homocysteine concentration is associated with vascular risk and is thus a risk factor for CVD. High alcohol consumption is associated with increased homocysteine concentrations (221), but low to moderate consumption, in particular red wine consumption, has been found to lower homocysteine concentrations in obese adults, where obese people have an increased risk of vascular morbidity and mortality (222). Moderate consumption has also been found to lower the levels, but not significantly, in the elderly (223).

- *Antimicrobial effects:*

Red wine and resveratrol have an *in vitro* activity against *Chlamydia pneumoniae*, which is associated with the development of CHD (224).

Red wine has also been found to have a bactericidal effect against *H. pylori* infections (225), and spirits and beer consumption, although not as effective as wine, also have this effect (226).

- *Anticarcinogenic effects:*

Certain flavonoids such as quercetin, kaempferol and myricetin (Figure 1.22) have been found to be clastogenic and mutagenic at high concentrations in short-term *in vitro* human cell experiments, and are therefore potentially carcinogenic (227). The mechanisms are not fully understood. Quercetin is known to be unstable in the presence of oxygen, subsequently reacting with DNA, and it is this instability that is thought to cause quercetin's cytotoxic effect (228). Red wine polyphenols (catechin, resveratrol (Figures 1.21 and 1.19) and quercetin) inhibited the proliferation of 3 different breast cancer cell lines (229). Inhibition of growth by low concentrations of various components of red wine such as gallic acid (Figure 1.18) quercetin, catechins and resveratrol on prostate cancer cell lines have been found, where prostate cancer is a very commonly-diagnosed cancer in the USA and in Europe (230,231). There are conflicting reports about the health benefits of quercetin as shown by for example *in vitro* as well as *in vivo* testing for DNA damage (8). These studies demonstrated both the mutagenic and cancer-preventive effects of quercetin. In a study investigating the growth effects of flavonoids on colorectal tumours, quercetin was a strong inducer of growth inhibition (mainly via inhibition of epidermal growth factor (EGF) receptor kinase, where EGF receptor plays an essential role in growth regulation) and apoptosis (232). Results from animal, human and cell culture studies show possible mechanisms whereby ethanol has an enhancing action on mammary carcinogenesis, such as an increase in circulating oestrogens and androgens and an increase in potential for invasiveness of breast cancer cells (233).

- *Decreased risk of type 2 diabetes mellitus :*

Clinical and experimental data show an association between oxidative stress and both types of diabetes mellitus (234). An analysis of the literature shows the protective effect from moderate and regular alcohol, in particular wine, consumption (235,236). Red wine

has been found to prevent meal-induced oxidative stress in diabetic subjects, which protects them from heart disease (237)

1.2.5. Bioavailability.

The health benefits of polyphenols depend on the particular polyphenols, the amounts consumed and their bioavailability. At high doses, or at higher levels obtained from an average vegetarian diet for example from supplements where gram doses are often recommended, they may be mutagenic or act as pro-oxidants that generate free-radicals (238). In the diet, phenolic acids account for approximately one-third and flavonoids two-thirds of the total intake of phenolic compounds (239,240), the total intake of which is usually quoted as 1–1.5g per day (although the worldwide figure is estimated to be much lower at approximately 70mg), depending upon the individual food choices.

The approximate dietary intake of flavonoids in the USA was described in 1976: catechins 22%, flavanols, flavones and flavanones 16%, anthocyanins 17% and biflavans (for example procyanidins) 45%. These figures continue to serve as reference data (241). The average human consumption of flavonoids is usually estimated at 1g/day per person (242), but by some is estimated at approximately 23mg/day (243). The most abundant flavonoids in the diet are the flavanols (catechins and proanthocyanidins) and the anthocyanins. The daily intake of catechins is estimated at 18–50mg (244), but the final percentage absorption varies widely between studies (245). Studies show the concentration of total catechins in plasma to be approximately 0.1–1.4 μ mol/L (244).

Bioavailability of the different polyphenols varies a lot and the most abundant dietary polyphenols are not necessarily those found in the highest concentrations in the tissues. After the consumption of between 10 and 100mg of a single phenolic compound, the maximum plasma concentration is still less than 1 μ g/L, and urinary excretion varies from 0.3–43% of the ingested concentration of polyphenols, depending on the particular polyphenol (244). The average concentration of polyphenols in red wine is 1200–1700mg/L (246,247) with the total phenolic acids and polyphenols ranging between 900–2500mg/L in red wine and 190–290mg/L in white wine (248).

Results from 97 studies show that gallic acid is a well-absorbed phenol, followed by catechins, flavones and quercetin glucosides and that the least well-absorbed are the

proanthocyanidins and the anthocyanins (244). There are very few results on the hydroxycinnamic acids and hydroxybenzoic acids (except gallic acid). Simonetti *et al* found an increase in the plasma concentration of caffeic acid (figure 1.17) (a hydroxycinnamic acid) after consumption of 200mL red wine, reaching a maximum by 60 minutes and baseline by 5 hours (246). Bioavailability has important implications for the efficiency of the polyphenols *in vivo* and for the establishment of physiological concentrations to be used in relevant investigations. It remains controversial as to whether ingestion of the polyphenols in moderate quantities of alcoholic beverages increases their concentrations in blood significantly. The polyphenol content of different red wines varies widely. Duthie *et al* reported 12.5µg/mL total phenols (as gallic acid equivalents) present in human plasma after consumption of 100mL red wine (249). Basal concentrations were reached within 4 hours. Bell *et al* (63) showed an increase in plasma total catechin concentration (with an average of 0.3% of the total mass consumed) after 120mL dealcoholised red wine consumption, with the peak at 1–2 hours, reaching baseline after 8 hours. A marked increase in human plasma quercetin concentration was found 3 hours after ingestion of quercetin, decreasing after 7 hours and returning to baseline after 20 hours (250). These returns to baseline within a day indicate the need for regular consumption of these antioxidant phenols. Studies with human colonic cell monolayers show that radiolabeled proanthocyanidin and procyanidin dimers and trimers can be absorbed and transported from the apical to the basal side of the cultures (251).

Modification of polyphenols, for example glycosylation, methylation and glucuronidation, greatly determines their absorption and bioavailability (252), for example there are marked differences in humans between absorption of quercetin and its glycosides (253,254). Quercetin glucosides are more efficiently absorbed than quercetin alone. Studies show a high inter-individual variability in absorption of quercetin (250,254). All flavonoids except flavanols are found in the glycosylated form in foods (241), and these glycosidic bonds hydrolyse with age. (+)-Catechin and epicatechin are found in wine exclusively as aglycones (248). Flavonoids found in plasma and urine are usually glucuronidated and/or sulphated with only trace amounts of the native forms. Urinary excretion of catechin was 3–10% of that consumed from 120mL red wine and was present as metabolites almost exclusively (sulphated, glucuronidated and methylated)

(255). Quercetin is generally not found in the plasma as the free form or the glucoside (256), but as methyl, sulphate or glucuronic acid conjugates, and the position of the conjugation is important in determining activity (257,258). Glycosides are the major circulating form of anthocyanins, possibly because of their instability in the aglycone form (241). Quercetin has a plasma half-life of 11–28 hours. Concentrations in most diets are low, at approximately 20–35mg per day. Ingestion of 50mg results in a plasma concentration of approximately 0.75–1.5 μ mol/L (239,244). Catechin has a much shorter plasma half-life (2–3 hours) than the other flavonoids and ingestion of 120mL red wine (35mg catechin) resulted in a maximum plasma concentration of catechin at 1 hour after ingestion of approximately 91nmol/L (259). It has been previously speculated that ethanol may improve absorption of polyphenols by increasing their solubility. However, in this study, the plasma concentrations of catechin metabolites were similar after consumption of red wine or dealcoholised red wine.

Of the total mass of polyphenols ingested, only a small percentage of the flavonoids are absorbed across the intestinal membrane. Recent information suggests that the absorption of catechins is approximately 3–5%, of flavonols 17–52%, of anthocyanins 4%, of flavanones 7–24% and of isoflavonoids 9–35% (260). There is a common pathway for polyphenol metabolism. Aglycones can be absorbed from the small intestine, but most of the polyphenols are in the form of glycosides or polymers that cannot be absorbed. These compounds must be hydrolysed by intestinal enzymes or by microflora in the colon before absorption. Colonic flora may further degrade the aglycones, producing several phenolic acids, depending on the structure of the original polyphenol. During absorption, polyphenols are conjugated in the small intestine and then in the liver, undergoing methylation, sulphation and glucuronidation. This is a very efficient process, and circulating polyphenols are found as conjugated derivatives, extensively bound to albumin. Polyphenols penetrate tissues, especially those where they are metabolised. Derivatives of polyphenols as well as polyphenols are excreted mainly in bile and urine. They may also be secreted into the duodenum via the biliary route, subjected to the action of bacterial enzymes mainly β -glucuronidase, and then re-absorbed. This recycling leads to a longer presence of polyphenols in the body (241,261,262).

An important factor that affects polyphenols and their bioavailability is food composition, for example proteins in the food may bind to polyphenols and thereby reduce their bioavailability, although the denaturation at gastric pH may result in a loss of protein binding. Polyphenol content in plants is affected by environmental factors (for example sun exposure, rainfall, soil type and plant stress). Storage also affects the content of easily-oxidised polyphenols Browning is one such effect. Food processing also affects the content, for example fruit juice processing often involves a clarification stage which removes certain flavonoids, and for this reason processed fruit juices often have a decreased flavonoid concentration (241).

Although wine chemistry is complex, detailed descriptions of the composition of the different wines would facilitate much-needed *in vitro* studies on bioavailability and metabolism of the specific components of the wine. This would in turn facilitate the recommendation of wine as an adjunct to a healthy diet.

1.2.6. Conclusions

Atherosclerosis is the dominant cause of morbidity and mortality in developed countries and is rapidly rising in developing countries (263). It is preferable to combat atherosclerosis by preventive strategies involving lifestyle, including a balanced diet, exercise, maintaining an ideal body weight, and no smoking. Epidemiological evidence suggests that moderate consumption (1–3 drinks per day) of alcoholic beverages, particularly red wine, is associated with an overall improvement in health, especially cardiovascular health. Although the epidemiological information is attractive, it is not adequately compelling for the deliberate inclusion of alcoholic beverages in the lifestyle of westernised subjects. Nevertheless, moderate consumption of affordable alcoholic beverages in otherwise healthy subjects will not increase the risk for disease, especially if it accompanies a healthy lifestyle. The consumption of large amounts of alcohol is clearly ill-advised. The consumption of alcoholic beverages during pregnancy clearly places the fetus at risk for developmental abnormalities, and alcohol is not recommended during periods of breast-feeding.

Although several lines of experimental evidence suggest mechanisms by which alcoholic beverages may reduce the risk of vascular disease, there is inadequate

understanding of atherogenesis to know whether these mechanisms apply to any given individual at risk and whether the alcoholic beverage will be of direct benefit. Scientific research will hopefully elucidate these mechanisms for specific intervention. If health benefits of alcoholic beverages are confirmed, a new avenue may be opened for the prevention and possibly the treatment of atherosclerosis.

Table 1.1. Summary of the beneficial effects of wine: epidemiological and experimental.

| | <u>↓ CVD Risk</u> | <u>↓ Stroke Risk</u> | <u>↓ Total Mortality Risk</u> |
|---|---|--|--|
| E P I D E M I O L O G I C A L | <ul style="list-style-type: none"> ▪ Vliegenthart R <i>et al</i>, 2004 ▪ Rimm EB <i>et al</i>, 1991 & 1996 ▪ Cleophas TJ <i>et al</i>, 1999 ▪ Mukamal KJ <i>et al</i>, 2003 ▪ Gaziano JM <i>et al</i>, 1999 ▪ Sato M <i>et al</i>, 2002 ▪ Marmot MG, 2001 ▪ Klatsky AL, 2002 ▪ Grønbaek M, 2004 <ul style="list-style-type: none"> ▪ Iacoviello L & Gaetano G, 2003 ▪ Agarwal DP, 2002 ▪ Klatsky AL <i>et al</i>, 1990 ▪ Stampfer MJ <i>et al</i>, 1988 ▪ Klatsky AL <i>et al</i>, 1986 ▪ Diem P <i>et al</i>, 2003 <ul style="list-style-type: none"> ▪ Woodward M & Tunstall-Pedoe H, 1995 ▪ Burns J <i>et al</i>, 2001 ▪ Hart CL <i>et al</i>, 1999 ▪ Grønbaek M <i>et al</i>, 2000 | <ul style="list-style-type: none"> ▪ Truelsen T <i>et al</i>, 1998 ▪ Klatsky AL <i>et al</i>, 1990 ▪ Stampfer MJ <i>et al</i>, 1988 ▪ Iso H <i>et al</i>, 1995 & 2004 ▪ Gill JS <i>et al</i>, 1986 ▪ Hansagi H <i>et al</i>, 1995 ▪ Wannamethee SG & Shaper AG, 1996 ▪ Hart CL <i>et al</i>, 1999 ▪ Berger K <i>et al</i>, 1999 ▪ Donahue RP, 1986 | <ul style="list-style-type: none"> ▪ Burns J <i>et al</i>, 2001 ▪ Diem P <i>et al</i>, 2003 ▪ Klatsky AL <i>et al</i>, 1990 ▪ Renaud SC <i>et al</i>, 1998 & 1999 ▪ Grønbaek M <i>et al</i>, 2000 ▪ Hart CL <i>et al</i>, 1999 |

| | | | | | | | | | | |
|--|---|---|---|---|--|--|---|--|--|--|
| E X P E R I M E N T A L | ↓ Cancer | ↓ LDL Oxidation | ↑ HDL Conc. | ↑ FMD | ↓ Type 2 DM | ↓ Platelet Aggregation | ↑ Bone Density (post Meno pausal ♀) | ↑ Cardio- protection | ↓ Inflamma- tory Markers | Others |
| | <p>Colon:</p> <ul style="list-style-type: none"> •Kuo S-M, 1996 •Soleas G-J <i>et al</i>, 2002 <p>Prostate:</p> <ul style="list-style-type: none"> •Romero I <i>et al</i>, 2002 •Kampa M <i>et al</i>, 2000 <p>Lung:</p> <ul style="list-style-type: none"> •Prescott E <i>et al</i>, 1999 <p>Breast:</p> <ul style="list-style-type: none"> •Pozo-Guisado E <i>et al</i>, 2002 •Zhang Y <i>et al</i>, 1999 •Kropp S <i>et al</i>, 2001 •Damianaki A <i>et al</i>, 2000 <p>Leukaemia:</p> <ul style="list-style-type: none"> •Surh Y-J <i>et al</i>, 1999 <p>General Effects:</p> <ul style="list-style-type: none"> •Jang M <i>et al</i>, 1997 •Doss MX <i>et al</i>, 2005 | <ul style="list-style-type: none"> •Chopra M <i>et al</i>, 2000 •Frankel EN <i>et al</i>, 1993 •Brito P <i>et al</i>, 2002 •Struck M <i>et al</i>, 1994 •van Golde PHM <i>et al</i>, 1999 •Fuhrman B <i>et al</i>, 1995 | <ul style="list-style-type: none"> •Goldberg DM <i>et al</i>, 1995 •Hansen AS <i>et al</i>, 2005 •Ellison RC <i>et al</i>, 2005 •Taskinen M-R <i>et al</i>, 1987 •Rakic V <i>et al</i>, 1998 | <ul style="list-style-type: none"> •Agewall S <i>et al</i>, 2000 •Duffy SJ & Vita JA, 2003 •Hashimoto M <i>et al</i>, 2001 •Djousse L <i>et al</i>, 1999 •Cuevas AM <i>et al</i>, 2003 | <ul style="list-style-type: none"> •Ajani UA <i>et al</i>, 2000 •Lazarus R <i>et al</i>, 1997 •Solomon CG <i>et al</i>, 2000 •Wanna-methee SG <i>et al</i>, 2002 | <ul style="list-style-type: none"> •Ruf J-C <i>et al</i>, 1995 •Pace-Asciak CR <i>et al</i>, 1995 •Russo P <i>et al</i>, 2001 •Wu JM <i>et al</i>, 2001 •Frémont L, 2000 •Pellegrini N <i>et al</i>, 1996 •Kontula K <i>et al</i>, 1982 •Renaud SC <i>et al</i>, 1992 •Pikaar NA <i>et al</i>, 1987 | <ul style="list-style-type: none"> •Fes-kanich D <i>et al</i>, 1999 •Ganry O <i>et al</i>, 2000 | <ul style="list-style-type: none"> •Fantinelli JC <i>et al</i>, 2005 •de Lorgeril M <i>et al</i>, 2002 | <ul style="list-style-type: none"> •Imhof A <i>et al</i>, 2001 •Estruch R <i>et al</i>, 2004 •Mukamal KJ <i>et al</i>, 2004 <p>↑ BP</p> <ul style="list-style-type: none"> •Puddey IB <i>et al</i>, 1985 •Xin x <i>et al</i>, 2001 •Zilkens RR <i>et al</i>, 2005 | <p>↓ <i>Helicobacter pylori</i> infection:</p> <ul style="list-style-type: none"> •Marimón JM <i>et al</i>, 1998 •Brenner H <i>et al</i>, 1999 <p>↓ <i>Chlamydia pneumoniae</i> infection:</p> <ul style="list-style-type: none"> •Schriever C <i>et al</i>, 2003 <p>↓ Cell ageing:</p> <ul style="list-style-type: none"> •Howitz KT <i>et al</i>, 2003 <p>↑ Plasma antioxidant capacity:</p> <ul style="list-style-type: none"> •Serafini M <i>et al</i>, 1998 <p>↓ Homocysteine:</p> <ul style="list-style-type: none"> •Dixon JB <i>et al</i>, 2002 •Koehler KM <i>et al</i>, 2001 <p>↑ eNOS:</p> <ul style="list-style-type: none"> •Wallerath T <i>et al</i>, 2003 <p>↓ Dementia risk:</p> <ul style="list-style-type: none"> •Ruitenberga A <i>et al</i>, 2002 •Truelsen T <i>et al</i>, 2002 •Mukamal KJ <i>et al</i>, 2003 <p>↑ Post Ischaemia-reperfusion protection:</p> <ul style="list-style-type: none"> •Shigematsu S <i>et al</i>, 2003 •Hung L-M <i>et al</i>, 2002 <p>↓ BP:</p> <ul style="list-style-type: none"> •Thadhani R <i>et al</i>, 2002 |

1.3 WINE PHENOLICS AND FLAVONOIDS

1.3.1. Background

Wine essentially comprises water, ethanol, glycerol, various acids, esters as well as many different phenols or phenolic compounds, a group of organic compounds that contains a hydroxyl group(s) bound directly to an aromatic ring (264). There are several hundred different phenolic compounds present in red wine, especially aged red wine, and these differ between cultivars and vintages due to variations in the different grapes and the different seasonal conditions; must preparation during crushing, pressing and skin contact; fermentation, also during skin contact and sulphite addition, and ageing (265). The total mass of phenols found in an average-sized glass of red wine is approximately 200mg compared with that in white wine, which is approximately 40mg. Phenols found in wine are also not identical to those found in grapes.

They have several important functions in wine including the colour of red wine, the astringent and bitter tastes of wine, the preservation of wine during ageing and, because phenolics oxidise readily, the browning of wine when exposed to air. A number of these phenolic substances have also been found to have antioxidant properties, for example the tannins, the non-flavonoids, for example stilbenes (resveratrol) and the flavonoids, for example flavanols, flavonols as well as anthocyanins (266). These compounds are of special interest for the antioxidant effects of wine that are investigated in this thesis.

Wine polyphenols may very broadly be divided into flavonoids and non-flavonoids, (classification of wine phenols has not yet been fully elucidated). Flavonoids make up the larger group. Albert Szent-Gyorgi, a Nobel Prize-winning biochemist, who labelled them "vitamin P", was the first to describe them in 1936, after working with citrus flavanones (242). They were found to enhance the function of vitamin C by improving its absorption and also by protecting it from oxidation. They often occur attached to glycosides and therefore tend to be water-soluble. There are more than four thousand known flavonoids, some found in wine as well as other beverages and fruits (tea, coffee, fruit juices, beer and chocolate) and to a lesser extent vegetables and cereals. They are characteristically

found in higher plants, in all parts of the plant. Approximately half of the total extractable phenols in the grape berry are found in red wines. The distribution in the grape berry is illustrated in Figure 1.14.

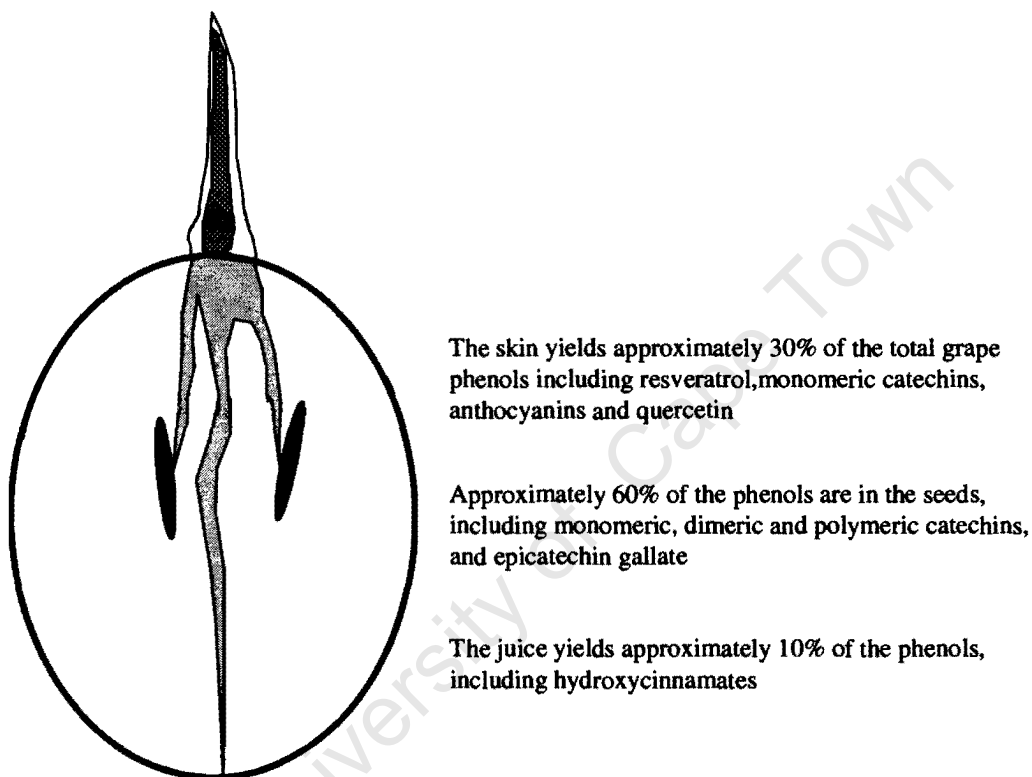


Figure 1.14. Diagram of a grape berry showing the distribution of phenols.

1.3.2. Classification and chemical structure

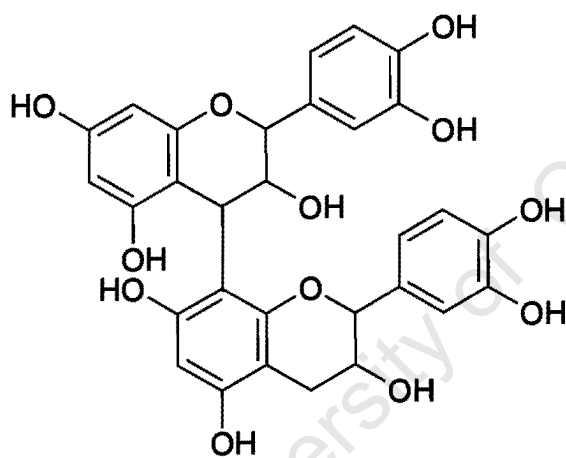
1.3.2.1. Tannins

A large number of wine phenols include polymeric tannins: condensed and hydrolysable tannins. The term “tannin” is mostly functional but it is also often used to describe high molecular-weight phenolic mixtures. Both wood (in particular oak) tannins and grape tannins are responsible for the presence of tannins in wine. Grape tannins are

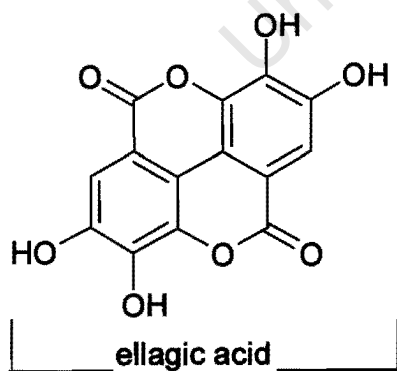
found mainly in the skins and seeds of the grape berry. Fresh tannins from young oak are bitter and astringent and may impart this flavour to the wine, and so “toasting” of the oak renders the tannins more acceptable. Oak tannin chemistry is poorly understood.

Condensed tannins are polymeric mixtures of flavonoids in which the polymer is made up of flavan units, usually flavanol, linked by 4:8 C–C bonds, for example proanthocyanidins (Figure 1.15).

Hydrolysable tannins, which in wine are derived mainly from wood extraction during wine maturation, can be hydrolysed to glucose and gallic acid (gallotannins) or ellagic acid (ellagitannins) (Figure 1.15). They are both formed during the Shikimate pathway (Section 1.3.3).



a)



b)

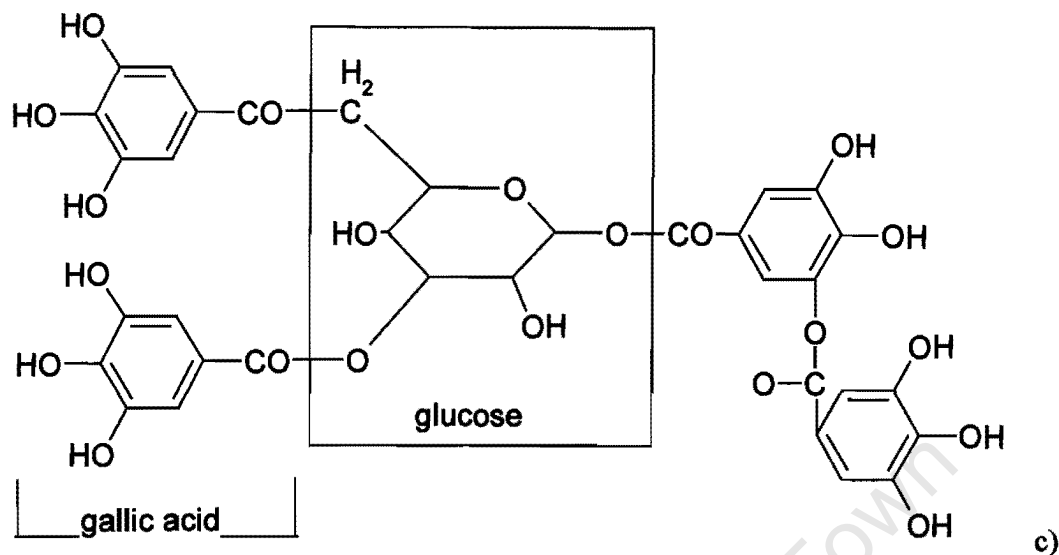
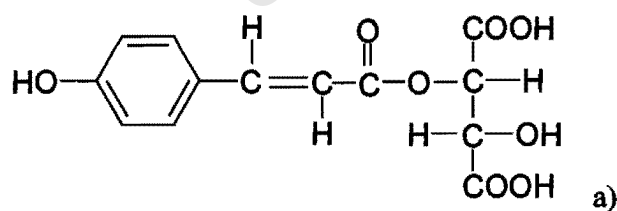


Figure 1.15. Chemical structures of a) a proanthocyanidin, b) ellagic acid and c) a gallotannin.

1.3.2.2. Non-flavonoids

The non-flavonoids include several classes such as the hydroxycinnamic acids, the hydroxybenzoic acids and the stilbenes.

Hydroxycinnamic acids are major phenols in white wine. In wine making, the most important effect of these compounds is their browning effect, producing desirable golden colours. The trivial names of the three most commonly found are caftaric, *p*-coutaric and fertaric acids and they occur in grapes as esters of tartaric acid:



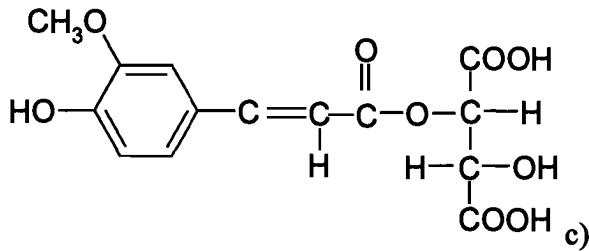
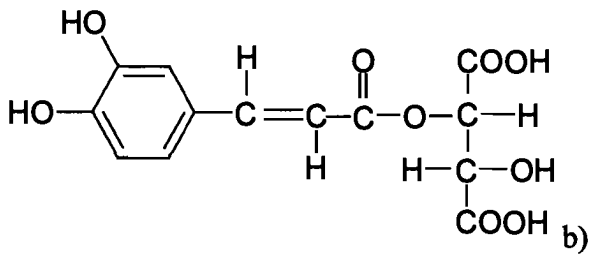
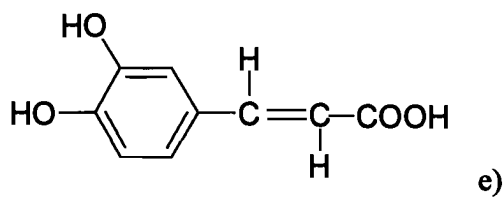
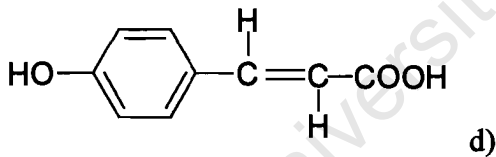


Figure 1.16. Chemical structures of a) p-coumaric, b) caffeic and c) ferulic acids as esters of tartaric acid.

They occur in white and red wine also in the free form (after hydrolysis) as p-coumaric, caffeic (the most predominant) and ferulic acids:



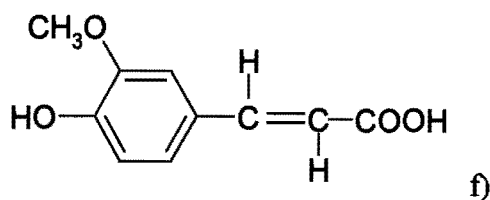


Figure 1.17. Chemical structures of d) p-coumaric, e) caffeic and f) ferulic acids.

Concentrations of total hydroxycinnamic acids range from approximately 130mg/L in white wines to 60mg/L in red wines (247).

The hydroxybenzoic acids are mostly degradation products that appear during wine ageing and are therefore not usually found in new wines. Hydrolysis again results in the free acids in wine. The most important is gallic acid, which is generated from hydrolysis of gallate esters of hydrolysable tannins. Concentrations of gallic acid range from approximately 1mg/L in white wines to 30mg/L in red wines (248).

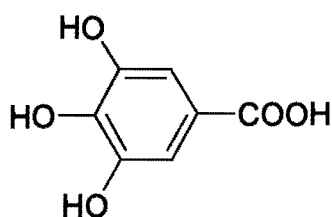


Figure 1.18. Chemical structure of gallic acid.

Stilbenes are minor phenols produced by the grapevine and are found in response to attack by fungi such as *Botrytis cinera* where oligomers of resveratrol called viniferins are the actual anti-fungal compounds. The main stilbene in wine is resveratrol and red wine contains much more of this compound than white wine, in several forms including the *trans* (predominant form) and *cis*-isomers. Aglycones and piceids (glucosides) are both found in white wine, rosé and red wine (88,99). The total concentration of all forms of resveratrol in white wines is approximately 0.5mg/L, in rosé wines is approximately 2.15mg/L and in red wines is approximately 7mg/L (247) although there is a wide variation (99). There have been many studies reporting that resveratrol has anti-cancer

properties (Section 1.2.3.3) as well as being able to reduce heart disease and in 2001 alone there were more than 200 publications concerning this compound.

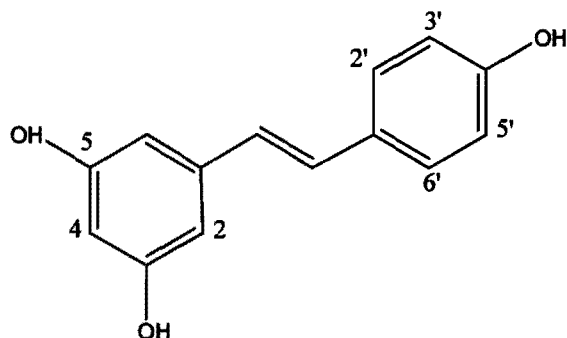


Figure 1.19. Chemical structure of resveratrol.

1.3.2.3. Flavonoids

1.3.2.3.1. General chemical structure of the flavonoids

The structure of flavonoids is based on a fifteen carbon, two benzene ring skeleton with a chromane ring carrying the second aromatic B ring in position 2 in flavonoids, position 3 in isoflavonoids and position 4 in neoflavonoids:

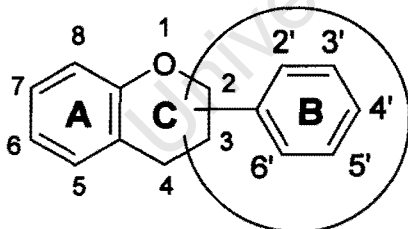


Figure 1.20. General structure of flavonoids.

The A ring in flavonoids from grapes and wine has a characteristic hydroxylation at the 5 and 7 position. Flavonoids are classified into different classes depending on the different substitutions and oxidation states on the C ring. In flavans the C ring is saturated; the flavones have a keto group at position 4 and unsaturation between positions

2 and 3; the anthocyanins have a fully aromatic ring with a positive charge and the flavanols (flavan-3-ols) have a hydroxyl substituent on the C ring in the 3 position.

Members of each class are defined by the B ring substitution pattern. Hydroxyl substitutions usually occur at position 4', and additional oxygen substitutions may occur at positions 3' and/or 5'. These oxygen substitutions may be hydroxyls or methoxyls. There may also be sugar conjugation on the oxygen. Isoflavonoids include isoflavones, isoflavanones and isoflavans.

1.3.2.3.2. Classes of flavonoids

The flavonoids make up most, approximately 85%, of the phenolic compounds of the seeded grape berry and may be divided into the following major classes: flavanols, flavonols and anthocyanins. Other classes include flavones, isoflavones and flavanones.

Flavanols form the largest class and include monomeric catechins as well as oligomeric and polymeric proanthocyanidins. Wine flavanols are different from the other flavonoid classes in that they are not found as glycosides. Monomeric flavanols are sometimes collectively known as "the catechins". These monomers include two stereoisomers, *trans* or (2R, 3S) (+)-catechin and *cis* or (2R, 3R) (-)-epicatechin. Both of these are found in grapes and wine but (+)-catechin is the predominant flavanol in red wines, the concentration depending to a large extent on the grape cultivar as well as climate. Biflavans, sometimes referred to as proanthocyanidins, are a group of catechin condensation products consisting of 1 molecule of a catechin and 1 of a flavandiols. There are 2 groups of flavandiols, those without and those with a hydroxyl in position C5. They have a protein-complexing and tanning activity (242). Their concentration in red wine ranges from 1–2.5g/L. An example of biflavans are the procyanidins, oligomers of (+)-catechin (catechin) and (-)-epicatechin (epicatechin). Monomeric, dimeric and polymeric flavanols are partly responsible for astringent and bitter mouth sensations and the astringency increases with the increase in polymerisation (267). Their interaction with salivary proteins may lead to a decrease in phenolic bioavailability (268). (+)-Catechin is partly responsible for colouring red wine (269). Both forms have catechol substitutions in the 3'4' positions on the B ring.

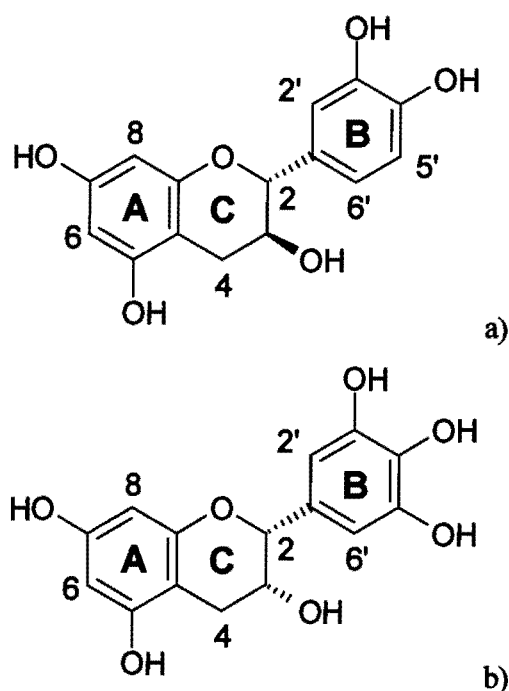


Figure 1.21. Chemical structures of a) (+)-catechin and b) epigallocatechin.

Gallocatechins, for example epigallocatechin and epicatechin gallate, have trihydroxy substitutions in the 3'4'5' positions. Concentrations of total monomeric flavanols range from 40–200mg/L in red wines (247), depending on seed extraction methods and methods of analysis.

Condensation of flavanol monomers via covalent linkage yields oligomers such as proanthocyanidins and polymeric condensed tannins (Section 1.3.2.1). Total oligomer plus polymer concentrations range between 10–50 mg/L in white wines, and 0.5–1.5g/L in red wines (247). Owing to their high molecular weight (270) these compounds do not seem to be absorbed from the gut (259) and therefore probably do not have many health effects. Older red wines sometimes have an insoluble precipitate that is formed by these large size polymers. This is most likely the reason for the decrease in concentration of phenols after ageing of red wines.

Flavonols are made up of a varied combination of glycosidic forms, such as the 3–glucosides and the 3–glucuronides, but essentially the main simple flavonoid structures within this class are quercetin, kaempferol and myricetin.

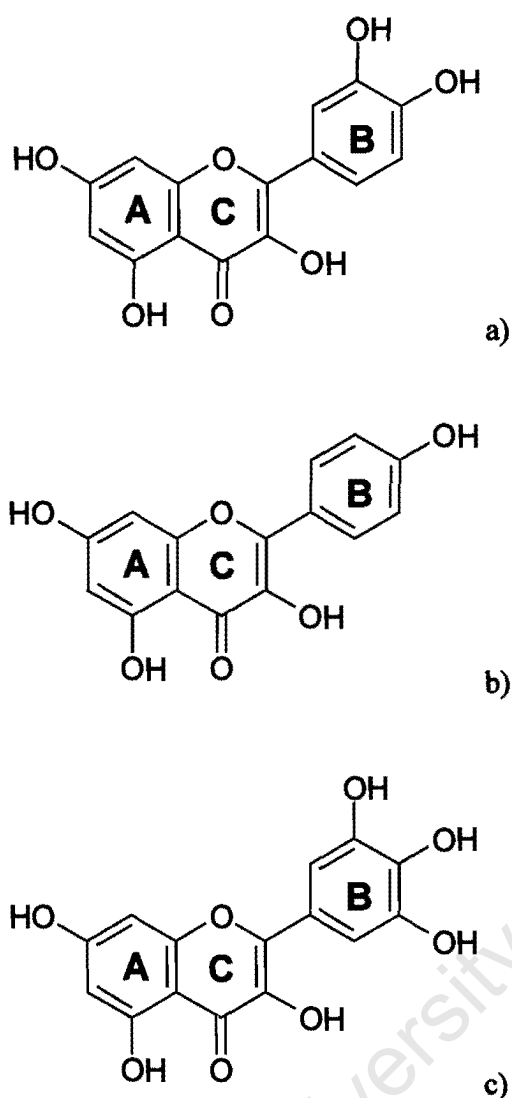
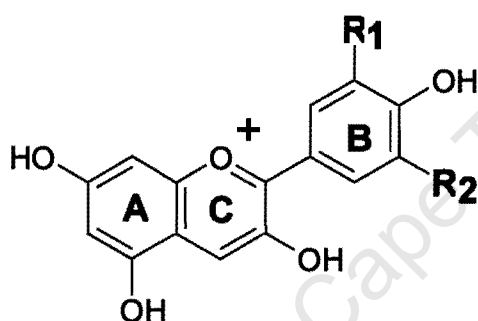


Figure 1.22. Chemical structures of a) quercetin, b) kaempferol and c) myricetin.

Levels of flavonols, in particular quercetin, in some red wines have been shown to increase dramatically with increased exposure of the grape berry to sunlight (8). Different skin extraction techniques affect flavonol concentrations, which range from trace amounts to 100mg/L in some red wines (259). The concentration of flavonols also seems to be an indicator of the quality of the wine as flavonols have been shown to have a bitter taste in alcoholic beverages (9).

Anthocyanins are found in the skins of red grapes and may exist in 2 forms: the red flavylium cation and a colourless hydrated hemiketal form. They show a red colour when in an acid solution, as in red wine pH of approximately 3.5 (269). Anthocyanins are able to covalently bond with the tannins in the wine to produce pigmented tannins that are stable and persist in red wines more than a few years old. In their simplest form of flavonoid ring structure, these compounds are known as anthocyanidins, which are unstable and found in very low concentrations in grapes or red wine. In wine there are five main structures: malvidin (the most abundant in red wines), petunidin, peonidin, cyanidin and delphinidin:



| | R ₁ | R ₂ |
|-------------|------------------|------------------|
| malvidin | OCH ₃ | OCH ₃ |
| petunidin | OH | OCH ₃ |
| peonidin | H | OCH ₃ |
| cyanidin | H | OH |
| delphinidin | OH | OH |

Figure 1.23. Chemical structures of the anthocyanidins in wine.

Anthocyanins are compounds with glycosides, and in wines the glycoside is 3–glucoside. There may be further substitution at the 6–hydroxyl group of the glucose, with ester linkage of acetyl, coumaryl or caffeoyl groups:

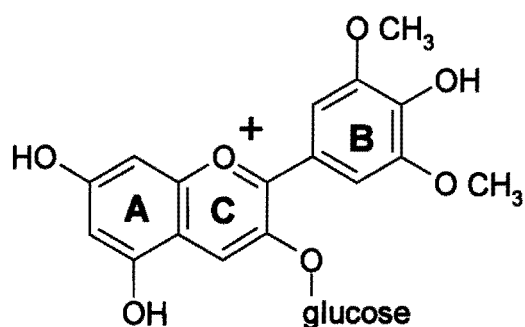


Figure 1.24. Chemical structure of malvidin-3-glucoside.

Different species of grape and different processing techniques result in variable concentrations of anthocyanins (247). Concentrations of anthocyanins in red wine range from 20–500mg/L (248). These levels have been found to decrease with increasing age of the wine: up to 97% decrease by eight months after fermentation (271).

Although the range of concentrations of total polyphenols in both white and red wines is large, the table below shows the means and/or ranges of quoted values (248):

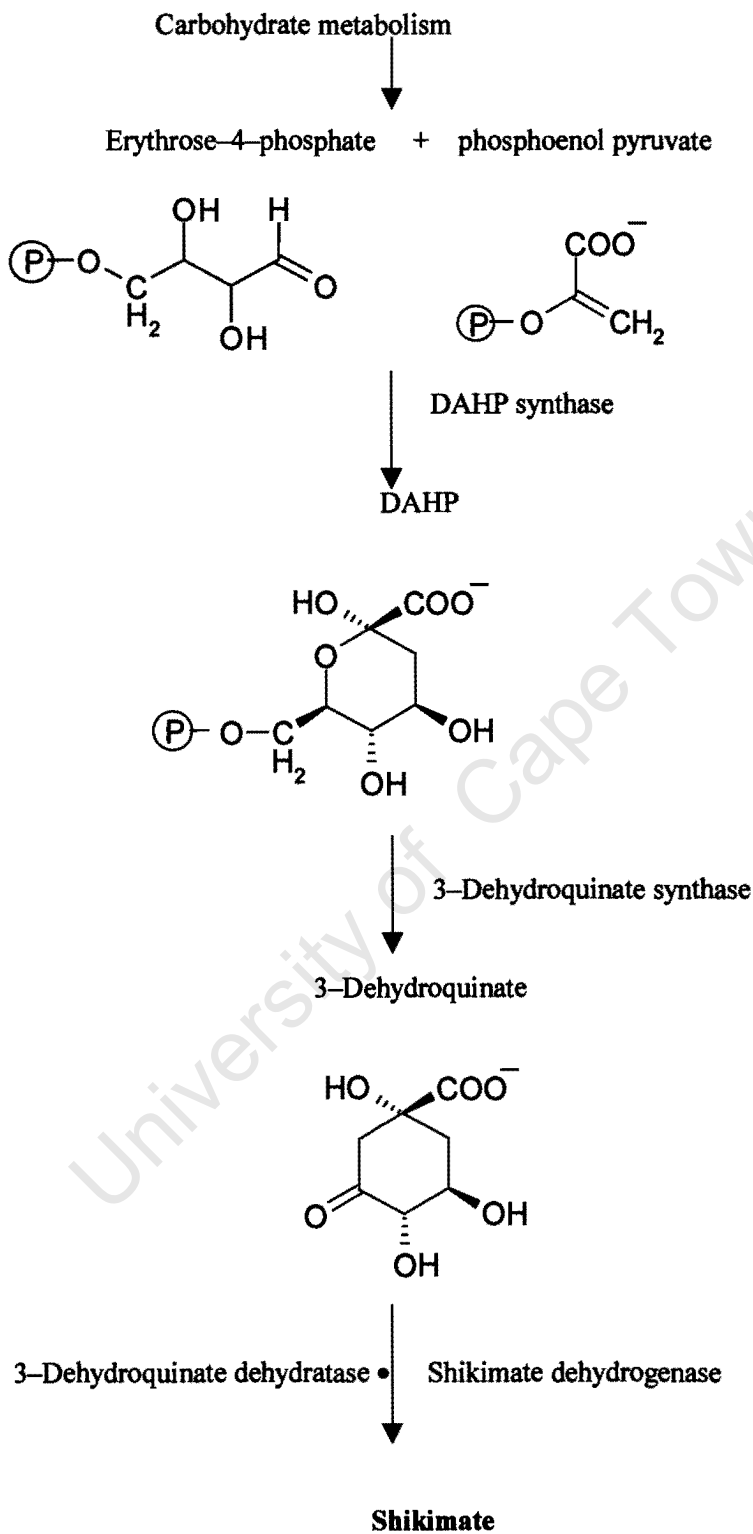
Table 1.2. Concentrations in mg/L of wine phenolic acids and polyphenols

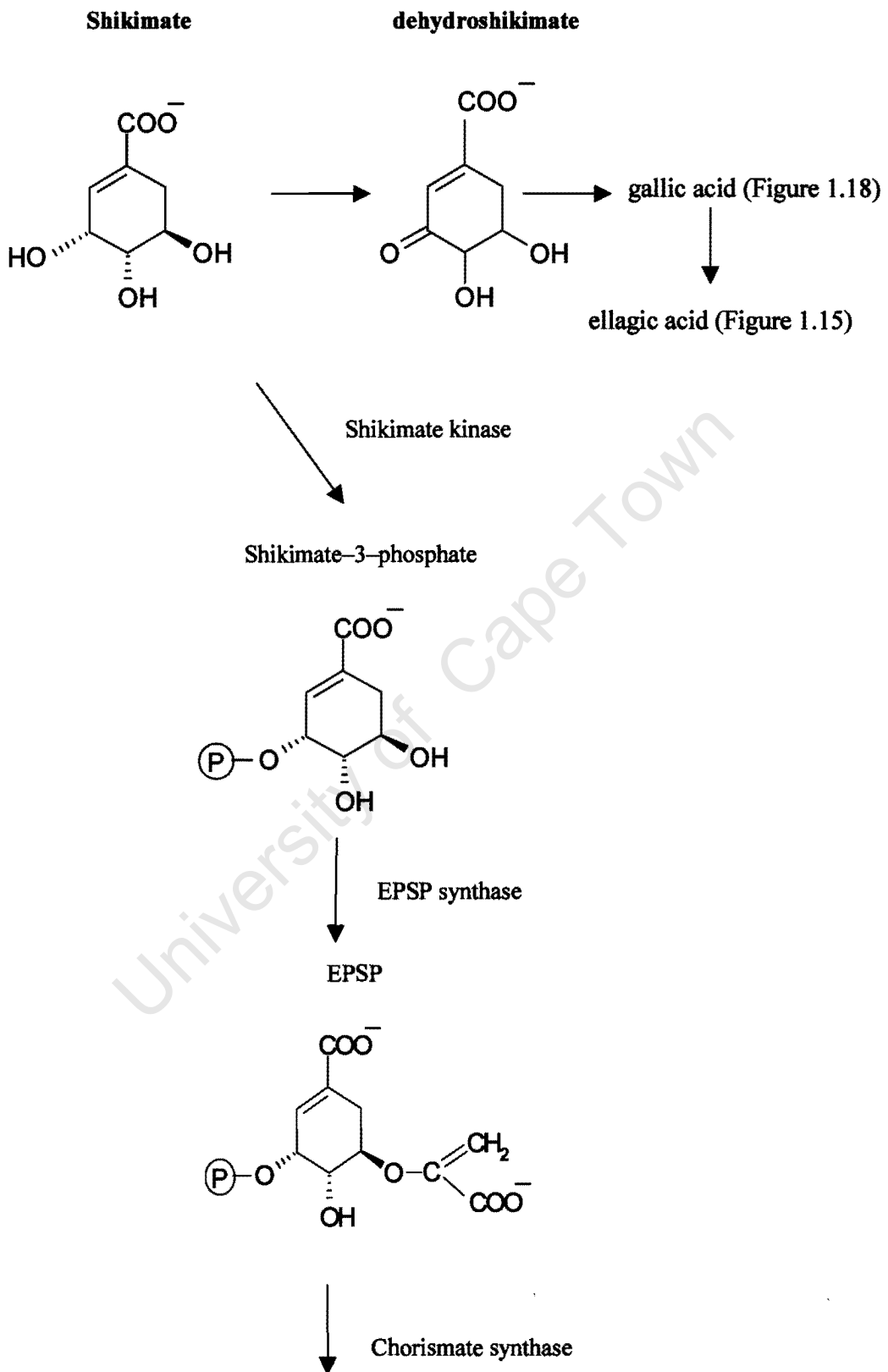
| | Red wine | White wine |
|--|------------------------|----------------------|
| Non-flavonoids | 240–500 | 160–260 |
| Hydroxybenzoic acids | 0–260 | 0–100 |
| p-Hydroxybenzoic acid | 20 | – |
| Gallic acid | 116 (26–320) | 1.4 |
| Total gallates | 40 (30–59) | 7 |
| Syringic acid | 5 (4.2–5.9) | – |
| Protocatechuic acid | 88 | – |
| Hydroxycinnamic acids | 162 (62–334) | 130–154 |
| <i>cis/trans</i> Coumaric acid | 20 (16–24) | 1.8 |
| <i>cis/trans</i> Caffeic acid | 25 (11–47) | 5 |
| Caffeic acid | 8.5 (3–18) | 2.8 |
| Coumaric acid | 12.6 (7.5–22) | 1.5 |
| Ferulic acid | 19 | – |
| Stilbenes | | |
| trans-Resveratrol | 1 (0.1–2.3) | 0.22 (0.003–2) |
| Flavonoids | 750–1060 | 25–30 |
| Flavonols | 98 (10–203) | trace |
| Quercetin | 18.8 (5–53) | 0 |
| Myricetin | 16.2 (2–45) | 0 |
| Kaempferol | 18 | 0 |
| Rutin | 6.8 (0.5–10.8) | 0 |
| Flavanols | 168 (48–440) | 15–30 |
| Catechin | 89 (27–191) | 17.3 (3–35) |
| Epicatechin | 57.3 (21.4–128) | 13.6 |
| Procyanidins | 171 (29–333) | 7.1 (5–10) |
| Anthocyanins | 281 (20–500) | 0 |
| Delphinidin 3-monoglucoside | 22 | 0 |
| Cyanidin 3-monoglucoside | 20 | 0 |
| Petunidin 3-monoglucoside | 18 | 0 |
| Peonidin 3-monoglucoside | 32 | 0 |
| Malvidin 3-monoglucoside | 93 (24–170) | 1 |
| Total phenolic acids and polyphenols: | 1200 (900–2500) | 200 (190–290) |

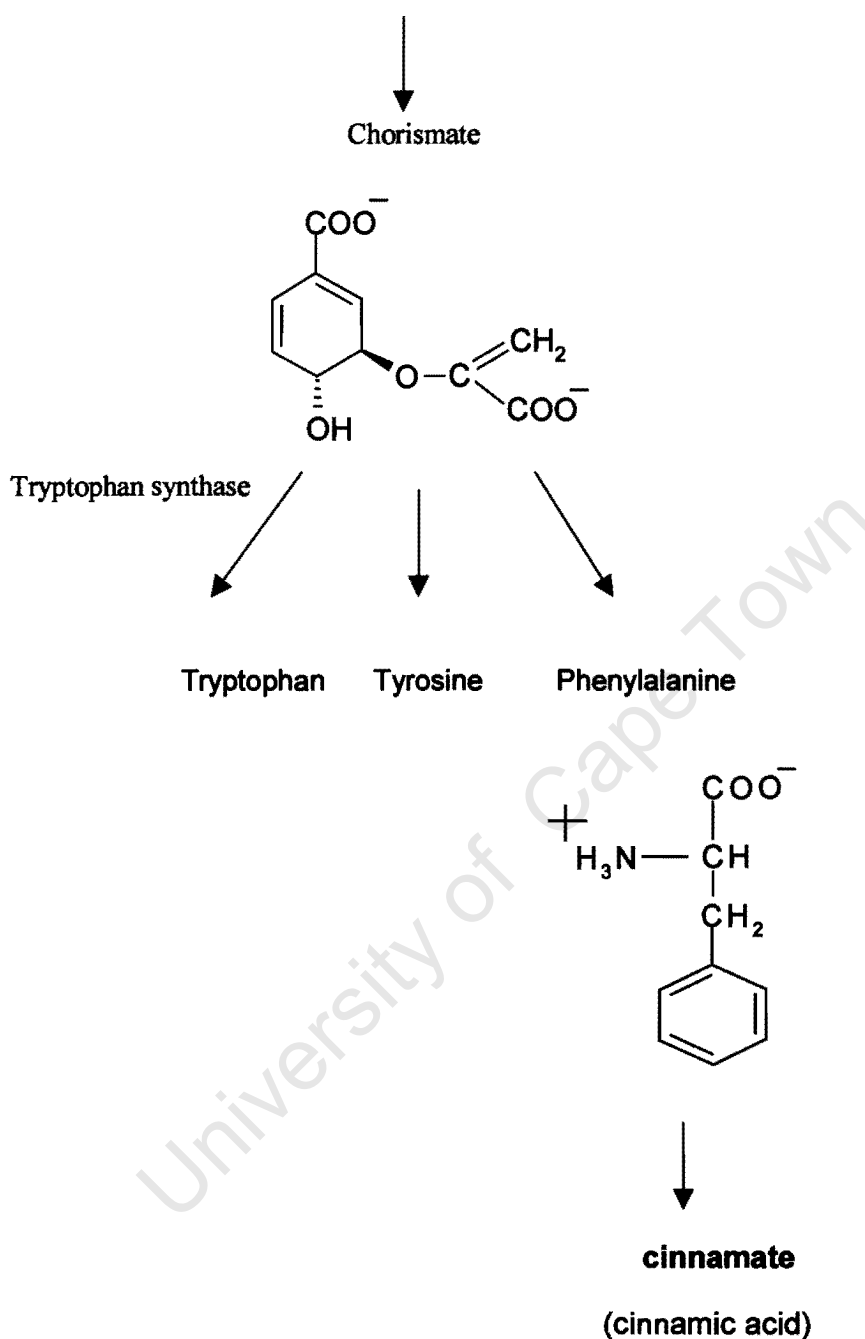
In some instances, only ranges are reported and in others, only mean values without a range are reported; – indicates no value found in the literature.

1.3.3. Biosynthesis of phenolic compounds, in particular the flavonoids

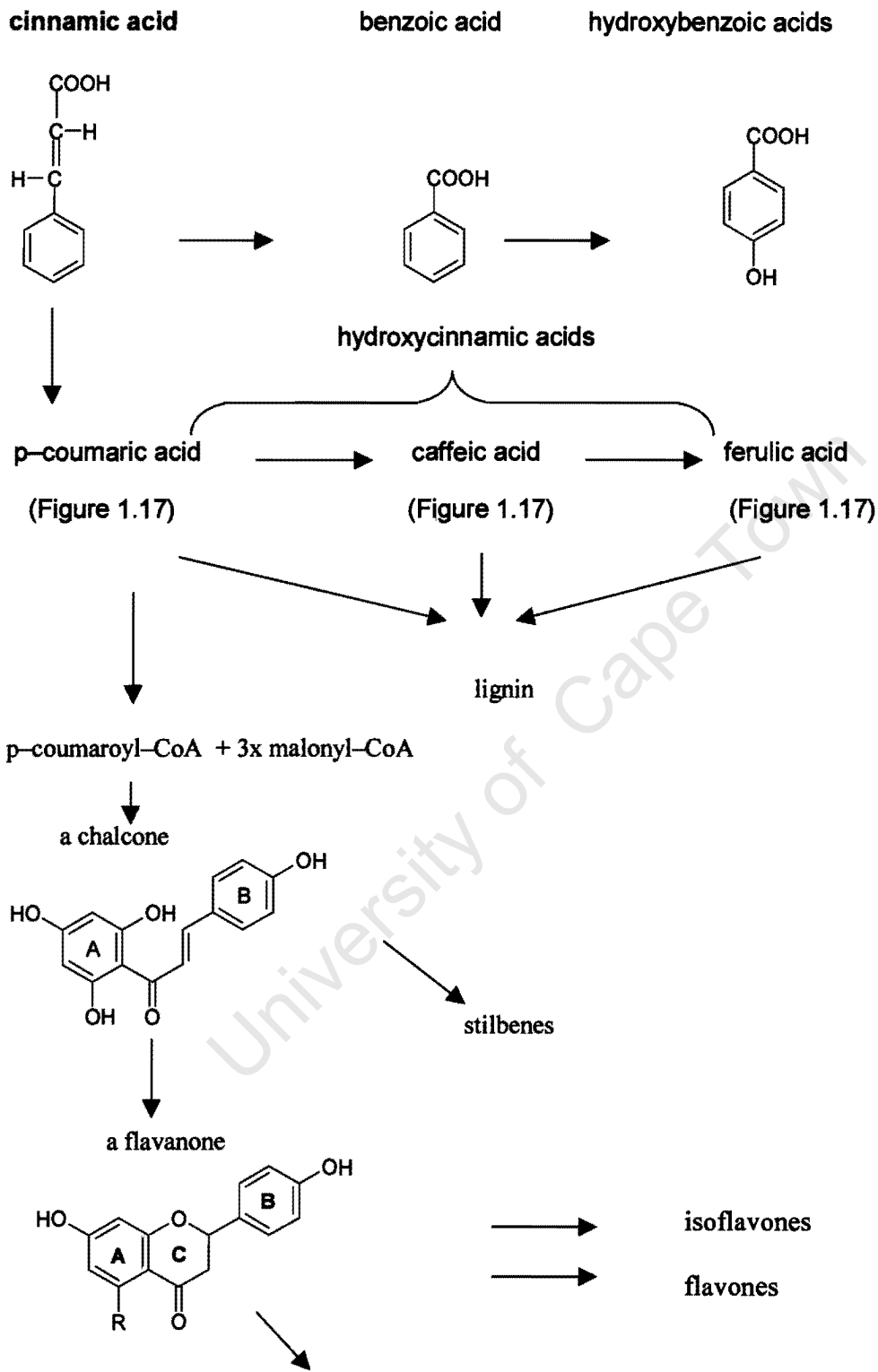
The biosynthetic pathway that leads to the generation of phenolic compounds in plants is known as the Shikimate pathway. This major pathway generates the aromatic amino acids: tryptophan, phenylalanine and tyrosine and can be divided into two main parts: the pathway from erythrose-4-phosphate and phosphoenol pyruvate, through 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) and shikimate, via 5-enolpyruvylshikimate-3-phosphate (EPSP) to chorismate, and then the pathway from chorismate, the common compound, to the three amino acids. The pathway from phenylalanine leads to the generation of the non-flavonoids and the flavonoids. It is well known that all classes of flavonoids are biosynthetically closely related. Chalcones are the first detectable C₁₅ compounds that are common to all the flavonoids, including the isoflavonoids. Stilbenes are also derived from chalcones. The pathway from phenylalanine leads to the generation of other phenyl compounds such as lignin.







The structures of the non-flavonoids and the flavonoids synthesized from cinnamate in wine are most often depicted in the protonated acidic form (272), and so this is adhered to here. However, for the purpose of this thesis, which mostly involves wine in physiological conditions, involving a pH of approximately 7.4, the dissociated form of structures from carbohydrate metabolism to cinnamate is used. Knowledge of the pK_a of the particular structures would determine in which of the forms the compound would be present.



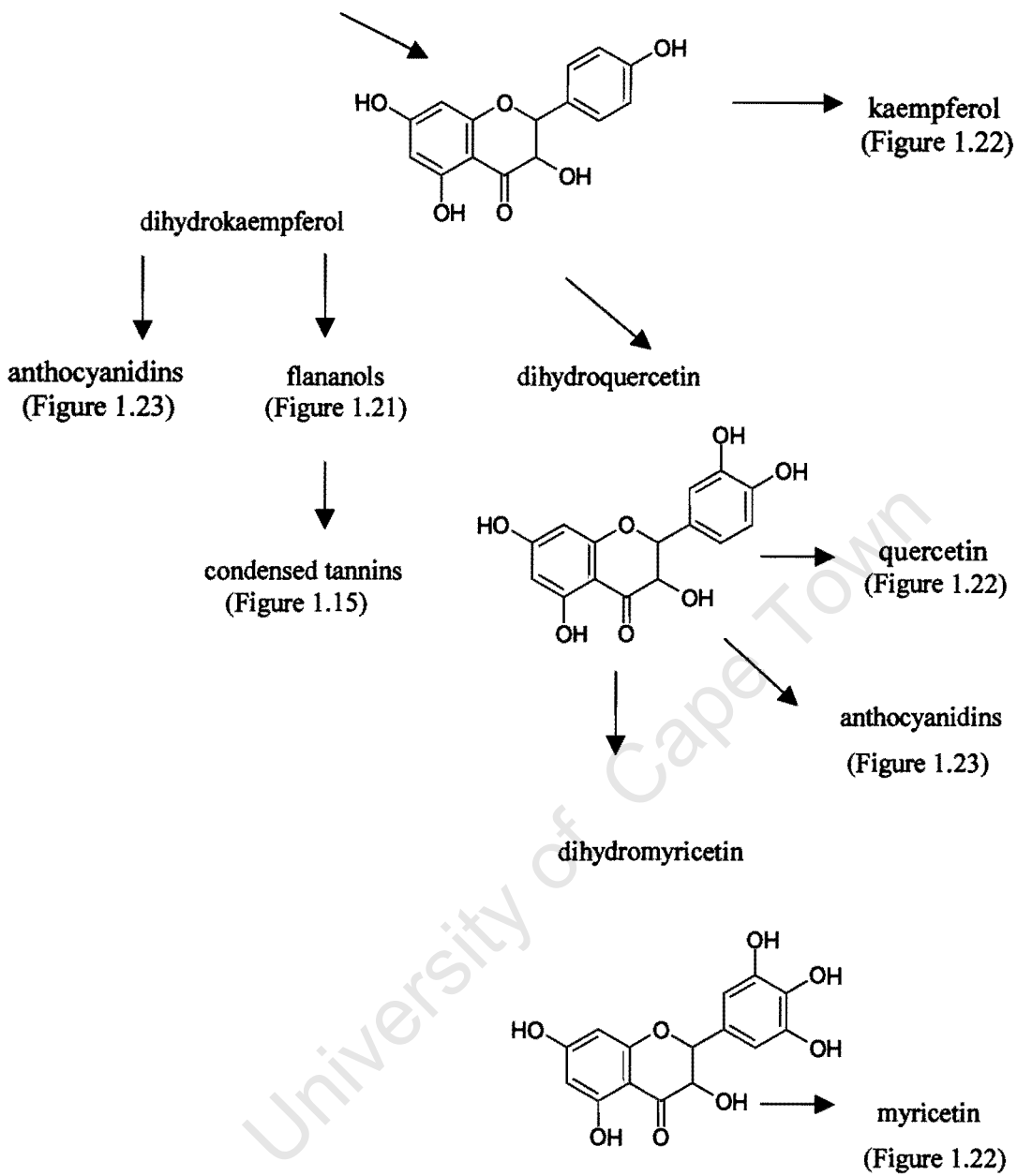


Figure 1.25. Biosynthesis of tannins, non-flavonoids and flavonoids (20,272-278).

1.4. FATTY ACIDS, THEIR PEROXIDATION, AND ANTIOXIDANTS

1.4.1. Fatty acids as lipids

Lipids are biomolecules that are insoluble in water but soluble in organic solvents of low polarity, for example chloroform. They may be classified into 2 broad categories: simple lipids, for example carboxylic acids (fatty acids) and sterols, and complex lipids, which are esters of long-chain fatty acids.

Fatty acids are major structural components in acylglycerols, which include triacylglycerols (TG) and phospholipids (PL) and make up the majority of lipids in the body. TG are the largest group of complex lipids and occur as esters of glycerol in which all three hydroxyl groups are esterified with a fatty acid. TG act as storage reserves of energy in animal cells. They provide approximately 40% of the energy requirements of man as part of a normal diet. Simple TG have all 3 fatty acids identical while the mixed TG have 2 or 3 different fatty acids, with an unsaturated fatty acid usually occupying position *sn*2 in biology. The structure of the different fatty acids is described in Section 1.4.2.1.

Phospholipids are major compounds in cell membranes. Fatty acids also form part of the structure of PL, the second largest group of complex lipids with one fatty acid in sphingolipids, such as sphingomyelin (which do not contain glycerol) or more fatty acids (phosphoacylglycerols, where the 3 hydroxyl groups of glycerol are esterified with 2 fatty acids and a phosphate group). The phosphate group of phosphoacylglycerols may itself be bound to one of several simple organic groups such as the choline in phosphatidylcholine. Phosphoacylglycerols include phosphatidic acid and phosphatidylglycerol, major PL such as phosphatidylcholine (lecithin), and phosphatidylethanolamine as well as minor PL such as phosphatidylserine, phosphatidylinositol and cardiolipin. Sphingolipids are all based on the amino alcohol sphingosine, are derivatives of ceramide and are found in large amounts in nerve tissue and brain. Glycosphingolipids, for example gangliosides, also contain sugar residues and make up 5–10% of lipids in plasma membranes. Phosphatidylglycerol is a precursor of cardiolipin, a lipid found largely in mitochondrial membranes.

Phospholipids form lipid bilayers in cell membranes. They are also components of prostaglandins, leucotrienes and ketone bodies. Prostaglandins and leucotrienes are important in physiological regulation. They are related structurally and metabolically; both being derived mainly from arachidonic acid, a C₂₀ unsaturated fatty acid containing four double bonds. Prostaglandins mostly prevent platelet aggregation and clotting and usually cause smooth muscle contraction of blood vessels. Leucotrienes are responsible for bronchostriction, modulation of immune hypersensitivity and are chemotactic agents (20). Ketone bodies act as an important energy source when carbohydrates are in short supply.

1.4.2. Fatty acid structure, classification and nomenclature

1.4.2.1. Structure and classification

Fatty acids consist of a hydrocarbon chain and a terminal carboxyl group with a general formula of:



They occur naturally as esters (for example triacylglycerols Section 1.4.1), and only small amounts of non-esterified “free” fatty acids are carried on albumin in plasma because of poor solubility, and this also protects cells from their detergent action. Chain length may be from 1 hydrogen atom (HCOOH methanoic (formic) acid) to 30 or more carbon atoms, although fatty acids mostly occur in the C₁₂₋₂₂ range. Short-chain fatty acids are more water-soluble. Long-chain fatty acids (>10 carbon atoms) occur commonly in TG and PL.

With the exception of C₅ (pentanoic (valeric) acid), only fatty acids with an even number of carbon atoms are found in substantial amounts.

Fatty acids are classified according to the number of double bonds: saturated when there are no carbon-carbon double bonds, monounsaturated (MUFA) when there is one double bond and polyunsaturated (PUFA) when there are two or more double bonds, up to six in important fatty acids. The double bonds in the more important naturally-

occurring PUFA are always separated by one methylene group: $-\text{CH}_2-$, and the double bonds are in the *cis* configuration, for example linoleic acid (Figure 1.26 and arachidonic acid ($20:4^{\Delta 5,8,11,14}$) (the Δ convention is explained in Section 1.4.2.2):

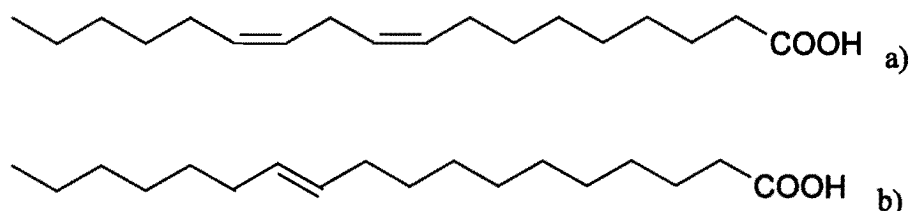


Figure 1.26. Structure of a) a *cis* dienoic unsaturated fatty acid, linoleic acid, and b) a *trans* monoenoic fatty acid, vaccenic acid.

Another group of PUFA has part or all of their unsaturation in a conjugated system with *cis* and *trans* double bonds (Figure 1.27), where a conjugated system has the double (or triple) bonds separated by a single bond. These are not commonly found in animal lipids, occurring mostly in some seed oils:

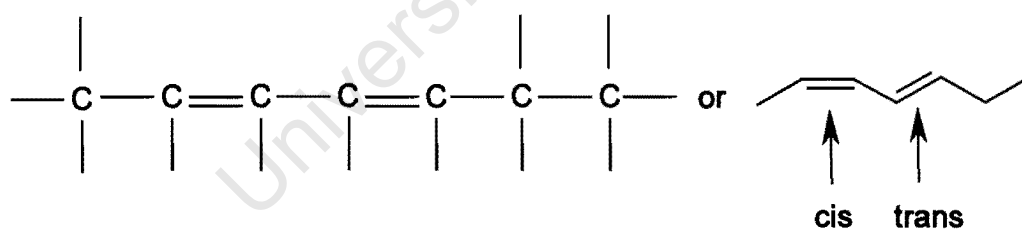


Figure 1.27. Conjugated unsaturation in a polyunsaturated fatty acid.

Fatty acids with conjugated unsaturation compared with their non-conjugated isomers exhibit a characteristic ultraviolet spectrum where the greater the extent of diene conjugation the greater the increase in absorption at 234nm (279), higher stability therefore higher melting points, commonly occurring *trans* unsaturation and they are more reactive towards free radical addition. A third category has double bonds that are

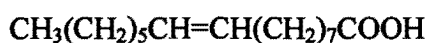
not totally in a methylene-separated system. They are known as the non-methylene-interrupted dienes (NMID) and an example is the fatty acid in tall oil ($18:3^{\Delta 5,9,12}$) (280). The *cis* configuration has restricted rotation around the double bond and this reduces close packing adjacent to the hydrocarbon chain which results in weaker intermolecular van der Waal's forces. Unsaturated fatty acids therefore have lower melting points than saturated ones. TG containing *trans* unsaturated fatty acids as opposed to *cis* fatty acids have their hydrocarbon chains more closely packed and so have higher melting points. Two highly unsaturated fatty acids found in fish oils are eicosapentaenoic acid (EPA): $20:5^{\Delta 2,5,8,11,14}$ or $20:5^{\Delta 5,8,11,14,16}$ or $20:5^{\Delta 5,8,11,14,17}$ and docosahexaenoic acid (DHA): $22:6^{\Delta 4,7,10,13,16,19}$.

The two most abundant saturated fatty acids in mammals are palmitic acid (16:0) and stearic acid (18:0), while oleic acid ($18:1^{\Delta 9}$) is the most commonly occurring unsaturated fatty acid. Linoleic acid ($18:2^{\Delta 9,12}$), linolenic acid ($18:3^{\Delta 9,12,15}$) and arachidonic acid are known as essential fatty acids because they are not synthesised by mammals but are important in the diet, being acquired from plants.

Fatty acids may also have branched chains or cyclic structures, although these are present in small amounts in humans.

1.4.2.2. Nomenclature

The most commonly-used systematic nomenclature (the IUPAC system) names the fatty acid the same way as the hydrocarbon with the same number and arrangement of the carbon atoms, with "oic" in place of the "e" at the end of the hydrocarbon name, for example the single carbon methane becomes methanoic acid (the common or trivial name is formic acid) as a fatty acid. This is the systematic name. "Anoic" denotes a saturated fatty acid while "enoic" denotes a double bond and therefore an unsaturated fatty acid, for example hexadecenoic (palmitoleic) acid:



There are several conventions denoting the position(s) of the double bond(s) in the hydrocarbon chain. In the IUPAC and Δ (delta) conventions, the double bond is

numbered from the carboxyl carbon (carbon number 1) whereas in the ω (omega) or n - (n minus) conventions, it is numbered from the terminal methyl carbon, for example:

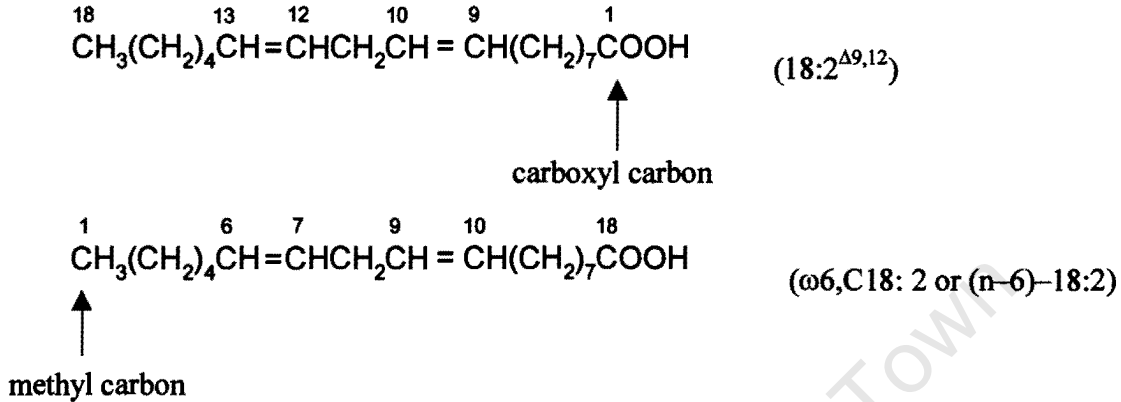


Figure 1.28. Double bond numbering conventions of octadecadienoic (linoleic) acid.

1.4.3. Fatty acid biosynthesis and metabolism

The main sources of fatty acids in humans are dietary and cellular stores. The exogenous pathway of TG metabolism refers to the metabolism of dietary lipids (including TG, PL and cholesterol esters) which are digested (hydrolysed) by pancreatic enzymes to fatty acids in emulsions with bile acids in the gut, followed by absorption into the enterocyte and reassembly into TG and subsequent incorporation into chylomicrons for transport. These lipoproteins are secreted into the lymphatic system and subsequently enter the systemic circulation for distribution to mainly the muscles and adipose tissue. Chylomicron remnants return to the liver. Chylomicrons contain mainly TG, and the fatty acid composition is that of the diet. Phospholipids, cholesterol ester and free cholesterol are minor constituents. The generally accepted composition is 88% TG, 8% PL and 3% cholesterol ester. The chylomicrons are the largest and least dense of the lipoproteins, and the structural apoproteins include Cii for lipoprotein lipase activation and apoE to mediate clearance. The half life is the shortest of all the lipoproteins. The endogenous pathway of TG metabolism refers to the secretion of VLDL, the TG-rich lipoprotein

assembled on apoB-100 in the liver. This lipoprotein is smaller and denser but is metabolised in a similar manner to chylomicrons. While chylomicrons will contain almost entirely dietary fatty acids, the VLDL can contain “recycled” diet-derived fatty acids as well as fatty acids synthesised in the body and returning from body stores (adipocytes). Exchange of neutral lipids between TG-rich lipoproteins and cholesterol ester-rich lipoproteins can occur by virtue of cholesterol ester transfer protein.

1.4.3.1. *De novo* biosynthesis (lipogenesis)

There are big variations among animal species with regard to their fatty acid pathways and the substrates for fatty acid synthesis. Most animal cells are able to synthesise fatty acids to a level that maintains lipids in membranes. However, liver cells engage in more extensive synthesis, which leads to storage of triacylglycerols in adipocytes. The main enzymes are expressed in higher levels in cells of liver, adipose tissue, brain, lung, kidney and mammary glands of higher animals. Insulin is an important hormone that stimulates lipogenesis via several mechanisms, one of which is the activation of acetyl-CoA carboxylase.

Fatty acids are synthesised from acetyl-CoA and the overall reaction of biosynthesis of palmitate is:



1 acetyl unit from acetyl-CoA +

7 acetyl units from malonyl-CoA

Figure 1.29. Overall reaction of palmitate biosynthesis.

Palmitate is the precursor of both saturated and unsaturated fatty acids. All mammalian PUFA are formed from elongation and/or desaturation of palmitoleic, oleic, linoleic and linolenic acids.

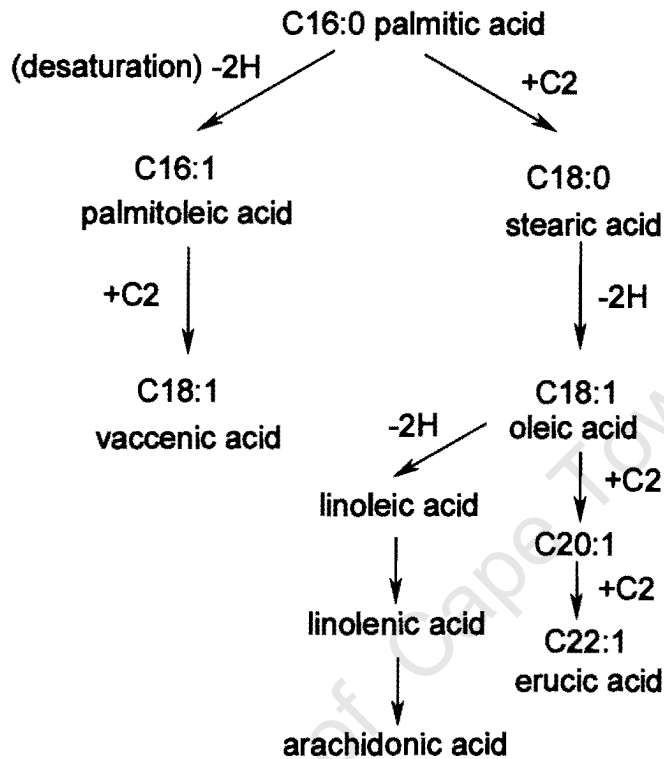
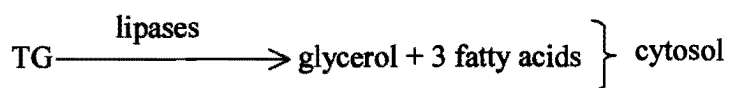


Figure 1.30. Biosynthesis of some unsaturated fatty acids from palmitic acid (281,282)

1.4.3.2. Fatty acid catabolism (oxidation)

Fatty acid degradation in man is an aerobic process which provides energy and which is brought about almost entirely by β -oxidation and to a far lesser extent α - and ω -oxidation.

It is initiated by hydrolysis of TG by hormone-sensitive lipases, which occurs when protein kinase A is stimulated by high concentrations of cyclic AMP (cAMP) to phosphorylate the lipases:



Fatty acids are then activated by esterification with CoA to fatty acyl-CoA thioesters by acyl-CoA synthetases, located on the outer mitochondrial membrane, with different synthetases activating different fatty acids depending on their chain length. These synthetases activate both saturated and unsaturated fatty acids. Fatty acyl-CoAs are impermeable to the inner mitochondrial membrane and are therefore transported as fatty acylcarnitine after the reaction catalysed by carnitine acyltransferase I and II (CAT I and II) located on the outer and inner surfaces respectively of the inner mitochondrial membrane. Carnitine is widely found in the body, particularly in muscle, kidney and liver and is synthesized from lysine.

β -oxidation, a cyclic process, takes place in the mitochondrial matrix, after re-formation of fatty acyl-CoA. The fatty acids are degraded stepwise from the carboxyl end by removal of 2 carbon atoms per turn of the cycle. The 2 carbon atoms are removed as acetyl-CoA and the β -carbon is oxidized:

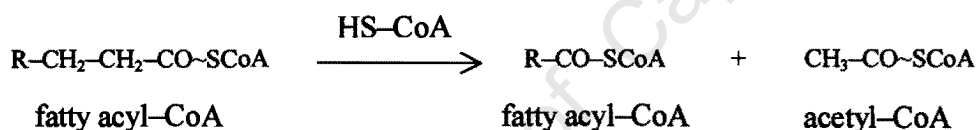


Figure 1.31. β -oxidation reaction.

β -oxidation of unsaturated fatty acids requires further enzymatic stages in order to move the double bonds. Complete oxidation of unsaturated fatty acids yields fewer ATP molecules than the corresponding saturated fatty acids due to the fewer hydrogens in the unsaturated molecules. α -Oxidation is a process that takes place in brain tissue, where there is removal of 1 carbon at a time from the carboxyl end of the fatty acid. CoA intermediates are not found. ω -Oxidation involves hydroxylase enzymes and cytochrome P450 in the endoplasmic reticulum and is a very minor pathway of fatty acid oxidation.

1.4.3.3. TG and PL assays

1.4.3.3.1. TG assays

Triacylglycerols are hydrolysed to free fatty acids and glycerol by the action of lipoprotein lipases. The glycerol is converted to glycerol-3-phosphate and ADP by glycerokinase and ATP. In the presence of oxygen, glycerophosphate oxidase converts the glycerol-3-phosphate to dihydroxyacetone phosphate and hydrogen peroxide. Two molecules of the peroxide, in the presence of 4-chlorophenol and 4-aminoantipyrine, are used by peroxidase to generate red quinoneimine hydrochloride and water. The absorbance of the red colour may be read spectrophotometrically at 500nm. The method is detailed in the Appendix .

1.4.3.3.2. PL assays

The phospholipid assay measures phosphatidylcholine, the major PL in blood. PL are hydrolysed by phospholipase D to choline and phosphatidic acids. In the presence of oxygen and water, the choline is converted to betaine and hydrogen peroxide by choline oxidase. Two molecules of the peroxide are converted by peroxidase in the presence of 4-aminophenazone and phenol to red 4-(*p*-benzoquinone-mono-imino)-phenazone and water. The red colour may be read spectrophotometrically at 500nm. The method is detailed in the Appendix .

1.4.4. Lipid peroxidation

Free radicals have been extensively researched in recent years. They are derived mostly from oxygen as reactive oxygen species (ROS) and are generated *in vivo* by various endogenous processes, exposure to various physiochemical conditions or pathophysiological states (283). Lipids, which are prone to free radical attack, may be altered by these free radicals, resulting in lipid peroxidation which has been implicated in various diseases.

Oxidation of the unsaturated fatty acids in lipids is one of the most fundamental chemical reactions and generates a very complex set of volatile oxidation products that can cause spoiling or rancidity in compounds such as foods. The oxidative decomposition

products such as lipid hydroperoxides (LOOH) and thiobarbituric acid reactive substances (TBARS) may also cause cellular damage in the body, as in atherosclerosis, cancer, various inflammatory diseases and ageing.

There are several mechanisms of lipid oxidation. *Lipid peroxidation* (or autooxidation) of lipids is a process where electrons are removed from the lipids by free radicals, resulting in the increased production of more free radicals. This involves a catalytic chain reaction where hydroperoxides are formed via loss of a hydrogen radical in the presence of trace metals, light or heat. Photooxidation of lipids occurs when the oxidation reactions are induced by light, resulting in the loss of 1 or more electrons as a result of photoexcitation. Oxidation by singlet and triplet oxygen proceeds by a different mechanism from free radical autooxidation.

Since the early 1960s a lot of research in this field has been carried out, leading to a considerable increase in the understanding of the process of lipid peroxidation and the role it plays in the decomposition of lipid hydroperoxides, which are the precursors of the volatile secondary products. Although there have been huge advances in this field, the methods used are often still very non-specific and unreliable. Lipid peroxidation is often studied under drastic conditions with simplistic models that do not mimic complex biological or food systems. For the purposes of the studies in this thesis, lipid peroxidation will refer to all the changes in PUFA that are found to differ from the usual biological state of these fatty acids, including conjugated dienes (CD) (in which there is no change in oxidation states), LOOH and more advanced products including aldehydes (TBARS) and fragments of long chain fatty acids.

1.4.4.1. The process of lipid peroxidation

The autoxidation of PUFA proceeds via a free radical chain reaction (Figure 1.32). Reversion can occur at the stage of the CD radical. Compared with the original biologically-derived fatty acid, the methylene-interrupted double bond is now altered to a CD and its conformation is no longer enzymatically determined and merely follows entropy. In this context a CD reflects free radical attack of a biological fatty acid and promotes the reaction with ROS.

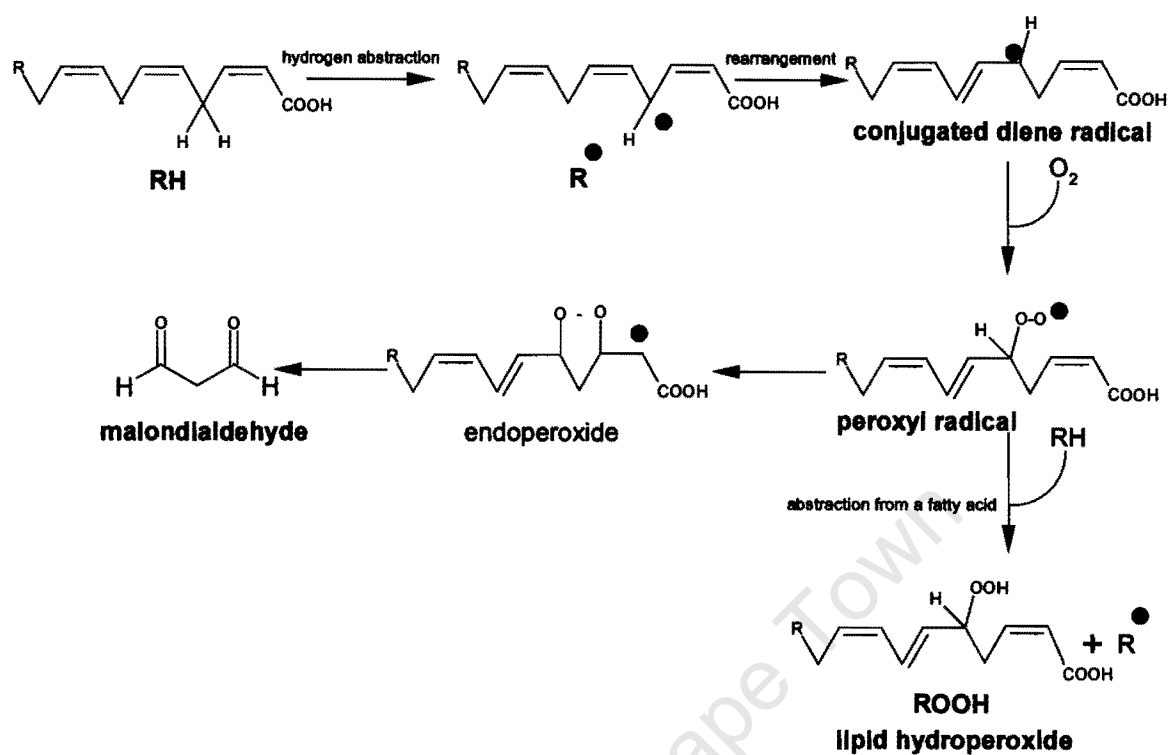
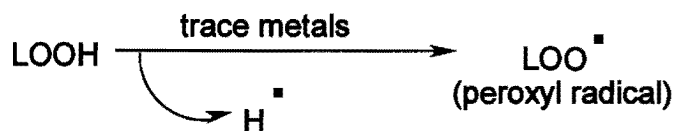


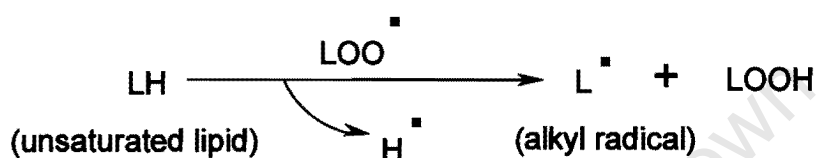
Figure 1.32. The overall process of lipid peroxidation (adapted from (284) and (285)).

The chain reaction may be described in terms of an initiation stage, followed by propagation, branching and a termination stage (286,287):

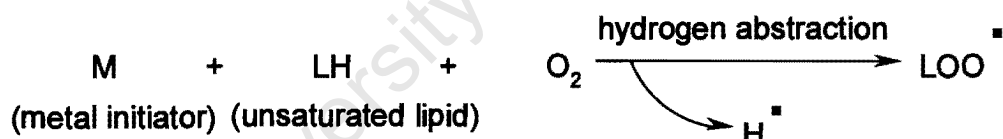
- The *initiation* process involves all the primary reactions that generate free radicals, resulting in a net increase in the number of active free radicals, as opposed to the propagation stage where the number remains the same, and the termination stage where there is a net decrease in the number of free radicals. One of the main products of lipid peroxidation, lipid hydroperoxides (LOOH), may also be a source of active free radicals, via a specific stage of the initiation process known as “degenerate chain-branching”:



The peroxyl radical, the principle chain-carrying free radical, may act as an initiator for oxidation in the following reaction:



Trace metal ions such as iron and copper, heat and ultraviolet radiation, may also act as initiators of lipid peroxidation, and when the oxygen concentration is high enough, peroxyl radicals are formed:



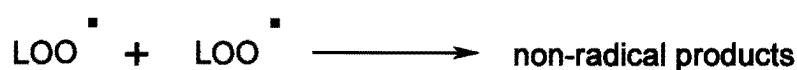
- The *propagation* stage:



The hydroperoxides are the precursors of the volatile secondary products such as MDA (Figure 1.33).

- The *termination* stage:

In the termination stage, the peroxy radicals react with each other and self-destruct, forming non-radical products.



1.4.4.2. Determination of the products of lipid peroxidation

Lipid peroxidation of fatty acids in food during storage and preparation is discussed in Section 2.1. Continuous monitoring of lipid peroxidation or collection of individual samples from the reaction are both used to determine the concentration of products. Evaluation of lipid peroxidation as CD and TBARS constitute the most popular assays. The methods below are all detailed in the Appendix.

1.4.4.2.1. CD and the CD assay

A CD is an alkene whose double bonds are separated by a single bond in the molecule: for example $\text{H}_2\text{C}=\text{CH}-\text{CH}=\text{CH}_2$. CD are the initial markers of oxidative stress. Molecules that contain CD absorb maximally at 215–250nm, depending on the presence of substituent groups nearby. Lipids with CD are characterised by a peak of absorbance at approximately 233nm, with a lesser peak at 260–280nm. The concentration of CD is determined by the extinction coefficient of $2.95 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. A potential problem with measurement of CD in biological samples is the interfering background absorption from PUFA, haem proteins, purines and pyrimidines.

1.4.4.2.2. LOOH and the LOOH assay

The steady-state concentrations of LOOH in human body fluids is very low (less than 100nM), as measured by methods that directly determine the concentration of “real” and not artefactual LOOH, as used by Holley and Slater with HPLC–chemiluminescence (288). The FOX (ferrous–oxidation xylenol orange) method, however, is ideal for comparison of concentrations and measures the oxidation of Fe^{++} to Fe^{+++} by LOOH in dilute acidic conditions. The antioxidant BHT is added to prevent further oxidation during the reaction. In the presence of xylenol orange, Fe^{+++} forms a coloured xylenol orange–ferric ion complex which absorbs maximally at 560nm. Phosphines also react specifically with hydroperoxides, and their fluorescent derivatives (for example diphenyl–1–pyrenylphosphine) have been used for assays as well.

1.4.4.2.3. TBARS and the TBARS assay

In the presence of iron and copper complexes, LOOH break down to produce a variety of aldehydes such as malondialdehyde (MDA). MDA is one of the most important TBARS in lipid peroxidation. TBARS produce a red product when they react with thiobarbituric acid (TBA) (Figure 1.33). The spectrophotometric method, although sensitive, is also non-specific because compounds such as bilirubin, amino acids, oxidised proteins, acids, aldehydes, ketones and esters may also react with TBA. Another potential problem is that significant amounts of MDA might be lost via volatilisation or cross-linking with proteins. Additionally, the heat required for the reaction can promote the formation of new TBARS during the assay. Ferric chloride is added to the assay to promote the oxidation reaction. The assay involves the heating of the sample at an acid pH. The coloured product is read at 532nm and the concentration of the TBARS is determined by the molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

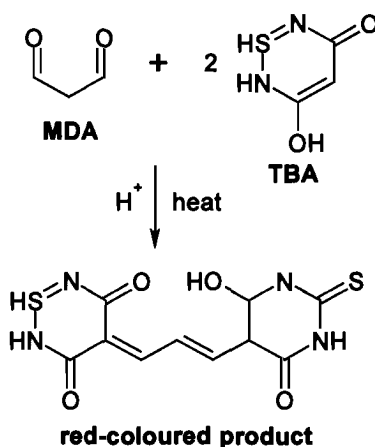


Figure 1.33. The TBARS reaction.

BHT, a free radical scavenger antioxidant, may be added to reduce *in vitro* lipid peroxidation. To improve the specificity of the MDA–TBA adduct, a number of HPLC methods have been devised where non–MDA contaminants are separated out during the chromatography stage. The determination of plasma MDA by a number of authors has shown the range of concentrations to be 590–4570nmol/L (285). The TBARS assay, despite its problems, has been found to correlate well with other markers of lipid peroxidation (289). Young and Trimble (289) found that plasma TBARS samples were stable during storage at 4°C for 10 days, at –20°C for 3 weeks and at –70°C for longer storage.

1.4.4.3. ANTIOXIDANTS

An antioxidant is a substance that significantly delays or prevents oxidative damage to a substrate, for example a PUFA. The interest in antioxidants increased in about 1990 when it became recognized that the beneficial health effects of beverages and foodstuffs such as red wine, tea, fruits and vegetables, depended largely on their antioxidant activities. This resulted in the development of a number of new methods to determine the total antioxidant capabilities of the individual antioxidants as well as that of blood serum. Antioxidants may be primary, reducing the rate of production of radicals that start the new oxidation chains during lipid peroxidation, reducing production of hydroperoxides or chelating the metal ions that catalyse oxidation reactions, or they may be secondary

antioxidants, which trap radicals, such as peroxy radicals (the main chain-carrying radicals during lipid peroxidation (290)) and hydroxyl radicals, directly and so reduce chain propagation and amplification. However, the majority of antioxidant compounds has multiple antioxidant properties. There are various *in vivo* antioxidants such as transferrin, superoxide dismutase, catalase, glutathione peroxidase and reductase, urate, vitamin E (tocopherols and tocotrienols) as well as various dietary compounds such as plant phenolic compounds, vitamin A and vitamin C. Flavonoids are regarded as “high-level” antioxidants based on their ability to scavenge free radicals such as superoxide and hydroxyl radicals, as opposed to the chain-breaking α -tocopherol which is considered a “low-level” antioxidant because it stabilises radicals that occur later in the oxidation process, such as peroxy radicals. To be an efficient radical scavenger, a flavonoid must have ortho-dihydroxy groups in the B-ring and secondly, there should be a 2–3 double bond conjugated with the 4-oxo group in the C-ring, which contributes to electron delocalisation in the B-ring, and thus stability (261). Thirdly, for maximum radical-scavenging ability, the flavonoid should have 3- and 5-hydroxyl groups. The antioxidant capacity of any antioxidant is determined by its reactivity with the oxidant substrate, its concentration, metabolism, mobility and distribution, its possible pro-oxidant effects under certain conditions, synergistic reactions with other antioxidant compounds, and the means of determining the antioxidant capacity (290,291).

Future antioxidant research includes the use of gene therapy to produce different antioxidants *in vivo*, the use of genetically engineered plants with higher concentrations of antioxidants, synthetic antioxidant enzymes and the use of functional foods with higher concentrations of antioxidants (283).

1.4.4.3.1. Antioxidant assays

Methods for determining the concentration of chain-breaking antioxidants, such as BHT and Trolox, may be direct or indirect. Direct methods determine the effect of the antioxidant-containing substance directly on the oxidation of the testing system, for example the effect of wine on the oxidation of LDL and the ORAC (oxygen radical absorbance capacity) method. Indirect methods include the TRAP (total radical-trapping antioxidant parameter), FRAP (ferric reducing ability of plasma), and the TEAC (trolox

equivalent antioxidant capacity) tests. These are methods that determine total antioxidant capacity.

- The TRAP (total peroxy radical-trapping antioxidant parameter) assay, as described by Wayner *et al* (292), measures oxygen consumption during thermally-controlled lipid peroxidation of ABAP (2,2'-azo-bis (amidinopropane hydrochloride)), a water-soluble peroxy radical generator. Trolox, a water-soluble α -tocopherol, is used as a standard in the assay. The TRAP assay has been found by some authors to be reliable and reproducible (291,293), while others found it an imprecise method, based on the instability of the oxygen electrode over the time of the assay (294).
- The FRAP assay, described by Benzie and Strain (295), measures the ability of antioxidants, in particular phenolics, to reduce ferric tripyridyltriazine to a coloured ferrous tripyridyltriazine complex. This is a simple assay but it is not the ideal assay for all antioxidants, such as glutathione, because not all antioxidants have this reducing ability.
- The TEAC assay, also referred to as TAS (total antioxidant status), and described by Miller *et al* (296,297), is based on the ability of antioxidants to inhibit the absorbance of ABTS (2,2'-azinobis-3-ethyl benzothiazoline-6-sulphonate).
- The ORAC assay described by Cao *et al* (298) and revised by Ou in 2001 (299) is the most recent and commonly-used of these assays. The details of the assay are described in the Appendix. The method is very specific, can test for numerous antioxidants, and is the only assay that takes the free radical action to completion. It uses a peroxy radical generator, AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) as a pro-oxidant. The calculated area under the curve is used to determine total antioxidant capacity.

The photography in this section was carried out by the author with the assistance of Natalie Payne at the Distell Winery, Stellenbosch, South Africa, unless otherwise stated.

CHAPTER 2. A NOVEL OIL–BOIL METHOD TO DETERMINE THE ANTIOXIDANT EFFECTS OF RED WINE

2.1. Background

Edible oils contain 98–99% triacylglycerols and 1–2% non-saponifiable compounds, such as sterols and fat-soluble vitamins. The chemistry of triacylglycerols is described in Chapter 1. Worldwide, the consumption of oils and fats has grown steadily over the last 25 years, with an average annual growth rate during 1999 to 2004 of 3.8% (300). The approximate total world production of major oils and fats from 1995 to 1996 was 82 million metric tons, with the production of canola oil being the highest, followed by sunflowerseed (sunflower) and cottonseed oils (301). In South Africa, sunflower oil is used extensively. In 1994 in the USA, approximately 2.5 billion kilograms of fats and oils of a total of 5.4 billion kilograms consumed, were used for frying or baking, with deep-fat frying at high temperatures being the most common method (302,303).

The safety of thermally-stressed unsaturated fatty acids has been the subject of research for many years. Food that contains PUFA is very susceptible to lipid peroxidation and the greater the degree of unsaturation of the fatty acid, the more susceptible it is to oxidation, particularly during cooking. Heating oils causes an increase in the concentrations of free fatty acids and polymers, and a decrease in antioxidant content (304). The concern when frying food in oil is the possible interaction of the food, especially protein, with the lipid peroxidation products, forming new compounds that are potentially toxic. The chemistry of autoxidation has been discussed in Section 1.4.4.1. Lipid peroxidation leads to the formation of hydroperoxides, which are unstable and decompose rapidly to form secondary products such as malondialdehyde. Lipid peroxidation relating to cooking processes may result in changes to the oils and fats. Apart from changes in palatability (rancidity), that may affect food preference, alterations from *cis* to *trans* isomers may occur. These changes are accepted as unfavourable to health. Whilst aldehydes may cause polymerisation that could merely make fats indigestible, these and other chemically reactive species may harm the lining of the alimentary tract and could potentially also be transported in the circulation where vascular endothelium may be affected adversely and may also harm the organs or tissues of final destination, including the liver, muscle and

adipose tissue. Early studies indicated toxicity, but unfortunately very few analytical details were given. Animal studies have shown that the products of lipid peroxidation are well absorbed via the lymphatic system into the systemic circulation (305): Sánchez-Muniz *et al* (306) found that oxidised lipids are actively absorbed in rats and Naruszewicz *et al* (307) found an increase in TBARS in human plasma after consumption of thermally-oxidised oil. Liver TBARS were found to be significantly increased in rats that ingested thermally-oxidised oil (308-310), while there was also evidence of significant liver necrosis (311). Liver metabolism was affected by consumption of oxidised oils (312). The absorption of fatty acids in heated oils was significantly lower than in unheated oils (313,314) and the authors ascribed this to an increase in polymer formation in the heated oils. Some studies found short-term changes in the gastrointestinal tract such as diarrhoea, while longer-term changes included growth retardation, liver damage and death (312,315-320). The susceptibility of tissue to iron-induced lipid peroxidation was increased by ingestion of heated sunflower oil (321). Malondialdehyde was found to be unequivocally mutagenic (322) and carcinogenic (323). Peroxidation products may also contribute to the pathogenesis of degenerative diseases, cancers and the ageing process (324). Consumption of a meal containing used cooking oil resulted in impaired endothelial function (325). Chapter 5 describes the effect of red wine on endothelial function of the brachial artery

Autoxidation is therefore a critical factor that affects the quality of oils. It is one that is difficult to prevent, given that it takes place even at low temperatures in the dark (326). In oils, this process is slow, until their antioxidant potential is exceeded. Oils have their own natural antioxidants and are also protected from oxidation by the addition of antioxidants such as the chain-breaking tocopherols, and BHT (327). When these are depleted, peroxidation accelerates and becomes rapid. The time taken for this to happen is known as the induction time, and this may vary between different oils. An increase in the unsaturated nature of the fatty acids in an oil reduces the induction time and increases the rate of oxidation (326).

Frying in oil is one of the most commonly-used cooking methods in food preparation. Frying conditions increase the rate of oxidation in oil, and antioxidants in the oils tend to decompose rapidly. Degradation products, such as polar triacylglycerols and polar and

non-polar polymers, tend to accumulate, and when the concentration of polar compounds reaches approximately 30% (the threshold concentration of di- and polymeric triacylglycerols in oils is 10% by mass (328)), the oil should be discarded. At this stage, the oil has usually darkened in colour and there is an increase in viscosity and foaming.

A large number of studies indicates the beneficial effects of alcohol in beverages, as described in Chapter 1. These effects are thought to be due, at least in part, to the presence of polyphenols. Section 1.2.3.1 describes a number of studies that found a greater beneficial effect from red wine compared with other alcoholic beverages. Wine, in particular red wine, contains a number of different polyphenols, including flavonoids, which have beneficial antioxidant effects and antioxidants play an important role in potentially preventing lipid peroxidation. Previous methods used to evaluate antioxidant activities of wine have generally been based on the ability of the wine to reduce *in vitro* oxidation of LDL. This is not a physiologically appropriate comparison as *in vivo* LDL will not be exposed to the same complement of chemistry. The antioxidant capacity of wines is determined by a number of factors including the grape variety, the wine processing techniques and by the reactions that occur during ageing of the wine. Some older wines have been found to have greater antioxidant capacity, attributed to the increase in tannins during ageing in oak (329). The objectives of this study were:

- i) To determine the concentration of 3 different peroxidation products in edible oils, (CD, LOOH and TBARS), and to select a suitable, probably PUFA, oil for use in further testing;
- ii) To determine the extent of lipid peroxidation in a thermally-stressed PUFA oil at 100°C, the temperature that would apply to cooking foods that contain water.
- iii) To establish an “oil-boil” method that would reliably determine and compare the total antioxidant effect of different wines in a food-relevant pseudomonophase using thermally-stressed PUFA oil. A macro-method using relatively large volumes of oil and wine was established first and then compared with a micro-method using smaller, more convenient volumes in order to accommodate larger sample numbers. To my knowledge, this method has not been used before in the published literature.

- iv) To determine the antioxidant capacity of each of the wines by the ORAC method, and to compare the results with the antioxidant capacity of the wines as determined by the macro and micro oil–boil methods.

2.2. Methods

2.2.1. Lipid peroxidation status of edible oils in South Africa

Thirty three commercially–available edible oils were purchased without reference to age or storage conditions, which would have reflected possible lipid peroxidation. These oils included 17 different brands of olive oil, 9 of sunflower oil, 2 of canola oil, 2 of sesame seed oil and 1 each of flax, grapeseed and peanut oil. The relative fatty acid compositions are shown in Table 2.1. The oils were classified as MUFA and PUFA according to the dominance of these fatty acids. The MUFA included the olive, canola and peanut oils, and the PUFA included the flax, sunflower, grapeseed and sesame oils. The spectrophotometric assays of the peroxidation products as described in the Appendix, were used to determine the concentration of the CD, LOOH and TBARS, both total and free (tTBARS and fTBARS), from 6 values, 3 determinations each on 2 days. The results were expressed per gram of oil.

Due to the popularity of sunflower oil as a first choice of cooking oil in South Africa, it was decided to use sunflower oil for further testing in re–heating, and in the macro and micro oil–boil experiments. Sample 4 of the sunflower oils was used as it is an oil that is readily available, inexpensive, and the peroxidation products in several different samples were consistent, with an inter–assay CV of < 10% for all the assays. The CD, LOOH and TBARS of this oil were similar to the means of all the 9 sunflower oils: per gram of oil, the CD content was 22.0 μmol in sample 4 compared with 21.0 μmol for the total mean; the LOOH content was 5.6 compared with 5.4 μmol ; the tTBARS content was 181.3 compared with 200.5 nmol and the fTBARS content was 0.6 compared with 1.5 nmol. The chemical composition of this oil was not declared by the manufacturer.

2.2.2. Lipid peroxidation in thermally-stressed PUFA oil, re-heated over 75 hours

Twenty five mL volumes of sample 4 sunflower oil were placed in 100 mL glass beakers (with a surface area of 2206 mm²) and heated on thermostatically-controlled Freed electric heater/stirrer modules for 3 hours whilst exposed to room air. One mL samples of each oil were taken before heating, at time 0 minutes (baseline) when the oil reached 100°C, and at 1, 2 and 3 hours. After 3 hours, the oil was removed from each beaker and stored at 4°C overnight in the dark. In order to simulate re-heating conditions, after 24 hours the oil was again heated to 100°C and 1 mL samples were taken at time 24, 25, 26 and 27 hours, relative to baseline. Heating was stopped, and the oils were stored overnight as before. The oils were again heated, and 1 mL samples of oil were taken at 72, 73, 74 and 75 hours. Concentrations of CD, LOOH and TBARS were determined for each of the 1 mL oil samples by the assays described in the Appendix.

2.2.3. Lipid peroxidation in thermally-stressed oil heated with copper and red wine

2.2.3.1. The macro-method

Twenty five mL of the oil were heated to 100 ± 5°C over 3 hours on Freed electric modules as above, alone, in a pseudomonophase with 25 mL deionised H₂O as a control, in a pseudomonophase with 25 mL of 20 µmol/L CuSO₄ in deionised H₂O, in a pseudomonophase with 25 mL red wine, and with 12.5 mL red wine with 12.5 mL 40 µmol/L CuSO₄. Heating was carried out in 100 mL glass beakers as in Section 2.2.2. Analyses of CD, LOOH and TBARS were carried out on 1 mL samples at baseline and at 3 hours. As the macro-method was established before the micro-method, it was considered necessary to determine the concentrations of all 3 peroxidation products, as only 1 product might not be representative of the peroxidation process. There was very good correlation between CD and LOOH, as shown in Figure 2.5. A maximum of 3 samples of wine could be tested in triplicate at the same time by this macro method, owing to the limited number of heating devices. The advantage of the macro-method was the ability to control the heating temperature of the different conditions, as heating of each condition was carried out on a separate heating module.



Figure 2.1. Heated oil and CuSO_4 solution on a Freed heater/stirrer module.

A total of 9 red wines was tested, each in triplicate. In addition, 2 wines, a Chateau Libertas and a Pinotage, were stored, corked, for 6 weeks in the dark at 4°C , and tested for their antioxidant capacity by the macro-method over this time. Twelve bottles of the same cultivar and vintage, a 1997 Chateau Libertas, were compared by the macro-method, as was the same cultivar but different vintages (1990, 1991, 1994, 1996, and 1999) of one of the wines, a Merlot.

2.2.3.2. The micro –method

This method was devised in order that productivity could be improved: by increasing the sample number, and decreasing the volume of oil and putative antioxidant liquid to a total of 1 mL per sample, in borosilicate 13 x 100 mm tubes, using the same conditions

and ratios of reactants as the macro-method. Magnets in each tube were used to create a pseudomonophase and a glass marble was placed at the top of each tube to reduce evaporation of these relatively small volumes, and limit the exposure to air over a surface area of 95 mm². Twenty samples of wine could be tested in triplicate at the same time by this method. Heating was carried out in a Pierce Reacti-therm heating/ stirring module and monitored by means of a digital Digitron thermocouple thermometer with a Physitemp micro-probe, for 2 hours at the following temperatures (mean ± SD): oil alone, 102.9 ± 1.8°C; oil with CuSO₄, 99.3 ± 3.1°C; oil with wine, 96.6 ± 1.1°C and oil with wine and CuSO₄, 96.6 ± 1.1°C. CD analysis was used to determine the antioxidant capability of the wine. Each sample was centrifuged at 14 000 x g for 10 minutes in a Jouan MR microfuge to separate the oil from the aqueous phase. CD analysis is quick and reliable but more importantly, it was established that it has good correlation with the LOOH assay ($r^2 = 0.62$) (Figure 2.5).

The antioxidant effect of each wine was determined as a percentage reduction of CD relative to the change brought about by oxidation by copper:

A = (concentration of peroxidation product from oil with Cu⁺⁺ at 180 minutes (macro) or 120 minutes (micro)) – (concentration of product at 0 minutes);

B = (concentration of peroxidation product from oil with Cu⁺⁺ and wine at 180 minutes (macro) or 120 minutes (micro)) – (concentration of product at 0 minutes);

C = (B/A x 100)

Antioxidant capacity of the wine = 100-C%.

2.2.3.3. The ORAC determination of red wine

The method of Ou *et al* (299), as described in the Appendix, was used. Briefly, the wine samples were diluted in phosphate buffer pH = 7.4. Fluorescein and AAPH were added and the reactions were allowed to go to completion, after which they were compared with a standard reaction of Trolox. This relatively more water-soluble derivative of tocopherol provides a useful reference. Samples were analysed in triplicate. The results were expressed as mmol trolox equivalents (TE)/L wine, and then compared with the antioxidant capability of the wines determined by the macro and the micro oil-boil methods.

2.2.4. Statistical analyses

Data are expressed as mean \pm SD. All statistical analyses were performed with the use of Graphpad PRISM software (version 3, San Diego, USA). The Student's t test was used to analyse the significance of paired data, and the unpaired t test was used to compare unpaired data. The PRISM software was also used to determine correlation coefficients. Statistical significance was accepted at $P < 0.05$.

2.3. Results

2.3.1. The concentrations of CD, LOOH and TBARS in edible oils

Table 2.2 shows the means of 6 values each of the inherent CD, LOOH and TBARS in 33 MUFA and PUFA oils. Considering all the oils, the mean \pm SD of the CD was 14.7 ± 7.0 $\mu\text{mol/g}$; of the LOOH was 5.8 ± 0.7 $\mu\text{mol/g}$, of the tTBARS was 264.9 ± 594.5 nmol/g and of the fTBARS was 2.5 ± 3.5 nmol/g oil.

Table 2.3 compares the peroxidation products of the oils separated into MUFA and PUFA oils. The CD and the LOOH were significantly different ($P = 0.008$ and $P < 0.001$ respectively), while the tTBARS and fTBARS were not ($P = 0.100$ and $P = 0.597$ respectively).

Figures 2.2 and 2.3 show the variation of CD and TBARS in the MUFA and PUFA edible oils. There was not much variation in LOOH in the oils and they were therefore not graphically presented.

The full range of peroxidation products (Table 2.2) showed that the CD ranged approximately 6-fold, from 5.3 to 32.4 $\mu\text{mol/g}$, LOOH approximately 2-fold, from 4.3 to 7.3 $\mu\text{mol/g}$, the tTBARS approximately 300-fold, from 9.8 to 3472.0 nmol/g and the fTBARS also varied widely, from 0 to 17.9 nmol/g oil.

Table 2.1. Saturated fatty acids (SFA), monounsaturated (MUFA) – and polyunsaturated fatty acids (PUFA) in edible oils (g %).
 The information in the table is adapted from Boskou & Elmadfa (326), Gunstone *et al* (280), and Ruf *et al* (330).

| | SFA | | | | | MUFA | | | | PUFA | | | | |
|----------------------|-------|---------|---------|---------|---------|---------|-------|-------|-------|--------|---------|------|-------|-------|
| | 14:0 | 16:0 | 18:0 | 20:0 | 22:0 | 16:1 | 18:1 | 20:1 | 22:1 | 18:2 | 18:3 | 20:5 | 22:1 | 22:2 |
| Olive oil | t | 7.5-20 | 0.5-5.0 | 0.7 | < 0.2 | 0.3-3.5 | 55-83 | < 0.5 | | 3.5-21 | < 1.5 | 1.0 | | |
| Canola oil | | 4.0 | 1.8 | | | 0.2 | 56 | 1.7 | 0.6 | 20.3 | 9.3 | | | |
| Peanut oil | 1.0 | 11 | 3.0 | | | | 49 | | | 29 | | 1.0 | | |
| Flax oil | | 3.9 | 3.6 | | | | 16 | | | 15 | 60 | | | |
| Sunflower oil | 0-0.2 | 5.6-7.6 | 2.7-6.5 | 0.2-0.4 | 0.5-1.3 | 0-0.3 | 14-39 | 0-0.2 | 0-0.2 | 48-74 | 0-0.2 | | 0-0.2 | 0-0.3 |
| Grapeseed oil | 0.3 | 5.5-11 | 3.0-6.0 | < 1 | | | 12-28 | | | 58-81 | < 1.0 | | | |
| Sesame oil | 0.1 | 8.0-10 | 4.0-6.0 | 0.5 | t | 0.1 | 34-44 | 0.6 | | 40-51 | 0.3-0.5 | | | |

t = trace

TABLE 2.2. Concentration of CD, LOOH and TBARS in MUFA and PUFA oils.

| | CD¹ | LOOH¹ | tTBARS² | fTBARS² |
|--------------------|-----------------------|-------------------------|---------------------------|---------------------------|
| Olive 1 | 10.4 | 5.6 | 75.0 | 2.0 |
| Olive 2 | 10.0 | 6.3 | 81.7 | 5.4 |
| Olive 3 | 11.4 | 6.0 | 42.4 | 1.6 |
| Olive 4 | 7.5 | 5.4 | 37.8 | 2.0 |
| Olive 5 | 7.8 | 5.5 | 36.8 | 3.2 |
| Olive 6 | 10.7 | 6.2 | 38.8 | 4.2 |
| Olive 7 | 9.4 | 6.2 | 59.7 | 4.6 |
| Olive 8 | 9.2 | 5.6 | 43.1 | 2.2 |
| Olive 9 | 6.5 | 6.1 | 35.6 | 2.8 |
| Olive 10 | 11.4 | 7.3 | 65.0 | 10.2 |
| Olive 11 | 5.3 | 5.7 | 93.4 | u |
| Olive 12 | 22.9 | 6.0 | 155.3 | u |
| Olive 13 | 8.4 | 5.6 | 23.9 | 1.2 |
| Olive 14 | 28.4 | 6.9 | 182.5 | u |
| Olive 15 | 12.0 | 7.2 | 9.8 | u |
| Olive 16 | 9.2 | 6.8 | 79.5 | 4.0 |
| Olive 17 | 8.9 | 6.9 | 93.8 | 2.4 |
| Canola 1 | 21.9 | 5.3 | 497.0 | u |
| Canola 2 | 16.3 | 5.6 | 672.8 | 1.8 |
| Peanut 1 | 15.9 | 5.7 | 216.3 | u |
| Flax 1 | 6.3 | 4.3 | 3472.0 | 17.9 |
| Sunflower 1 | 18.3 | 5.9 | 212.1 | 4.2 |
| Sunflower 2 | 12.3 | 5.9 | 187.1 | 3.4 |
| Sunflower 3 | 28.0 | 6.0 | 181.3 | 1.8 |
| Sunflower 4 | 22.0 | 5.6 | 181.3 | 0.6 |
| Sunflower 5 | 32.4 | 5.6 | 229.2 | 1.4 |
| Sunflower 6 | 22.7 | 5.2 | 190.3 | 1.0 |
| Sunflower 7 | 16.0 | 5.3 | 183.3 | 0.6 |
| Sunflower 8 | 22.5 | 4.8 | 168.6 | 0.2 |
| Sunflower 9 | 14.6 | 4.9 | 271.0 | 0.2 |
| Grapeseed 1 | 15.6 | 5.3 | 240.0 | 0.4 |
| Sesame 1 | 13.9 | 4.9 | 182.3 | 0.8 |
| Sesame 2 | 17.5 | 5.6 | 503.4 | 1.8 |
| Mean | 14.7 | 5.8 | 264.9 | 2.5 |
| SD | 7.0 | 0.7 | 594.5 | 3.5 |

Each concentration is a mean of 6 values. The MUFA are olive, canola and peanut oils; the PUFA are flax, sunflower, grapeseed and sesame oils. u = concentration cannot be determined; ¹ = μmol/g; ² = nmol/g oil.

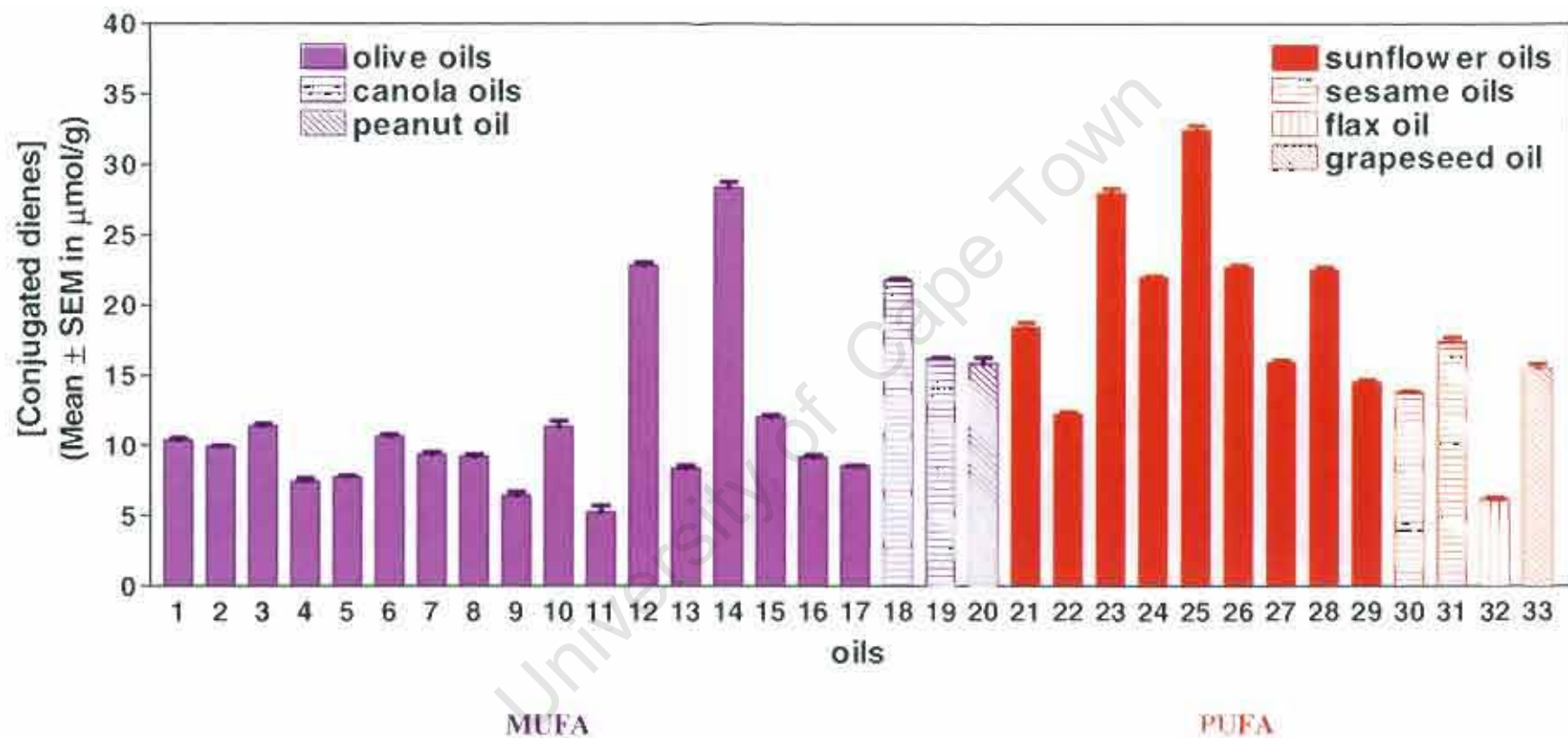


Figure 2.2. CD in edible oils, MUFA and PUFA; sample 24 is sunflower oil sample 4.

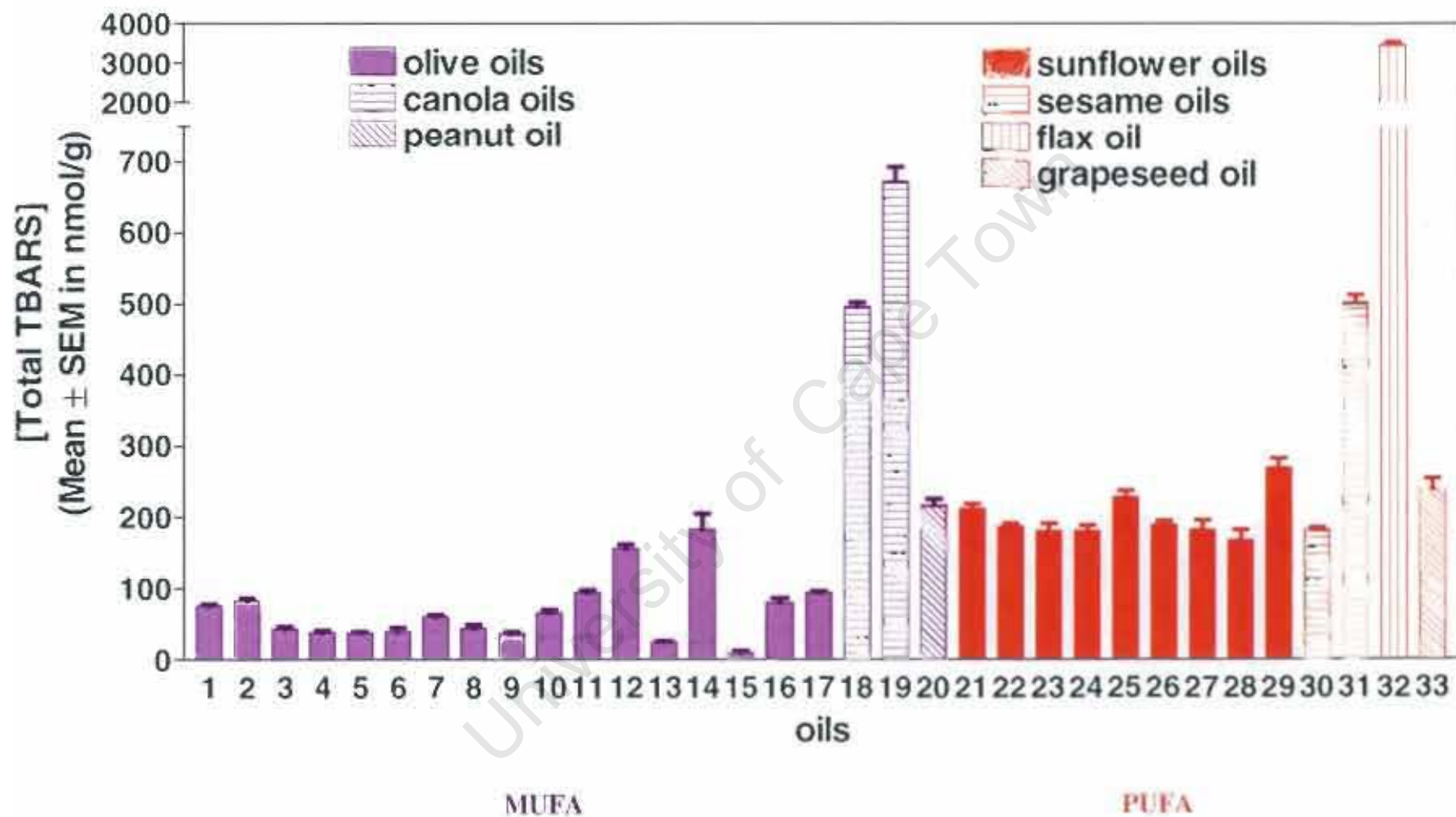


Figure 2.3. TBARS in edible oils, MUFA and PUFA; sample 24 is sunflower oil sample 4.

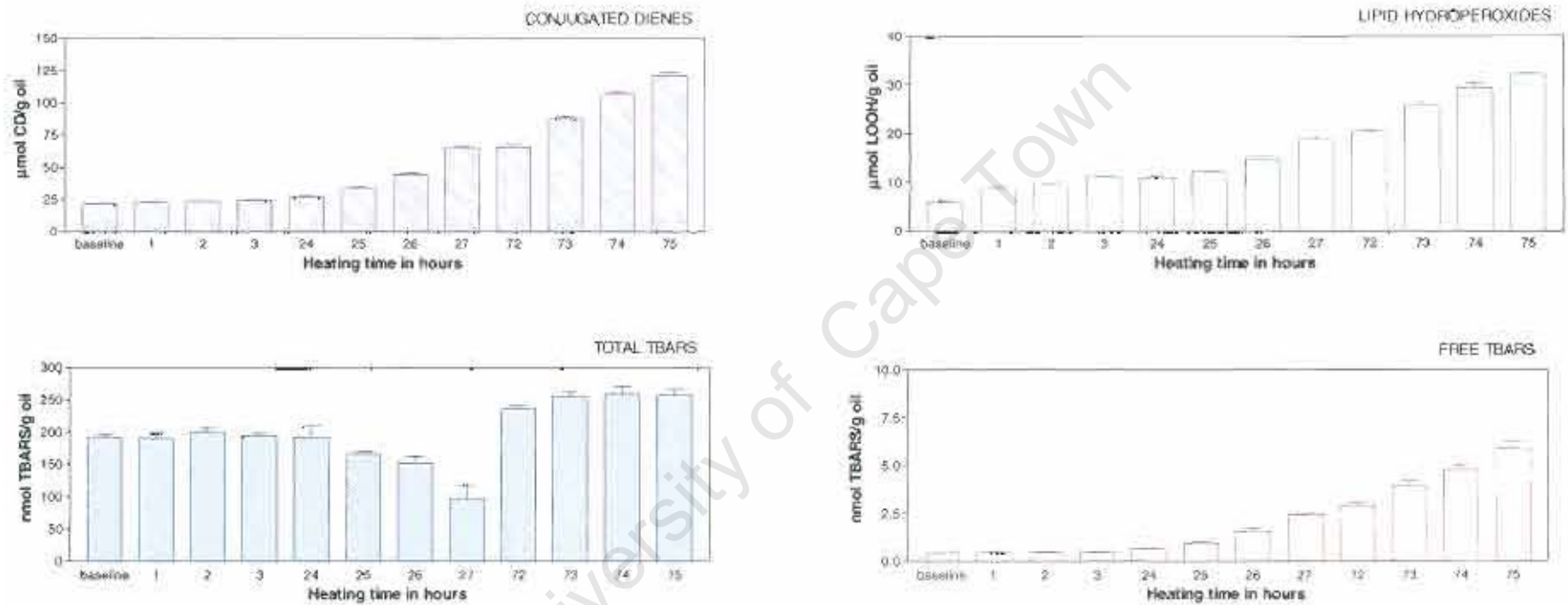


Figure 2.4. Concentrations of peroxidation products in re-heated PUFA oil over 75 hours. Results are expressed as mean \pm SEM of triplicates.

2.3.3. Lipid peroxidation in thermally-stressed oil heated with copper and red wine: the oil-boil method

Nine different red wines were tested for their antioxidant capabilities in protecting thermally-stressed PUFA sunflower oil against lipid peroxidation. These wines are listed in Table 2.4.

Table 2.4. Red wines used in the macro and micro oil-boil experiments

| Sample | Cultivar | Vintage |
|--------|--------------------|---------|
| 1 ▪ | Chateau Libertas | 1982 |
| 2 | Shiraz | 1984 |
| 3 Δ | Merlot | 1991 |
| 4 Δ | Merlot | 1994 |
| 5 ▪ | Pinotage | 1996 |
| 6 | Shiraz | 1996 |
| 7 | Cabernet Sauvignon | 1997 |
| 8 * | Chateau Libertas | 1997 |
| 9 Δ | Merlot | 1999 |

* used to determine inter-bottle variation

Δ used to determine variation between different vintages of the same cultivar

▪ used to determine the effect of selective ageing of wine on antioxidant capacity

The intra- and inter-assay CVs respectively from the combined macro- and micro-analysis of the antioxidant capability of red wines were as follows: CD < 2% (intra-assay) and < 4% (inter-assay); LOOH < 4% and < 10%, tTBARS < 4% and < 12% and fTBARS < 2% and < 4%. A scatter plot was used to determine the correlation between CD and LOOH from the macro-method (Figure 2.5). There was good correlation: $r^2 = 0.62$ ($P = 0.024$). The antioxidant capabilities of the red wines using the macro- and the micro-methods were therefore compared using CD only.

2.3.3.1. The macro –method

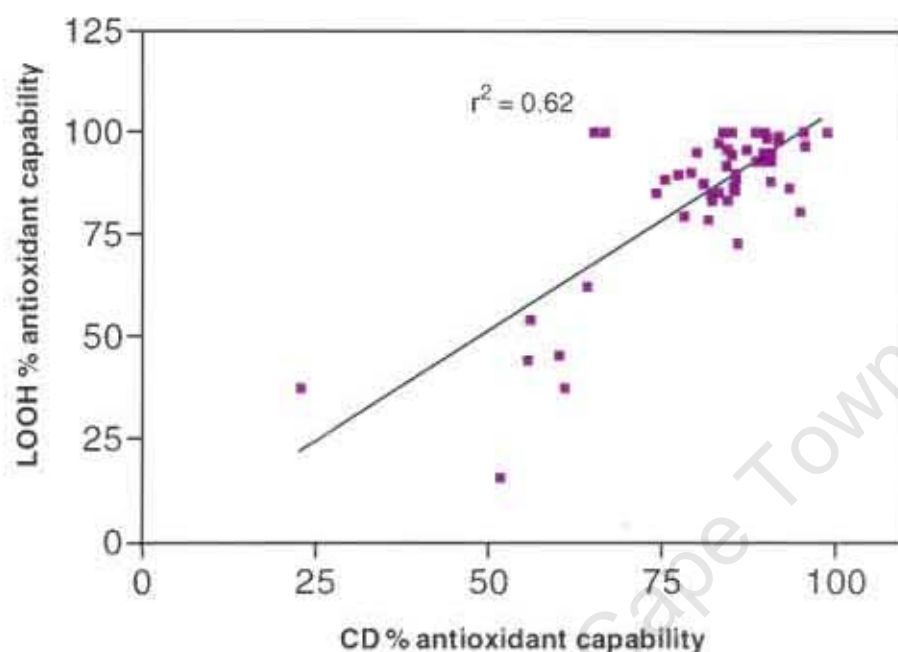


Figure 2.5. Correlation between CD and LOOH using % antioxidant capability of red wines from the macro oil-boil method; $r^2 = 0.62$, $P = 0.024$.

The protective effect of red wine against lipid peroxidation in thermally-stressed sunflower oil was determined, and the cumulative results are shown in Figure 2.6, expressed as percent change, mean \pm SD, of the concentration of specific product, relative to the readings of the uncooked oil. The baseline values of the oil are those determined when the oil just reached 100°C. Heating the oil in a pseudomonophase with deionised H₂O as a control showed the following percent differences compared with heating oil alone: CD -4%, LOOH +23% and total TBARS +0.2%. Conjugated dienes, LOOH and TBARS were determined for the macro-method. The micro-method CD results are shown in the block.

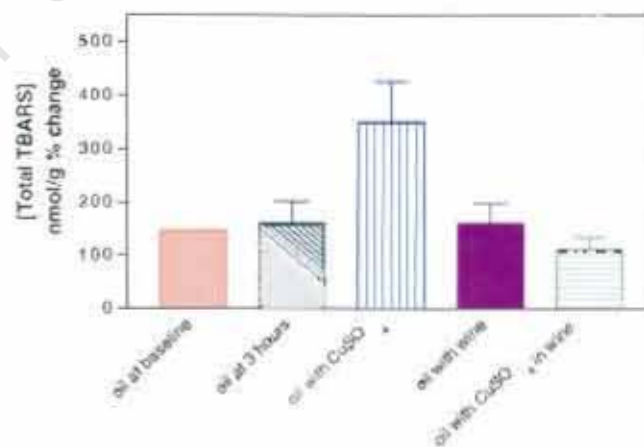
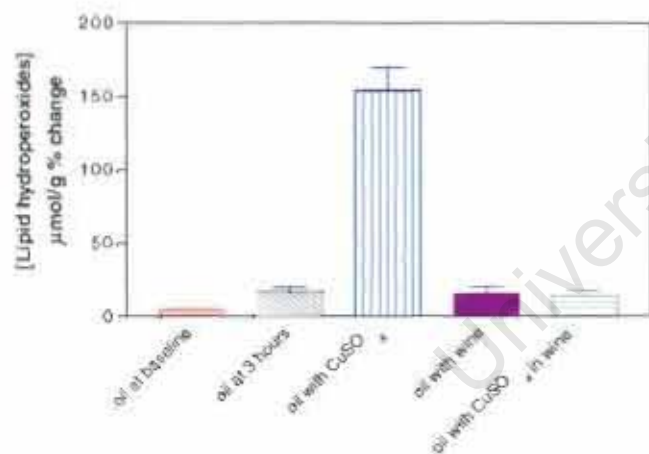
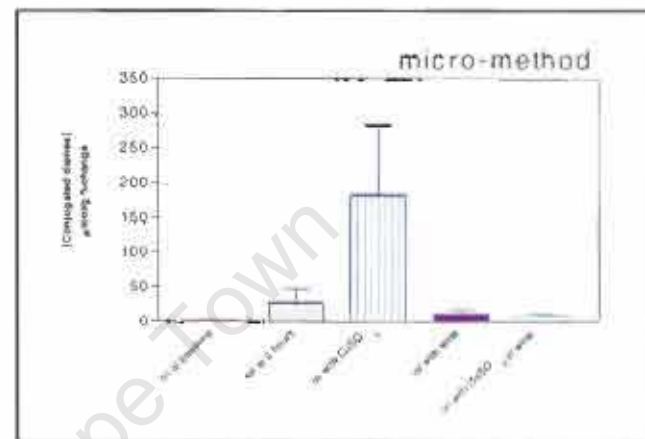
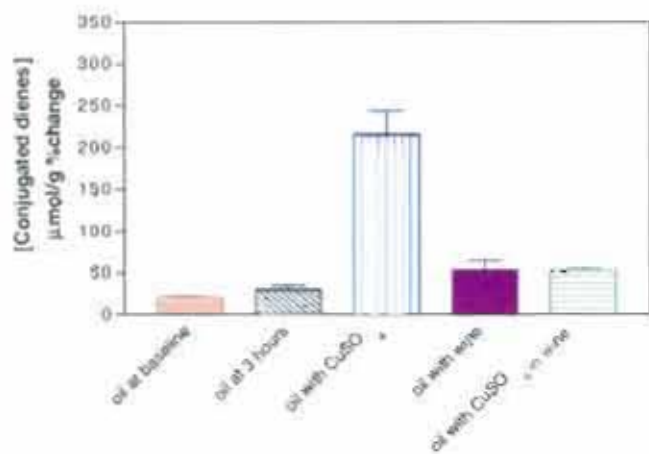


Figure 2.6. The antioxidant effect of 9 red wines on heated sunflower oil as shown by the macro-and micro-methods: mean \pm SEM.

A comparison of the inhibition of CD, LOOH and TBARS formation in thermally-stressed sunflower oil by red wine, measured by % antioxidant capability by the macro-method, is shown in Table 2.5.

Table 2.5. Antioxidant capability of 9 red wines measured by their protection against the increase of the specific peroxidation products during the macro oil-boil.

| n = 9 | Macro-method ¹ |
|--------|---------------------------|
| CD | 83.8 ± 2.8 |
| LOOH | 89.6 ± 8.2 |
| tTBARS | 79.1 ± 31.1 |
| fTBARS | 79.2 ± 17.2 |

¹ = % antioxidant capability relative to copper, mean ± SD

The results of selective ageing of 2 of the wines, relative to the freshly-opened wine, showed little change of antioxidant capacity, with an average $6.2 \pm 15.3\%$ decrease of the antioxidant strength measured for CD, an increase of $1.6 \pm 2.2\%$ for LOOH, and an average decrease for tTBARS of $17.3 \pm 20.2\%$ by 6 weeks. Inter-bottle variation of the same cultivar and vintage showed little change of CD and LOOH, with the CD ranging from 77–91%, LOOH 73–95%, tTBARS 22–100% and fTBARS 0–100%. Five different vintages of Merlot also showed little change, with CD ranging from 81–95%, LOOH from 77–100%, tTBARS from 59–100% and fTBARS from 56–96%.

2.3.3.2. The micro-method and the ORAC determination

Table 2.6 shows the mean (\pm SD) ORAC values of 9 red wines and also shows the correlation with the antioxidant capabilities of those wines, measured by the macro and micro oil-boil methods. The ORAC values ranged from 17.5 to 34.0 mmol/L TE. There was no significant correlation between ORAC and the macro- or the micro-methods ($r^2 = 0.13$, $P = 0.348$ and $r^2 = 0.06$, $P = 0.523$ respectively). There was little correlation

between the CD as determined by the macro and the micro-methods themselves, $r^2 = 0.001$, $P = 0.934$ (Figure 2.7).

Table 2.6. Mean \pm SD and correlation of ORAC, macro and micro oil boil CD results from 9 different samples of red wine.

| n = 9 | ORAC ^a | Macro ^b | Micro ^b |
|--|-------------------|--------------------|--------------------|
| 1 | 17.5 | 85 | 99 |
| 2 | 18.6 | 81 | 83 |
| 3 | 29.1 | 84 | 92 |
| 4 | 24.5 | 85 | 81 |
| 5 | 34.0 | 79 | 98 |
| 6 | 24.1 | 84 | 96 |
| 7 | 21.4 | 87 | 90 |
| 8 | 24.9 | 88 | 90 |
| 9 | 23.6 | 81 | 82 |
| Mean \pm SD | 24.2 \pm 5.1 | 83.8 \pm 2.8 | 90.1 \pm 6.9 |
| Correlation with ORAC: r^2 | | 0.13 | 0.06 |
| P value | | 0.348 | 0.523 |

^a = mmol/L trolox equivalents

^b = % antioxidant capability relative to copper, as determined by CD

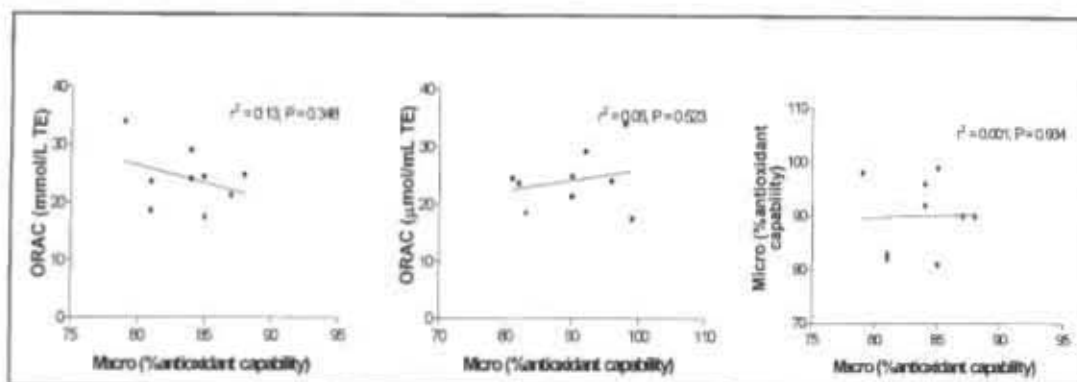


Figure 2.7. The correlation between the macro and the micro oil–boil assays and their correlation with the ORAC of the 9 red wines used in the oil–boil assays.

2.4. Discussion and conclusions

Foods that contain PUFA are susceptible to lipid peroxidation, particularly during cooking, when thermal–stressing of the fatty acids may cause the production of potentially toxic compounds such as CD, LOOH and TBARS. Edible oils containing PUFA are used universally, and there are concerns that the heating of these oils, especially in the fast–food industry, may result in detrimental health effects such as atherosclerosis and cancer. Red wine, with its antioxidant polyphenols, may abrogate these effects, either during its cooking with oil *in vitro*, or while protecting food such as meat when it is cooked after having been marinated in wine (Chapter 3).

The methods that are used to evaluate the process of lipid peroxidation in foods are very often non–specific, insensitive and unreliable (Section 1.4.4). The main aim of this study was to use a simple, novel method to evaluate the antioxidant capacity of 9 different locally–produced red wines, 5 different popular cultivars of different vintages, in their ability to protect against lipid peroxidation in a simulated cooking environment using thermally–stressed PUFA sunflower oil. Because flavonoid concentrations have previously been found to decrease during heating of wine (331), and also because food, excluding fats, generally contains water, care was taken not to heat the oils in this study to over 100°C. This was one of the problems with the micro oil–boil method (discussed below). The advantage of the oil–boil method, in particular the macro–method, is that it is fairly robust, uses inexpensive, easily–available chemicals, and standard laboratory equipment, it determines total antioxidant capacity, and the temperature used during the

assay is relevant to cooking conditions where water is involved. The macro-method, although more reproducible, is not able to process as many samples as the micro-method. The latter, however, was very difficult to control for temperature, because a number of different samples with different conditions were heated in the same heating block. Another factor to consider is that deionised water is used in the laboratory, while water used for cooking elsewhere may contain chlorine (used in the purification of water) or metal ions such as Fe^{++} , which may affect the peroxidation of the lipids in food. A control of oil heated with deionised H_2O showed no protection against the production of lipid peroxidation products, evidence that the observed antioxidant properties may be attributable to the wine. It was difficult to maintain consistent stirring of the samples with magnets during the micro-method, because different positions in the heating block promoted different stirring rates. Reducing evaporation in the macro-method samples, as was done with the micro-method samples, where glass marbles were used, was difficult owing to the rather large surface area (2206 mm^2).

There was variation in the inherent peroxidation status within the same classes of edible oils, as can be seen in Table 2.2. Samples 12 and 14 of olive oil, for example, had much greater concentrations of CD and tTBARS than the average olive oil samples (approximately 3 and 2.5 times higher respectively), as did flax oil sample 1, which had a far higher concentration of tTBARS and fTBARS than the average PUFA concentrations (approximately 7 and 6 times higher respectively). The current trend for favouring olive oil might therefore not necessarily be good for avoiding peroxidation products. There are a number of factors that could influence the peroxidation status of an oil: the fatty acid composition, the different oil processing methods, different storage conditions (pre-processing (seeds), and post-processing as well as during the processing), and different chemicals added to the oils such as tocopherols.

Heating sunflower oil at 100°C intermittently over 75 hours allowed the determination of the extent of peroxidation during moderate heating conditions, relevant to re-heating of oil 3 times (the popular recommended number). The concentration of all the products increased over 75 hours relative to the baseline, with little change during the first 3 hours, except for the LOOH, which increased by 86%. Free TBARS increased the most over 75 hours (1080%), although at low concentrations relative to the tTBARS. The CD and

LOOH increased by approximately the same amount (447% and 435% respectively), while the tTBARS increased the least (34%), owing perhaps to their decrease in concentration between 24 and 27 hours, due possibly to increased evaporation of these volatile products. This depletion of tTBARS was short-lived however, and their concentration had increased again by 72 hours.

The 9 wines tested showed a consistently powerful antioxidant capacity for both the macro and the micro oil-boil methods, for all the peroxidation products for the macro-method (79.1–89.6%) and for CD for the micro-method. Ageing did not seem to change the protective ability of the 2 wines tested nor was there much variation between bottles of the same cultivar and vintage, for the CD and LOOH. Different vintages of the same cultivar also showed little change. It would be interesting to compare the antioxidant results of white wines, beer and spirits, with those of the red wines. There was little correlation between the antioxidant capacities of the wines as tested by the ORAC method and the macro- and micro-methods, although the wines showed little variation for both oil-boil assays. It is possible that diluting the wine might show bigger differences. The number of samples was low, and it is possible that a larger number of wines would show a better overall correlation between the macro- and the micro-methods. The low correlation with the ORAC values is possibly just evidence that the methods for determining antioxidant capacity are often very different, and measure different parameters of the peroxidation process. The ORAC assay measures the ability of an antioxidant to scavenge peroxy radicals, while the macro- and micro-methods rely on the ability of the wine to chelate the copper, which would otherwise promote peroxidation. A previous study compared 3 methods for determining total antioxidant capacity of serum, the ORAC, and the TEAC and FRAP assays, and found that there was no correlation between the ORAC and the TEAC or between the FRAP and TEAC assays (294).

The relevance of these oil-boil assays to cooking conditions makes them appealing for testing food. Their relevance might allow the testing for antioxidant capacity in a range of aqueous foods, even homogenates of water-based foods, and not merely beverages. A whole new avenue of nutritional investigation for lipid peroxidation could follow from applying this method.

CHAPTER 3. THE EFFECTS OF MARINATING RED MEAT WITH RED WINE

3.1. Background

Man is the only animal that consumes food exposed to higher than physiological temperatures. This is probably a relatively recent evolutionary phenomenon. Previously, cooking may have had an advantage owing to prevention or limitation of microbial species. Longer life expectancy now may bring out adverse effects of lipid peroxidation in foods. Atherosclerosis may be significantly influenced not only by dietary fat and plasma lipoproteins, but also peroxidation status. This stimulated the investigation of peroxidation in meat and the effect of marinating in an antioxidant beverage.

Wine is often used as one of the main ingredients in the marinating of meat. One of the purposes of the marinade is to enhance the flavour of the meat, and the alcohol and acid in the wine also serve to tenderise the meat.

Meat, a complex food, undergoes significant biochemical changes during processing, storage and cooking. Brown *et al* showed that cooking procedures can dramatically affect the lipid peroxidation of meat (332). They compared the cooking of beef at low (100°C for 20 minutes) and high (250°C for 22 minutes) temperatures and found that the MDA concentration in the meat following the low temperature cooking was 3 – 4 times higher than in the meat exposed to the high temperature. This they attributed to the higher degree of volatilisation of MDA at high temperatures or the increased formation of non-TBA reactive MDA-protein adducts. Lipid peroxidation is in itself a complex process involving the degradation of PUFA via the production of products such as CD, LOOH and TBARS including MDA (333), that are considered harmful to human health (322,323) (Section 1.4.4). The products may cause colour, nutritional and flavour deterioration (rancidity) of foodstuffs. Minamoto *et al* (310) reported that ingestion of products of autoxidised linoleic acid stimulated lipid peroxidation in rat liver. Studies on the mechanisms of lipid peroxidation in meat are most often directed towards cooked rather than raw meat (334), because uncooked meat does not undergo peroxidation as readily. Cooking promotes this process, mostly by the release of free ferric iron, which acts as a pro-oxidant (335). There is still no consensus on the role of haem-iron versus non-haem iron. Liu concluded that the catalytic activity for peroxidation in beef

homogenate was due to both types of iron (336,337). An increase was found in non-haem iron after cooking, believed to parallel the decrease of haem iron when the haem molecule from myoglobin, the red meat muscle pigment, is broken down during cooking (338).

Several factors have been found to influence the rate and degree of lipid peroxidation in meat. The pre-mortem factors include animal species (339), sex (334) and age of the animal. Type and quantity of the animal feed are also important and grass or pasture feeding has been found to increase the concentrations of n-3 unsaturated fatty acids (including linolenic acid) and conjugated linoleic acid (CLA) in beef, compared with concentrate feeding (340,341). CLA is formed during reticulorumen hydrogenation of linoleic acid and through endogenous desaturation of *trans*-11 octadecaenoic acid (342). CLA, although a CD, has been shown to have anti-atherogenic, anti-carcinogenic and anti-thrombotic properties (343), with rumenic acid (the *cis*-9, *trans*-11 isomer) being the main naturally-occurring CLA in food.

Post-mortem factors include the type of muscle studied. Wilson *et al* found that red muscles are more susceptible to lipid peroxidation than white muscles (344). Badiani *et al* found that the peroxide value was lower in the *infraspinatus* beef muscle compared with the *semitendinosus* muscle (345). Conditions during storage, such as the temperature, time and oxygen concentration, are also important. A temperature of 4°C has been found to be the most important factor for keeping lipid peroxidation to a minimum, compared with oxygen concentration and storage times (346). A correlation between the meat haem-protein pigments, myoglobin (major) and haemoglobin (minor) and lipid peroxidation has been shown (347). Packaging methods, for example aerobic or vacuum packaging, are also important, with packaging materials often being permeable to oxygen. Levels of oxygen in the packaging correlate with the extent of oxidation of the meat, and vacuum packaging, with its lower oxygen concentration (approximately 1%), can greatly extend the shelf-life of raw beef (348). The concentration of volatiles, for example butane, pentane, hexane, heptane and octane, increases with storage in aerobically packaged meat and there is a positive correlation with these volatiles and TBARS (339). Irradiation of meat has been found to generate free radicals (349).

Increased concentrations of TBARS have been found in irradiated meat compared with non-irradiated meat (339).

The degree of unsaturation of the fatty acids plays an important role in the peroxidation of meat (338,339). In beef, lipids constitute approximately 10% of the mass, of which almost one half is unsaturated. Beef lipid comprises 41–86% saturated fatty acids, 4–54% monoenoic, 1–5% di- and trienoic, and 0–0.3% tetra- and hexaenoic fatty acids (326). Phospholipids, in particular phosphatidylethanolamine and to a far lesser extent phosphatidylcholine, are the major molecules that undergo lipid peroxidation in cooked beef. Phospholipids are known to possess a PUFA on *sn*2 (Section 1.4.1). Triacylglycerol also undergoes lipid peroxidation, but to a lesser extent (338). Oleic, linoleic and arachidonic acids make significant contributions to increased rancidity in cooked beef (350). The presence of pro- and antioxidants (both endogenous and exogenous) is important. High concentrations of vitamin E (provided by grass grazing for example) have been found to inhibit lipid peroxidation (351). Nitrite acts as a potent antioxidant in cooked meat systems (338). Cooking practice introduces varying times and temperatures that will influence lipid peroxidation. Microwave cooking has been associated with less peroxidation of meat lipids than oven roasting (345) and also produces fewer fatty acid compositional changes than broiling (352).

The aim of this study was:

- i) to determine whether marinating with red wine protects red meat against lipid peroxidation by analyzing CD, LOOH and TBARS in control and marinated meat cooked in a microwave oven.

3.2. Methods

3.2.1. Study design

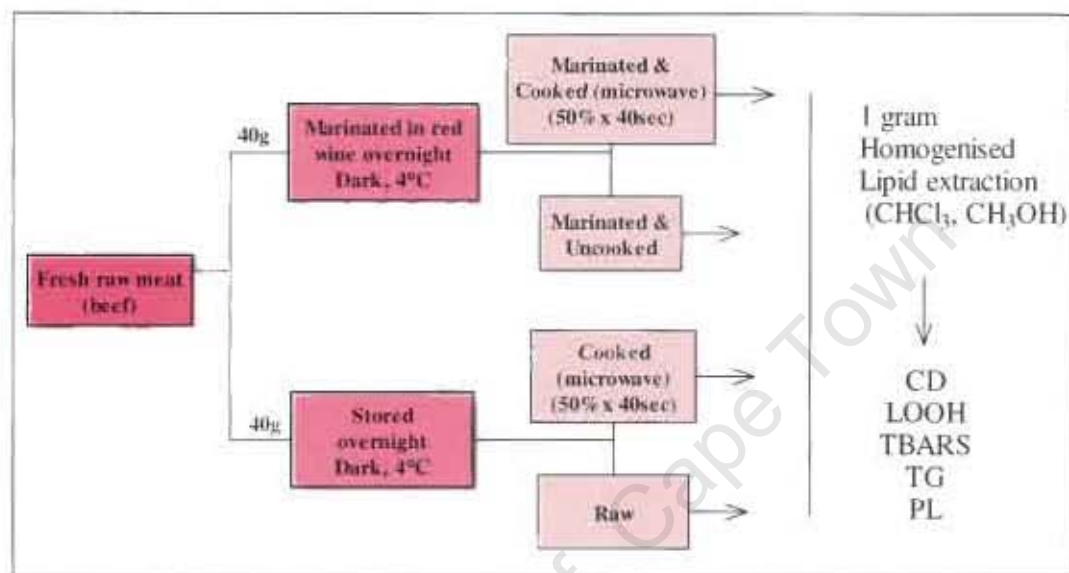


Figure 3.1. Experimental design of the meat preparation and subsequent analysis.

Twenty five different samples of fresh, unfrozen beef were purchased from two retail outlets. The samples had been stored in the shop at 4°C in a polystyrene base with a clingfoil wrapping. They included various cuts of beef for example porterhouse steak, rump steak and beef fillet and were trimmed of all surface adipose tissue. Two portions of 40g each were cut from each sample of meat, taking care that they had a similar appearance in size and content, with the exclusion of tissue other than striated muscle. One portion was marinated in 70mL red wine at 4°C in the dark for 24 hours, while the other portion was stored under the same conditions, but unmarinated. The South African red wines used in the study all had 12–14% alcohol by volume. After marinating, each portion was divided into 2 x 20g samples, resulting in 4 x 20g samples from each original piece of beef. One sample from each of the marinated and unmarinated portions was cooked in a microwave oven (output power 1000W, frequency 2450 MHz, Model R-341B, Sharp, Thailand) for 40sec at 50% power, sufficient to cook but not over-cook the

meat. Microwave cooking was selected for its moderate and reproducible cooking conditions. The time of cooking was established by asking laboratory staff not involved in the project, to declare an agreeable taste. Each sample was subsequently placed on ice for 5min to terminate the cooking reaction. The microwaving procedure resulted in a core temperature of $90 \pm 3^{\circ}\text{C}$ (determined by means of a digital thermometer: Digitron thermocouple, with a Physitemp micro-probe) immediately after cooking.

3.2.2. Meat samples

From each of the samples, a 1g portion was cut from the centre and minced finely with scissors and then finely ground in 3mL phosphate buffer (75mmol/L, pH 7.4 containing 0.5mmol/L EDTA and 1.5mmol/L NaN_3) with a mechanical homogenizer (VirTis handishear). Aliquots of 200 μL from each sample were frozen at -20°C under nitrogen for gas chromatography (GC) analysis of fatty acid methyl esters. Lipids were extracted from the remainder of the sample by the method of Bligh & Dyer (353), a modified method of Folch *et al* (354), detailed in the Appendix. Briefly, lipids were extracted into a monophasic mixture of chloroform and methanol, which was finally separated into two phases by the addition of chloroform and phosphate buffer: an upper aqueous phase containing the non-lipid compounds and an organic phase containing the purified lipids. The organic layer was dried under nitrogen gas and the lipid extract was then re-suspended in 200 μL chloroform and divided into 10 x 20 μL aliquots in micro-reaction vials. The extracts were again dried under nitrogen headspace as above and stored at -20°C for lipid and lipid peroxidation analyses.



Figure 3.2. A hand-held handishear used for homogenising meat.

3.2.3. Lipid analyses

Meat TG and PL concentrations were determined using enzymatic colorimetric kits, as described in the Appendix. The concentration of TG and PL was determined from known standards supplied in the kits. The inter-assay CVs for both TG and PL methods were < 3%.

3.2.4. Lipid peroxidation analyses

Twenty five meat samples were analysed in the raw, cooked and cooked, marinated state. For technical reasons, not all 25 samples were completely analysed for raw marinated state, but at least 17 samples were analysed at all the stages. Concentrations of lipid peroxidation products were measured in meat lipid extracts that had been dried, by the following spectrophotometric methods using a GBC UV/VIS analyser (CD) and Labsystems Multiskan MS Analyser (LOOH and TBARS). Concentrations were

calculated using the appropriate molar extinction coefficients. The methods are detailed in the Appendix.

Briefly, CD were measured at 234nm after appropriate dilution in cyclohexane. The inter-assay CV was < 2%.

To determine LOOH, lipid extracts were assayed in the presence of xylenol-orange and Fe^{2+} in the FOX assay. The inter-assay CV was < 5%.

TBARS were measured by reaction with TBA in the presence of ferric chloride in a glycine buffer at pH 3.6 containing sodium dodecyl sulphate. The inter-assay CV was < 12%.

3.2.5. GC analysis

The fatty acid methyl ester (FAME) analysis was adapted from the method described by Christie (355). The sample was placed in a stoppered tube and dissolved/suspended in 1ml toluene, after which an internal standard (C17:0, Sigma Chemical Co, St Louis, MO, USA) was added. Two ml methanolic hydrochloric acid (5% HCl v/v) was added and the sample was left overnight in a waterbath at 50°C. After removal from the waterbath, 5ml NaCl solution (5% w/v) was added before extracting the esters twice with 5ml n-hexane. The hexane was then washed with 4ml potassium bicarbonate solution to remove unesterified fatty acids and dried over anhydrous sodium sulphate after which it was evaporated under a stream of nitrogen. Once the sample was dry it was reconstituted in n-decane and injected into the GC for analysis.

GC analysis was performed on a Varian CP-3800 gas chromatograph (Varian Analytical Instruments, Walton-on-Thames, UK). The gas chromatograph was fitted with a 30m FAMEWAX column (Restek Corp, Bellefonte, PA, USA) ID 0.32mm and film thickness 0.25µm. Helium was used as carrier gas at a constant flow rate of 2ml/min. The injector and flame ionisation detector temperatures were set at 250°C. The column oven temperature was programmed as follows, 155°C held for 2 minutes, then ramped to 180°C at a rate of 5°C/min from where it was ramped up to 240°C at 10°C/min where it was held for 7 minutes. Split injection was used with a split ratio of 1:5. Identification of fatty acid methyl esters was done by comparing retention times to that of known

standards (Sigma Chemical Co, St Louis, MO, USA). Additional unsaturated fatty acid identification was derived from the formula for equivalent chain lengths, using retention times for C16:0 and C18:0 (356).

3.2.6. ORAC determination of red wine

The method as described by Ou *et al* (299), and detailed in the Appendix, was used. Briefly, the wine samples were diluted in phosphate buffer pH = 7.4. Fluorescein and AAPH were added and the reactions were allowed to go to completion, after which they were compared with a standard reaction of Trolox. The results were expressed as mmol/L TE.

3.2.7. Statistical analyses

Data are expressed as mean \pm SD. All statistical analyses were performed with the use of Graphpad PRISM software (version 3, San Diego, USA). The Student's t test analysis for paired values was used for normally distributed data. The Mann-Whitney test was used to compare the raw and marinated raw data. The Wilcoxon matched pairs test for nonparametric data was used to compare ratios of UFA to SFA in the small number of samples analysed by GC. Statistical significance was accepted at $P < 0.05$.

3.3. Results

3.3.1. Lipid analyses

Per g of raw meat, the mass of total PL was 2.6 ± 1.2 mg and TG 1.1 ± 0.5 mg. After cooking, the concentrations increased significantly to 4.2 ± 1.5 and 1.4 ± 0.6 mg/g ($P < 0.0001$ and $P = 0.0005$ respectively).

3.3.2. Lipid peroxidation analyses

The lipid peroxidation parameters displayed large variation. All the measurements were retained for analysis.

Although not the primary interest of the investigation, the raw unmarinated meat was compared with the raw marinated meat for lipid peroxidation products. The results given in Table 3.1 indicate a significant decrease in CD after marinating raw meat. Per mg of lipid, raw meat contained the following: CD 113.2 ± 56.7 , LOOH 51.0 ± 68.3 and TBARS 23.2 ± 21.4 nmol. Marinating lowered the CD to 69.9 ± 33.9 nmol/mg lipid (-38.3% change), LOOH 43.2 ± 55.2 nmol/mg lipid (-15.3% change) and TBARS 22.1 ± 17.9 nmol/mg lipid (-4.7% change) ($P = 0.009, 0.868$ and 0.918 respectively). Cooking altered the peroxidation status of unmarinated meat in nmol/mg lipid: CD 124.3 ± 111.2 , LOOH 54.5 ± 95.7 and TBARS 39.2 ± 47.3 ($P = 0.441, 0.716$ and 0.031 respectively) whereas cooked, marinated meat displayed the following results: CD 86.8 ± 46.1 , LOOH 40.0 ± 55.8 and TBARS 31.8 ± 32.6 nmol/mg lipid, as seen in Table 3.2. The trend to lower CD by marinating was not significant ($P = 0.057$). There were no differences in the LOOH and TBARS ($P = 0.172$ and $P = 0.209$ respectively).

Table 3.1. Lipid peroxidation products and their changes in raw meat, with and without marinating.

| nmol/mg lipid | Raw unmarinated n = 25 | Raw marinated n = 17 | P value |
|---------------|---------------------------|-----------------------------|---------|
| CD | 113.2 ± 56.7 | 69.9 ± 33.9 (-38.3%) | 0.009 |
| LOOH | 51.0 ± 68.3 | 43.2 ± 55.2 (-15.3%) | 0.868 |
| TBARS | 23.2 ± 21.4 | 22.1 ± 17.9 (-4.7%) | 0.918 |

The difference of the mean values is given in parentheses.

Table 3.2. Lipid peroxidation products and their changes in cooked meat, with and without marinating.

| nmol/mg lipid n = 25 | Cooked unmarinated | Cooked marinated | % change ¹ (% change based on the mean) | P value |
|-------------------------|--------------------|------------------|---|---------|
| CD | 124.3 ± 111.2 | 86.8 ± 46.1 | -14.2 ± 26.7 (-30.2%) | 0.057 |
| LOOH | 54.5 ± 95.7 | 40.0 ± 55.8 | 46.8 ± 187.9 (-26.6%) | 0.172 |
| TBARS | 39.2 ± 47.3 | 31.8 ± 32.6 | -5.0 ± 53.4 (-18.9%) | 0.209 |

¹ = calculated as individual % changes of the 25 samples. The difference of the mean values is given in parentheses.

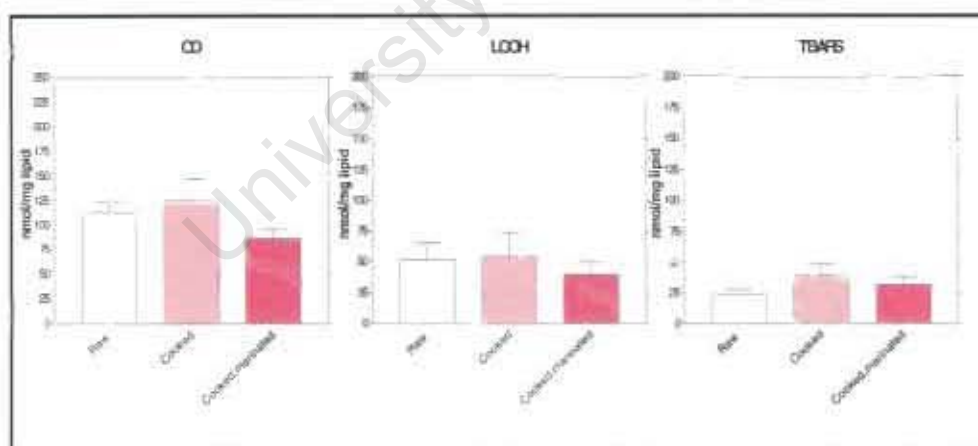


Figure 3.3. Lipid peroxidation products, CD, LOOH and TBARS, in beef after cooking with and without wine marinating. Values are in nmol/mg lipid, mean ± SEM (n = 25).

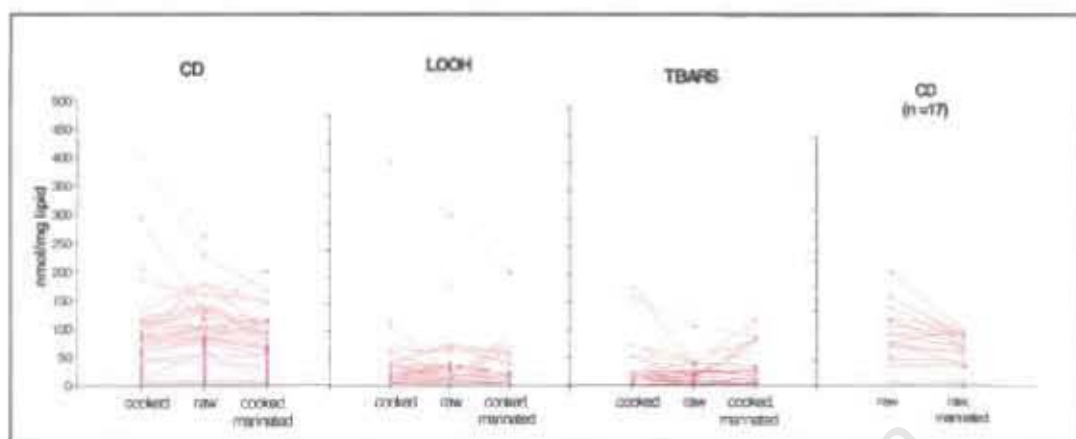


Figure 3.4. Individual lipid peroxidation products CD, LOOH and TBARS in beef, indicating changes from the raw state during cooking without and with marinade. The same samples of meat are labeled similarly for all 3 products.

3.3.3. Comparison of unsaturated fatty acids to saturated fatty acids by GC

When large amounts of UFA are removed from the samples by oxidative damage, the ratio of long chain PUFA : SFA will decrease. Owing to the low amounts of C20 and C22 UFA as well as their relatively poor separation on the column, only the ratios of C18:1, C18:2 and C18:3 were compared to C18:0. These results are shown in Table 3.3. There was no statistically significant difference in any of the UFA: SFA ratios. There was very little loss of PUFA from the meat into the marinade (< 0.5%). There appeared to be mainly longer-chain PUFA (C18–C24). No further analysis was done on this owing to the very low concentrations present.

Table 3.3. Comparison of the ratios of unsaturated fatty acids to saturated fatty acids by GC.

| | Raw | Cooked unmarinated | Cooked marinated | P value (cooked unmarinated vs cooked marinated) |
|-------------|-------------|--------------------|------------------|--|
| C18:1/C18:0 | 1.09 ± 0.07 | 1.13 ± 0.08 | 1.00 ± 0.12 | >0.99 |
| C18:2/C18:0 | 0.10 ± 0.01 | 0.08 ± 0.01 | 0.10 ± 0.03 | >0.99 |
| C18:3/C18:0 | 1.06 ± 0.05 | 1.08 ± 0.05 | 1.16 ± 0.07 | 0.50 |

Values are the medians ± SEM of 3 samples of meat each.

3.3.4. ORAC determination.

The ORAC values of the red wines used in the marinating of the meat samples ranged between 21 and 36 mmol/L TE, with a mean and SD of 29.2 ± 7.7 mmol/L TE.

3.4. Discussion and conclusions

An increase in PL and TG concentrations in the meat after microwave cooking was found in this study, as in the study described by Badiani *et al* (345), ascribed to the moisture loss caused by cooking. This is in contrast to Igene & Pearson (338), who found that cooking produced a significant decrease in total PL, and little difference in TG. Lipid extraction may be made more difficult by changes in consistency or by polymerisation. The method described in this chapter is able to homogenise very coarse samples so as to make for the best efficiency of extraction.

There were 2 main findings from this study. Firstly, there was wide variation in the inherent lipid peroxidation studies in the meat for all the products. Gas chromatography FAME analysis was not done systematically in the meat samples to relate the changes to fatty acid composition. Secondly, wine showed a trend towards lowering the CD in the

meat after it had been cooked. This appeared to be evident even after the marinating but before cooking.

As expected, cooking increased the concentration of all 3 peroxidation products, but only the TBARS were increased significantly ($P = 0.031$). A harsher form of cooking, for example oven roasting, might increase the production of lipid peroxidation products and make it easier to assess the impact of the antioxidant effects of the wine. The decrease in cooked meat of LOOH and TBARS, compared with those in marinated cooked meat, was not significant, although there was a trend to decrease the CD by marinating the meat in wine ($P = 0.057$). This seems to suggest a protective effect from the wine.

Red meat is commonly consumed and was therefore the meat of choice for this study. Poultry might resist lipid peroxidation due to having mainly MUFA, but fish is of interest due to having highly PUFA. However, it is not traditional to marinate chicken and fish, although the former may be cooked in wine. Meats are susceptible to fatty acid oxidation, due to relatively high concentrations of unsaturated fatty acids in membrane phospholipids as well as exposure to haem- and non-haem iron. Although the concentrations of oxidised lipids in foods are usually lower than that required to generate acute toxicity responses, the cumulative long-term effects of ingestion of small quantities of these compounds are not known (357). Lipid peroxidation is associated with flavour deterioration (358) and discolouration (334) in uncooked meat. The total lipids in beef constitute on average 10%. In this lipid fraction an average of 5% are PUFA (although in Italy this figure can be as much as 20% (359); 54% are monounsaturated and 41% are saturated (360). Phospholipids play a major role in lipid peroxidation of beef, compared with triacylglycerols which play a minor role (344).

Lean beef varies between 6-10% fat. The portions of beef selected for the study were lean fillet, and when 1g portions of the meat were subjected to the Folch method of lipid extraction, the mean \pm SD of the extracted lipid mass was 0.015 ± 0.005 g ($n=8$), or 15-25% of the anticipated fat mass. The 3.7mg TG + PL mass extracted from the meat was 25% of this extracted lipid. This loss was unexpected but was not specifically investigated, and cannot be explained. It is consistent throughout the extractions for all the meat samples and, therefore, does not influence the comparisons of lipid peroxidation products for raw, cooked and cooked marinated meat. Although the Folch extraction

procedure in our hands is quantitative, losses may have occurred. It is also possible that re-dissolution of fat was incomplete for an aqueous assay, despite this previously having been optimised. The PL assay is for phosphatidylcholine and may also therefore underestimate.

Other studies have found major differences in the quality attributes of beef (345,346,361). The different beef muscles vary considerably in their cooking properties, such as their cooking times. Also, controlling factors as the chill temperature and the time of ageing may reduce variation (362). The contact with oxygen, pre- and post-cooking, may also be responsible for the oxidative instability of cooked meat (348,363,364) and even when meat is cooked in an oxygen-free atmosphere, lipid peroxidation products can still be detected (348). Vacuum-packing is the most favourable method for packaging meat in order to reduce lipid peroxidation (364). The high variability in lipid peroxidation recorded in this study might warrant a study design including the tracking of the meat from the abattoir to the shop or the butchery.

True marinade recipes contain spices, oils and vinegar that can influence lipid content, change reactions with different pH and provide additional antioxidants. The effect of wine was specifically evaluated in this study. Its ability to penetrate the meat was not studied, but while it is possible that small molecules may diffuse in, it is unlikely that penetration of cell membranes may occur. Perforating the meat might have enhanced penetration, but it was not done because it is not traditional. The volume of marinade covered the meat, excluding oxygen owing to its poor solubility in water. No analysis was undertaken to assess the penetration of alcohol.

Oxidation of lipids in the meat may produce lipid hydroperoxides and aldehydes that may react with the meat protein. The aldehydes in particular are a group of very active products and they easily react with the amine groups of lysine, cysteine and glutathione, particularly in the presence of water, causing the formation of insoluble macromolecules such as 2,4,6-trimethyl-1,3,5-dithiazine and 2-pentyl-pyridine (326). The amounts of peroxidation intermediates measured in these studies may thus under-represent the peroxidation in that measurements apply only to these products in the lipid extract. No methods were set up to determine reacted peroxidation products with protein or nucleic acids.

The ORAC values of the wines used in this study were very similar to those used in the oil-boil study (29.2 ± 7.7 and 24.2 ± 5.1 mmol/L TE respectively).

The choice of microwave as a cooking method established reproducibility and a gentle and diffuse means of cooking the meat. Grilling is difficult to reproduce and results in layering of temperatures which are often extreme. The choice of cooking container is important, with copper and iron containers possibly providing pro-oxidative stress. In this setting, wine may chelate the pro-oxidant ions, as for example with the $10\mu\text{M}$ copper used in Chapter 2.

Variability is largely dependent on the meat and the storage conditions, and is possibly modified by wine during the marinating process but not much during cooking. Conjugated dienes, being the most stable, seem to be the most reliable product for studying the change in lipid peroxidation. The variability shown in Figure 3.4 indicates in general very little difference in the change from raw to cooked meat, with and without marinating. However, a few samples behaved very differently. For CD, the 2 highest raw sample displayed very different behaviour. These highly variable samples did not behave similarly with respect to other parameters. In 2 of the top 3 LOOH values, the marinade seemed to make a difference. The apparent impact on TBARS by marinade is less. Taken together, the trend that emerges is that CD and LOOH, being specific intermediates in the chain of events, may reflect actual peroxidation better than TBARS, which appear to reflect a reserve of oxidative PUFA yielding reactive scission products. This is in keeping with the lack of change in GC found.

Future studies in this field might include better compositional analysis of the meat, the use of post-abattoir samples and more intense cooking conditions.

CHAPTER 4. THE EFFECT OF THE CONCOMITANT CONSUMPTION OF RED WINE AND POLYUNSATURATED FATTY ACIDS IN EDIBLE OIL ON THE PEROXIDATION STATUS OF CHYLOMICRON LIPIDS AND THE PLASMA CATECHIN AND ORAC CONCENTRATIONS

4.1. Background

Atherosclerosis is a leading cause of mortality in the western world, and lipid peroxidation is an important factor in its pathogenesis (365). Red wine contains phenolic compounds that have antioxidant properties (264) that can inhibit *in vitro* oxidation of human low density lipoproteins (LDL) (187,366) and human high density lipoproteins (HDL) (367). Despite pro-oxidant effects ascribed to ethanol, van Golde *et al* (189) found that the polyphenols in red wine more than compensated, leading to a prolonged lag-time of copper-induced LDL oxidation. *Ex vivo* inhibition of LDL oxidation by red wine consumption with meals has also been demonstrated (190,368). Red wine polyphenols were reported to decrease lipid peroxidation in LDL isolated from subjects 3 hours after ingestion of a meal enriched with the antioxidant phenols (369). Hydrophilic antioxidants may spare lipophilic antioxidants. Reduced *ex vivo* LDL oxidation after consumption of red wine taken without meals was found by Covas *et al* (370). In contrast to these results, Sharpe *et al* (209) and de Rijke *et al* (371) found that LDL isolated from subjects after daily consumption of red wine for 10 days and 4 weeks respectively, showed no change in susceptibility to copper-induced oxidation.

The decrease in susceptibility of LDL to lipid peroxidation has been proposed as a reason for a lower incidence of atherosclerosis amongst red wine drinkers. Catechins are water-soluble flavonoids and include monomeric catechins such as (+)-catechin, the predominant flavanol in red wine. Catechins possess antioxidant properties (78,372) and exert a more potent antioxidant effect than flavonols and polymeric anthocyanidins as measured by prolongation of lag time in *in vitro* copper-induced peroxidation studies of LDL (373).

Chylomicrons (CM) are short-lived, large lipoproteins that contain products of fat digestion, which they transport in blood. Approximately 88% of their mass is TG, 8% is PL and 3% is cholesterol ester (374). Despite their high content of (dietary) TG that is

susceptible to lipid peroxidation, there have been few studies on the lipid peroxidation products in CM.

Postprandial impairment of endothelial function has been found to occur after consumption of meals rich in fat (237,375-377). Endothelial dysfunction is regarded as an important early event in, or marker of, atherosclerosis (378,379). A study by Williams *et al* (325) highlights the deleterious influence of lipid peroxidation products on endothelial function postprandially. This chapter describes the antioxidant effects of red wine on CM derived by a standardised protocol.

The objectives were:

- i) to compare the lipid peroxidation status of a PUFA-containing oil that was ingested by volunteers in the form of a milkshake with that of the lipids in the CM;
- ii) to compare the lipid peroxidation status and susceptibility of these CM to copper-induced peroxidation, with and without concomitant consumption of red wine. A range of spectrophotometric assays was used to describe early and later products of lipid peroxidation, as a single assay is not considered sufficient to act as marker for the process (285,380);
- iii) plasma ORAC and catechin concentrations were determined to establish whether antioxidant capacity or content was influenced by wine consumption in the postprandial phase.

This appears to be the first investigation of the effects of red wine on the lipid peroxidation status of CM as no such studies have been found in the scientific literature.

4.2. Methods

4.2.1. Study design

Fifteen healthy non-smoking volunteers who had no significant dyslipidaemia (confirmed by lipid biochemistry: Results) and who were not taking any medication or vitamin supplements, were recruited. There were 11 males and 4 females, with a mean age of 35 ± 8 years and a BMI of 25.5 ± 4.4 kg/m². They refrained from consuming any alcoholic beverage and from taking strenuous exercise for 24 hours before the start of the study and they fasted for 12 hours. Subjects ingested a fat-containing meal over 10

minutes randomly on days 1 and 3, with or without wine. The fat bolus was in the form of a milkshake according to the oral fat tolerance test (OFTT), with an equivalent lipid mass of PUFA (sunflower) oil in the place of cream (381,382). They ingested 70mL (64g) oil/m² body surface area (382,383), which contained 43g PUFA, 13g MUFA and 8g saturated fats: total 2380kJ. A time point of 3 hours was selected for studying CM as this is generally accepted as a reliable time for isolating CM and reflecting plasma catechins (384,385,63). The concentrations of CD, LOOH and TBARS in 6 samples of the PUFA oil were determined by the methods described below. The study used a commercially available blended red wine for which, owing to availability, 3 vintages were used. The alcohol by volume varied from 12–14%. The ORAC values were 24.8 ± 10.1 mmol trolox equivalents (TE)/L. The volume of wine consumed was calculated to deliver 0.35g alcohol/kg body mass. The study was approved by the University of Cape Town Research and Ethics Committee and written consent was given by the participants.

4.2.2. Blood samples

Blood samples were taken before and 3 hours after consumption of the meal with and without wine. Blood was taken by venepuncture into EDTA-containing (1g/L) vacutainer tubes and placed immediately on ice. Plasma was separated by centrifuging at 2000 x g for 15min at 4°C in a Beckman GS–6R centrifuge. Baseline and 3 hour plasma aliquots were stored under nitrogen at –20°C for future catechin and ORAC testing.

4.2.3. Lipid and lipoprotein studies

CM were separated from 3 hour plasma samples by density–gradient ultracentrifugation as described by Redgrave *et al* (386). Briefly, the density of the plasma was adjusted to 1.063g/mL by the addition of NaBr (BDH Laboratory Supplies), with gentle mixing for 30 minutes at 4°C. A density gradient of NaBr–NaCl was prepared by layering in an ultracentrifuge tube (Ultra–clear centrifuge tube, 14 x95 mm: Beckman Instruments, Palo Alto, CA) in the following order from the bottom of the tube: density–adjusted plasma 4.0mL, NaBr–NaCl 3.0mL d=1.040g/mL, NaBr–NaCl 3.0mL d=1.020g/mL and NaCl 3.0mL d=1.006g/mL. Ultracentrifugation was performed at 100 000 x g for 42 minutes at 10°C. The chylous supernatant was then quantitatively aspirated to 1.5mL.

Plasma total cholesterol and PL concentrations were determined using enzymatic colorimetric kits purchased from Boehringer Mannheim (Germany) (total cholesterol: CHOD-PAP method) and TG from Roche Diagnostics (Germany) (GPO-PAP method) on a Labsystems multiskan MS analyser. CM TG and PL concentrations were determined by the same methods, as described in the Appendix, for standardisation as reference mass for lipid peroxidation.

4.2.4. *In vitro* oxidation

CM isolated from subjects after they had consumed the meal, without and with wine, were tested for their susceptibility to copper-induced oxidative stress by the method of Esterbauer *et al* (387). Briefly, the CM samples were first diluted to the same lipid concentration to control the impact of the intervention, then diluted 5-fold with 9g/L NaCl, and CuSO₄ was added (final concentration 10µmol/L). This suspension was incubated at 37°C and studied over 27 hours. Beginning at time 0, 200µL was removed, placed on ice and EDTA (final concentration 5mmol/L) terminated the oxidation reaction. There was an excellent correlation between CD and LOOH in this study (Figure 4.1: $r^2 = 0.96$, $P < 0.0001$), confirming what had been previously published (387). Consequently only CD were assayed for evaluation of the oxidative susceptibility of CM.

4.2.5. Lipid peroxidation analyses

Concentrations of CD, LOOH and TBARS were measured in oil and CM by the spectrophotometric methods described in detail in the Appendix.

Briefly, CD were measured at 234nm after appropriate dilution in cyclohexane (387,388). After mixing the CM suspension with cyclohexane, separation was enhanced by centrifugation (14 000 x g for 10 minutes at 4°), and the supernatant was assayed for CD. The inter-assay CV was < 2%.

To determine LOOH, oil and the CM suspension were assayed in the presence of xylenol orange and Fe²⁺ in the ferrous oxidation/xylenol orange (FOX) assay that was adapted to enhance the solubility of non-polar compounds by including chloroform. Absorbance of the resulting Fe³⁺-xylenol orange complex was measured at 560nm (389,390). The inter-assay CV was < 5%.

TBARS were measured according to the method of Asakawa *et al* (391). The samples were reacted with ferric chloride in a glycine buffer at pH =3.6 and thiobarbituric acid (TBA) reagent with sodium dodecyl sulphate. Absorbance was read at 532nm and the inter-assay CV was < 12%.

4.2.6. Plasma catechin analysis

A spectrophotometric method (392) was used to determine the plasma catechin concentrations after ingestion of the meal, with and without wine. Purchased (+)-catechin was used as a standard. The method is detailed in the Appendix. The intra- and inter-assay CVs were < 2% and < 5% respectively.

4.2.7. Plasma ORAC analysis

The total antioxidant capacity of plasma against oxidative stress was assessed by the ORAC method as described by Cao *et al* (298,393) , and modified by Ou *et al* (299), detailed in the Appendix. Briefly, the loss of fluorescence of fluorescein in the presence of the peroxy radical generator AAPH reflects the antioxidant activity and can be compared with a standard (Trolox). The reaction was followed from the addition of AAPH until completion. The intra-assay CV was 4.5% and the inter-assay CV was 7.9%.

4.2.8. Statistical analyses

Data are expressed as mean \pm SD. All statistical analyses were performed with the use of Graphpad PRISM software (version 3, San Diego, USA). The Student's t test was used to analyse the data, with the exception of the comparison of the oil and CM lipid peroxidation data, where the Mann-Whitney test was used. Statistical significance was accepted at $P < 0.05$. PRISM software was also used to determine the AUC values for ORAC analysis.

4.3. Results

4.3.1. Lipid biochemistry

The volunteers were normolipidaemic. The fasting plasma total cholesterol was 4.45 ± 0.85 mmol/L, plasma TG was 1.27 ± 0.65 mmol/L and plasma PL 1.63 ± 0.41 mmol/L. Plasma TG increased significantly after consumption of the meal without and with wine to 2.46 ± 1.55 mmol/L ($P = 0.0005$) and 2.72 ± 1.83 mmol/L ($P = 0.0010$) respectively, as well as PL to 1.99 ± 0.62 mmol/L ($P = 0.0031$) without wine and 1.98 ± 0.57 mmol/L ($P = 0.0003$) with wine. The changes in plasma TG and PL concentrations after consumption of the wine with the meal compared with the meal alone were not significant ($P = 0.22$ and $P = 0.91$ respectively).

Neither the TG nor the PL in the CM fraction changed significantly on consumption of wine. The TG concentration was 3.47 ± 3.53 mmol/L without wine, and 3.62 ± 4.47 mmol/L with wine, $P = 0.85$ and the PL concentration was 0.46 ± 0.42 and 0.42 ± 0.48 mmol/L without and with wine respectively, $P = 0.60$.

4.3.2. Lipid peroxidation status

A comparison of the peroxidation status of the oil and the CM from the volunteers without the influence of red wine showed a significant decrease in the concentration of CD and TBARS in the CM. The CD concentrations were 16.5 ± 2.3 in the oil and 1.5 ± 1.0 $\mu\text{mol/g}$ lipid in the CM, $P = 0.0005$, and the TBARS concentrations were 16.6 ± 4.4 and 3.4 ± 4.2 $\mu\text{mol/g}$, $P = 0.0005$ in the oil and CM respectively. The LOOH concentrations were very varied in the CM but not significantly different from the oil: 2.2 ± 0.2 and 29.4 ± 42.7 $\mu\text{mol/g}$, $P = 0.199$ in the oil and CM respectively. There was no change in concentration of CD from the original oil and equivalent amount in the fatty meal after its preparation, $P = 0.25$. The peroxidation status of the CM after ingestion of the meal, with and without concomitant wine consumption, is shown in Table 4.1. There is no significant change after including wine in the meal.

Table 4.1. Peroxidation status of lipid in CM from OFTT without (-) and with (+) wine at 3 hours.

| | - wine ¹ | + wine ¹ | P value |
|--------------|---------------------|---------------------|---------|
| CD | 1.46 ± 1.01 | 1.75 ± 1.70 | 0.52 |
| LOOH | 29.43 ± 42.74 | 14.75 ± 15.34 | 0.15 |
| TBARS | 3.40 ± 4.23 | 2.79 ± 3.06 | 0.52 |

¹μmol/g TG + PL, mean ± SD, (n=15)

4.3.3. *In vitro* oxidation

Figure 4.1 shows the kinetics of the oxidation of CM as measured by CD and LOOH in the sample, which indicated close correlation between CD and LOOH. Each time point reflects a single value for each assay. The CD analysis extended over 26 hours, and the LOOH were analysed to 12 hours, to allow a comparison of CD and LOOH for their ability to reflect oxidative change in CM. As expected, the CD changes occurred ahead of LOOH changes.

Figure 4.2 shows the summarised responses from 15 subjects of CM to copper-induced lipid peroxidation over 27 hours as measured by CD. Table 4.2 shows the lag times and the calculated AUC from these experiments. Neither the lag times nor the AUC from the CM with and without wine consumption showed a significant difference: P = 0.54 and P = 0.49 respectively).

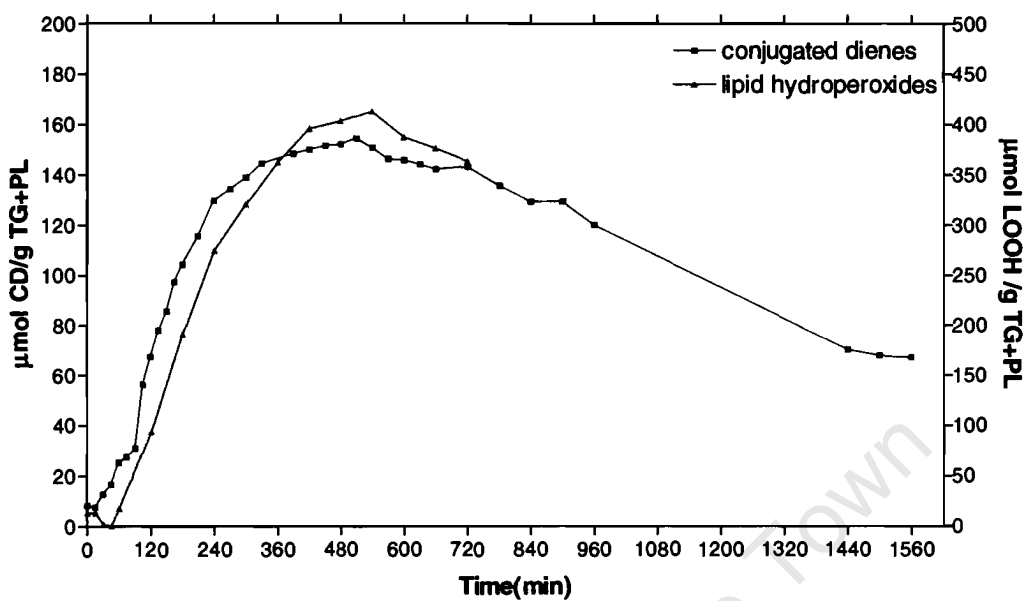


Figure 4.1. A comparison of the kinetics of the copper-dependent oxidation of CM as measured by CD and LOOH.

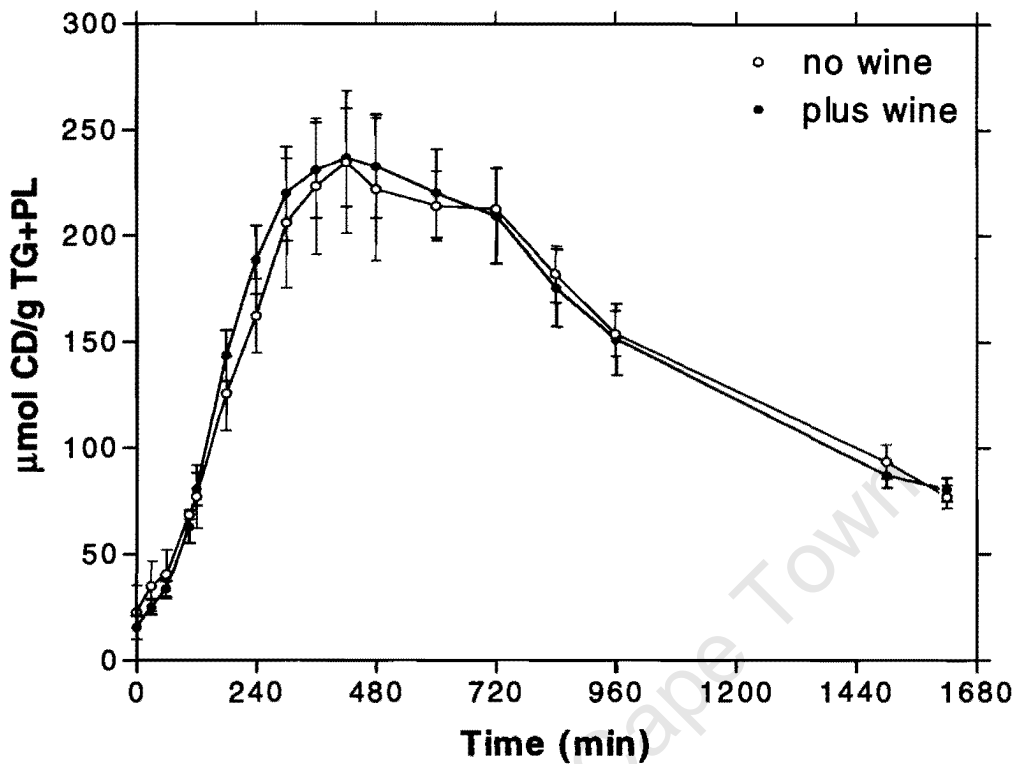


Figure 4.2. Comparison of the kinetics of copper-dependent oxidation of CM from 15 subjects as determined by CD, mean and SEM.

Table 4.2. Lag times and AUC of copper-dependent oxidation of CM from OFTT without (-) and with (+) wine at 3 hours.

| | - wine | + wine | P value |
|------------------------------|-------------|-------------|---------|
| Lag times ¹ | 60 ± 26.6 | 60 ± 23.4 | 0.54 |
| AUC ² by 27 hours | 1.91 ± 0.37 | 2.00 ± 0.53 | 0.49 |

Values are expressed as mean ± SD (n=15)

¹ min, ² μmol.min/g TG +PL x 10⁴

4.3.4. Plasma catechin and ORAC analyses

Three hours after consumption of the OFTT meal alone, the plasma catechin concentration increased from the baseline of 0.20 ± 0.24 to 0.27 ± 0.22 $\mu\text{mol/L}$, and to 0.41 ± 0.23 $\mu\text{mol/L}$ after consumption of wine with the meal. After controlling for the baseline values, the consumption of red wine significantly increased the catechin concentration: $P = 0.001$. After controlling for the plasma ORAC values at baseline (9.59 ± 2.94 mmol/L TE), the values after the meal alone (11.76 ± 2.99) were compared with those after the meal with wine (11.48 ± 3.21 mmol/L TE). The difference was not statistically significant: $P = 0.53$.

4.4. Discussion and conclusions

Given the paucity of published information about lipid peroxidation products in CM, this study took a novel approach by comparing the lipid peroxidation products in a PUFA-rich edible oil with the CM produced after its consumption without and with red wine as an antioxidant. There are 3 chief findings from the study. Firstly, there is an altered profile of lipid peroxidation products between the oil and CM: CD decreased, LOOH were very variable, but not statistically significantly increased, and less TBARS were generated in CM. Secondly, the consumption of red wine increases plasma catechin concentration at the time of chylomicronaemia but the total antioxidant capacity of plasma appears not to be altered. Thirdly, the consumption of red wine together with PUFA-rich edible oils does not influence the lipid peroxidation status and susceptibility of CM.

In this study, a small number of subjects acted as their own controls to explore whether the concomitant consumption of wine and PUFA affected the lipid peroxidation state of CM. Although only a single postprandial time-point was used to evaluate changes from the baseline state, the timing was appropriate for adequate CM collection for studies and for increasing plasma catechins that might be expected to influence oxidation status and susceptibility. A popular commercially available blended red wine with known antioxidant capacity was used in a dose that effected a significant increase in concentration in plasma catechins. Although a limited effect may be overlooked by this study, a dramatic effect of wine on postprandial (CM) lipid peroxidation was not found.

The PUFA-containing oil and the CM in this study contained low concentrations of peroxidation products that pertain to normal human nutrition. Additional complexity might be introduced by antioxidant additives to the oil but such additives are often not declared in South Africa. It was noted that the sunflower oil used in this study had a higher inherent concentration of total TBARS compared with the sunflower oils described in chapter 2 ($16.6 \pm 4.4 \mu\text{mol/g}$ lipid from the oil used in this study and $0.2 \pm 0.03 \mu\text{mol/g}$ oil described in chapter 2), although there was not much variation in the CD and LOOH concentrations. As discussed in Chapter 2, the inherent oxidation status depends on factors including fatty acid composition of the oil, which was not declared on the bottles of oil under study. Different storage conditions, as well as antioxidant additives could also result in different ratios of the peroxidation products in the different oils.

The majority of studies have concentrated on the effects of wine on LDL to investigate protection against atherosclerosis by an antioxidant action. Oxidation of LDL is thought to play an important causative role in atherosclerosis (394,395). Frankel *et al* (187) were the first to show that red wine protected LDL from oxidation *in vitro*. The results of many subsequent studies have been inconsistent. A number of studies have shown that wine and its phenols have significant antioxidant activity as measured by increase in resistance of LDL to lipid peroxidation (375,396-399) whereas other studies found no change (400,401).

Although PUFA are protected against peroxidation in their natural state, processing and storage may permit lipid peroxidation. The products of lipid peroxidation are reactive and potentially harmful. After ingestion, PUFA are absorbed and incorporated into CM, which have a short half-life of approximately 35 minutes compared with that of LDL (approximately 2.5 days). That postprandial metabolic phenomena are associated with atherogenesis was first proposed by Zilversmit (402). Subsequently there has been confirmation of penetration and retention of CM remnants in carotid arteries of animals (403). CM remnants produced in response to a meal rich in PUFA have been found to be more injurious to functional responses by arteries when compared with a meal rich in saturated fatty acids (404). Further associations between CM and atherosclerosis have been made by Stender *et al* (405) whose *in vivo* study showed that CM remnants deposit

in arterial walls. Van Lenten *et al* (406) showed that CM remnants are able to promote cholesterol esterification and cholesterol ester accumulation in monocyte–macrophages. Ventura *et al* (407) reported increases of reactive oxygen species and plasma malondialdehyde concentration after a high fat meal. Consumption of red wine during the meal reduced this oxidative stress. Staprāns *et al* (408) showed that the CD and TBARS in the diet were reflected in the CM but they were unable to detect LOOH. An increase in plasma TBARS was found after consumption of thermally–oxidised oil compared with a minimal increase after consumption of fresh oil (307). Furthermore, CM isolated after ingestion of oil containing high levels of oxidised fatty acids, were more susceptible to copper–induced lipid peroxidation.

The different distribution of the lipid peroxidation products in the fat meal and the CM in the present study is interesting. The lower concentrations of CD and TBARS could be explained by a dilution with endogenously synthesised or exchanged saturated or neutral lipids. A change in lipid peroxidation status during the preparation of the meal was excluded as a contributing factor to the difference in peroxidation status. Further studies will be required to evaluate whether there is on–going peroxidation in the alimentary tract, or differential absorption of peroxidised fatty acids into the enterocyte, or differential utilisation of fatty acids for re–esterification into glycerolipids. It is possible that LOOH may be produced during oxidative stress in the enterocyte or lipoprotein. The short residence time of CM probably will not permit significant compositional changes attributable to lipid exchange between lipoproteins or modification due to enzymes.

There was no significant difference in the peroxidation status, as measured by concentrations of CD, TBARS and LOOH, in the CM collected 3 hours after the fatty meal without and with red wine consumption (Table 4.1). There was also no difference in the susceptibility of these CM to copper–induced peroxidation. Interestingly, 1 individual displayed much greater susceptibility. This was not investigated further but may be speculated to be due to a difference in absorption of intrinsic antioxidants to CM (tocopherol).

De Beer *et al* (409) found that the flavanol concentration of red wines is a good predictor of antioxidant capacity. The antioxidant activities of the major South African red wines were evaluated for their ability to inhibit *in vitro* microsomal lipid

peroxidation (410) and also demonstrated a wide variation in antioxidant capacity. The median ORAC value of the red wines in this study compared favourably with that of Maxwell *et al* (411) and Dávalos *et al* (412). Although the mean plasma ORAC values in this study increased after consumption of the meal, with and without wine, the individual changes were not significant. A study by Alberti–Fidanza *et al* (413) showed similar plasma values, with the ORAC reaching a maximum at 90 minutes and sustaining it up to 360 minutes after ingestion of the wine. The results described in the present study reflect an appropriate time point for the plasma ORAC determinations as catechin concentrations increase. Although there is clearly an increase of plasma catechins with wine ingestion, these do not appear to provide significant additional antioxidant activity at this point in time. The similarity of lipid peroxidation products in CM derived from the meals with and without wine is likely due to the fact that the water–soluble antioxidants in wine will not protect lipids against peroxidation. These antioxidants may penetrate tissues where lipoprotein oxidation or cellular responses may be favourably affected. Even if the antioxidant molecules are water–soluble, they could prevent oxidation of lipoproteins by providing better buffering of reactive oxygen species in this milieu and spare lipid–soluble antioxidants and thus extend protection to lipoproteins indirectly. It could be speculated that antioxidant activity in tissue fluids may rely more on small molecules than large proteins that are not expected to enter tissue fluids. Plasma antioxidant activity may reside in phenols of amino acids in proteins as suggested by Ninfali and Aluigi (414).

Although each of the 15 subjects displayed the same trend in their results, a larger cohort might identify individuals whose CM are more susceptible to oxidative stress. An optimal time for the study of CM was selected. Additional time points may provide better information about antioxidant activity of plasma and the consistency of the lipid peroxidation status of CM. Although wine in general has good antioxidant activity and the same cultivar was selected, there was a variation in ORAC values in the bottles of wine used in this study. Testing subjects with wines that have identical and higher ORAC values might be preferable. Since bioavailability of the antioxidants may also be affected by nutrient interactions (for example insoluble complex formation of tannins and proteins (398)), findings such as these may not apply to the free–living state.

The studies in this chapter indicated little direct benefit on the peroxidation status of lipids in CM with the consumption of wine, despite increasing catechins and possibly other antioxidant compounds. The findings are however limited to a small number of subjects, and a single blended red wine and edible oil. Although no significant peroxidation is found between ingestion of fat and secretion of chylomicrons, this investigation can compare chylomicrons and ingested oil for changes. Should such changes occur, they are likely to be detected as the circulating time of chylomicrons is short, preventing significant oxidation or exchange during residence time in plasma. Since there is no *a priori* expectation of discriminant uptake of oxidised or normal fatty acids for chylomicron assembly, any sample would be appropriate, but systematic examination of such phenomenon could be done on future studies.

The absence of an effect in the plasma does not exclude a beneficial effect in tissues. Final proof of antioxidant benefits would require an outcome study rather than a mechanistic study. Nevertheless, the findings in this study suggest that there is a need to further explore the path that PUFA follow in digestion, absorption and lipoprotein metabolism, and possible differences amongst individuals in susceptibility. When such information is available, better directed studies can be undertaken, including studies with edible oils that have undergone more peroxidation.

CHAPTER 5. THE EFFECTS OF ACUTE WINE CONSUMPTION ON FLOW-MEDIATED DILATATION OF THE BRACHIAL ARTERY

5.1. Background

The vascular endothelium is a cell layer that lines all blood vessels. It occurs between the lumen and the vascular smooth muscle and secretes potent vasorelaxing substances such as nitric oxide (NO) and vasoconstricting substances such as endothelin-1.

Prior to the 1970s, it was thought to be relatively inert. Since then, however, research has shown that it is capable of a number of important functions including control over vascular growth and arterial tone (415). In 1986, Ludmer *et al* reported that endothelial dysfunction was associated with atherosclerosis (416), and this has subsequently been confirmed (417,418). Endothelial dysfunction has been demonstrated in asymptomatic children and young adults with risk factors for atherosclerosis, such as familial hypercholesterolaemia, lipoprotein(a) and smoking (419,420). An association between coronary risk factors (hyperlipidaemia, diabetes mellitus, hypertension and smoking) and endothelial dysfunction was described by Hashimoto *et al* (421). Endothelial dysfunction has also been shown to be reversible under certain conditions: Clarkson *et al* found oral treatment of young hypercholesterolaemic adults with L-arginine, a precursor of NO, improved endothelium-dependent dilatation significantly (422). It has been reported that oxidative stress has a role to play in impaired endothelial function, from the action of some reactive oxygen species (ROS) that have not been efficiently removed by endogenous antioxidant systems. These ROS might decrease the local production of NO by reacting with scavenging NO itself, thereby producing peroxynitrite. Alternatively, ROS might affect the expression levels of eNOS (423).

Research has shown that flow-mediated dilatation (FMD) is endothelium-dependent (424): in arteries with healthy endothelium, increased blood flow causes dilatation of the artery (425) via release of NO (426,427). This does not occur during endothelial dysfunction where there is an impaired vasodilatation response (428). Determination of FMD of the brachial artery is a non-invasive, ultrasonographic-imaging method for detecting endothelial dysfunction. The technique was first described by Celermajer *et al*

in 1992 (419). Arterial diameter is measured in response to an increase in shear stress caused by increased blood flow after occlusion of the artery, causing endothelium-dependent dilatation, and to sublingual trinitroglycerine (TNG), an endothelium-independent donor of NO. FMD has been shown to be stable and reproducible and to correlate well with invasive intra-arterial testing of coronary endothelial function (429). Changes in arterial diameter of 0.1 to 0.2 mm can be detected accurately (420,430). Guidelines for the technique have been published (426). The objectives of this study were:

- i) To determine the brachial artery diameter, blood flow and FMD after acute consumption of red wine
- ii) To determine the effect of ice and TNG on the brachial artery diameter.

5.2. Methods

5.2.1. Study design

Sixteen healthy, non-smoking volunteers, who were not taking any medication, were recruited into the study. There were 11 males (aged 23–48) years and 5 females (aged 19–43) years. Informed consent for the study was obtained from the subjects after the procedure had been explained to them. They refrained from exercise within 4–6 hours before the study. Testing was performed in the morning, after a 12-hour fast, in a quiet room. An Acuson 128 ultrasound machine equipped with a 7.5 MHz Acuson 7 transducer was used to generate images of the artery, which were recorded on a Sony VHS video recorder. The blood pressure and pulse rates were recorded at baseline, 30, 60 and 120 minutes by means of an Accutorr Plus Datascope electronic blood pressure recorder.

Each subject consumed the same cultivar and vintage of red wine as 0.35 g ethanol/kg body mass over 5 minutes. This was considered a moderate volume of wine, and was the same mass of ethanol used in the chylomicron study (Chapter 4).



Figure 5.1. Assessment of flow-mediated dilatation of the brachial artery by ultrasonographic imaging.

5.2.2. FMD assessment

The standardised protocol of Celermajer *et al* (419) was adapted for the study, and the same sonographer assessed the FMD throughout the study. The inter-assay CV for the technique was < 20%.

The brachial artery is imaged at the cubital fossa in the longitudinal plane. For assessment of FMD, a blood pressure cuff is placed distally around the forearm and inflated to 50mm Hg above systolic pressure for 5 minutes to cause ischaemia and subsequent dilatation of downstream vessels. When the cuff is released, there is a brief high flow state through the brachial artery into the dilated vessels. This increases shear stress which in turn causes the brachial artery to dilate in normal individuals. Additional manoeuvres were used to evaluate the range of arterial size. While the left hand is used for FMD measurements, the right hand is immersed in ice for 30 seconds in order to determine the minimum arterial diameter. This response may be absent in subjects with

autonomic neuropathy, but none of the study patients had neuropathy. TNG was sprayed sublingually, at a total dose of 0.8 mg, in order to determine the maximum vasodilator response. Images were recorded continuously during this time (Figure 5.2).

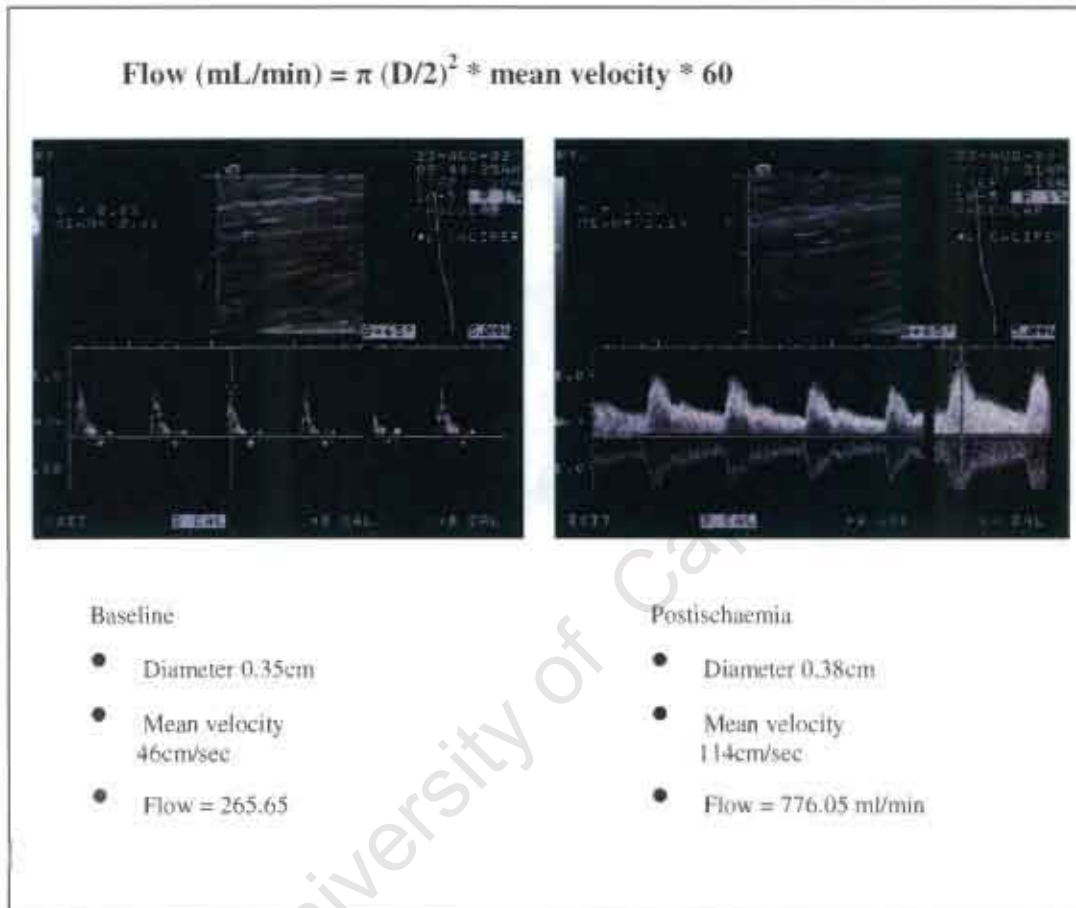


Figure 5.2. Ultrasonographic images of the brachial artery before and after ischaemia.

The B or brightness mode displays a longitudinal image of the artery, allowing the measurement of diameter, while the spectral Doppler allows the measurement of flow in m/sec. The flow in mL/min can then be calculated.

The brachial artery diameter, blood flow (metres/second) and FMD were all measured at baseline, and at 30, 60 and 120 minutes after consumption of the wine. The minimum and maximum lumen diameters were determined at baseline and at 60 minutes after wine consumption.

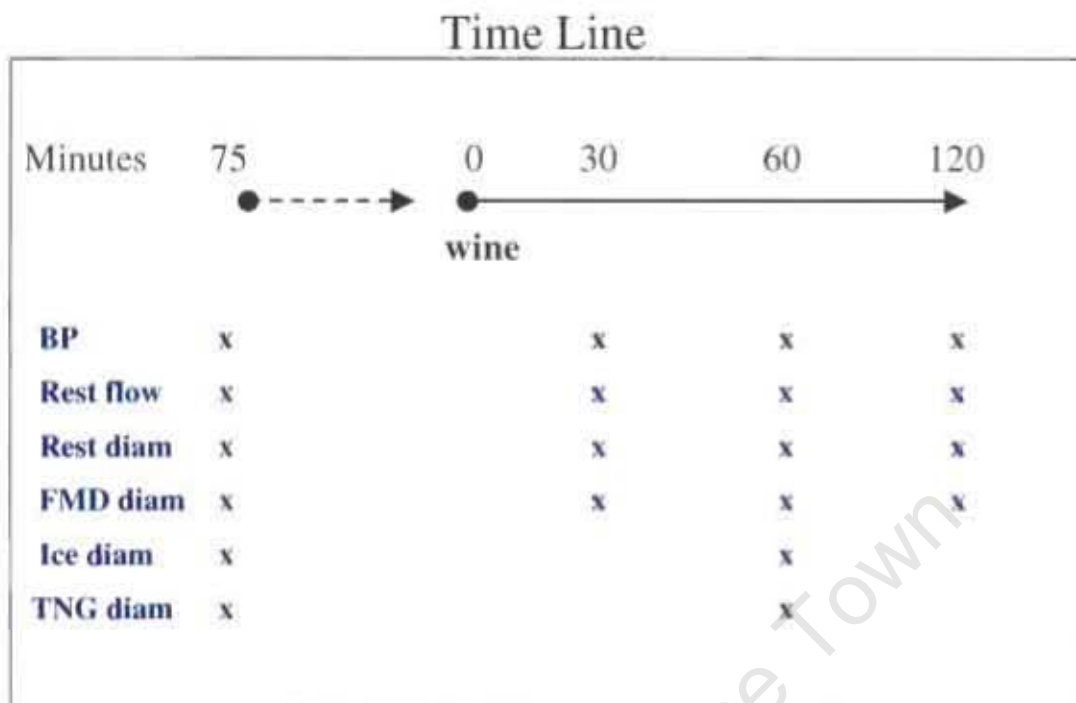


Figure 5.3. The protocol for the FMD study.

5.2.3. ORAC analysis

The antioxidant capacity of the red wine consumed by the volunteers was determined by the method of Ou *et al* (299), described in detail in the Appendix.

5.2.4. Statistical analyses

All data are expressed as mean \pm SD. All statistical analyses were performed with the use of Graphpad PRISM software (version 3, San Diego, USA). Unpaired t tests were used to compare the results from 11 males and 5 females. Comparisons of groups at the 4 time points were analysed by repeated measures Analysis of Variance (ANOVA). The Student's t test was used to compare the vasoconstrictory and vasodilatory changes at baseline and 60 minutes. Statistical significance was accepted at $P < 0.05$. PRISM software was also used to determine the AUC values for ORAC analysis, and to generate the Box and Whiskers plots.

5.3. Results

There were no significant differences in the age, baseline systolic blood pressure, baseline diastolic blood pressure, pulse rates, arterial diameters, FMD percent change or blood flow between males and females (unpaired t tests $P = 0.074, 0.684, 0.223, 0.489, 0.378, 0.201,$ and 0.098 respectively) and therefore the data for males and females were analysed together. The trends for differences in age and blood flow were not interpreted as being meaningful in altering the parameters of interest for the study.

TABLE 5.1. Comparison of FMD and related measurements from 16 subjects before and after wine consumption.

| | Fasting | Wine | | | P value |
|------------------------|--------------|---------------|---------------|----------------|----------|
| | <u>0 min</u> | <u>30 min</u> | <u>60 min</u> | <u>120 min</u> | |
| n = 16 | | | | | |
| Systolic BP mm Hg | 114 ± 6 | 114 ± 10 | 112 ± 12 | 110 ± 15 | 0.40 |
| Diastolic BP mm Hg | 67 ± 7 | 67 ± 14 | 64 ± 11 | 61 ± 10 | 0.05 |
| Heart rate beats/min | 65 ± 10 | 61 ± 8 | 63 ± 11 | 65 ± 10 | 0.34 |
| Diameter mm | 3.8 ± 0.6 | 4.4 ± 0.6 | 4.4 ± 0.6 | 4.5 ± 0.6 | < 0.0001 |
| Flow mL/min | 249 ± 122 | 258 ± 114 | 301 ± 132 | 303 ± 166 | 0.60 |
| FMD % change | 10.82 ± 4.61 | 6.08 ± 2.91 | 5.59 ± 4.24 | 7.46 ± 2.67 | < 0.0001 |
| Diameter after ice, mm | 3.8 ± 0.6 | | 4.3 ± 0.6 | | < 0.0001 |
| Diameter after TNG, mm | 4.7 ± 0.6 | | 4.8 ± 0.6 | | 0.07 |

5.3.1. Blood pressure and pulse rate determination

Neither the systolic blood pressures nor the pulse rates varied significantly from baseline to 2 hours after wine consumption, ($P = 0.397$ and $P = 0.338$ respectively). The trend for the diastolic blood pressures to decrease was not significant ($P = 0.054$), (Table 5.1).

5.3.2. Brachial artery diameter, calculated blood flow and FMD assessment

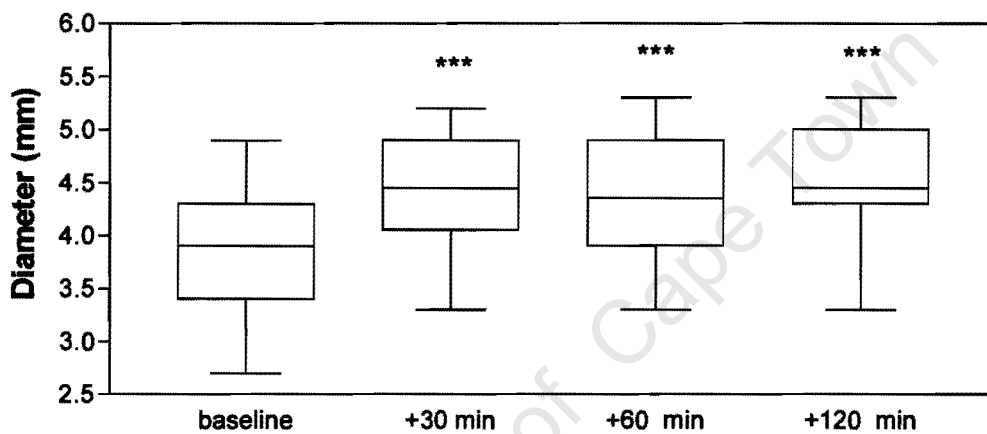


Figure 5.4. The resting brachial artery diameter at baseline and over 2 hours after wine consumption. * denotes a significance of $P < 0.0001$, relative to the baseline.**

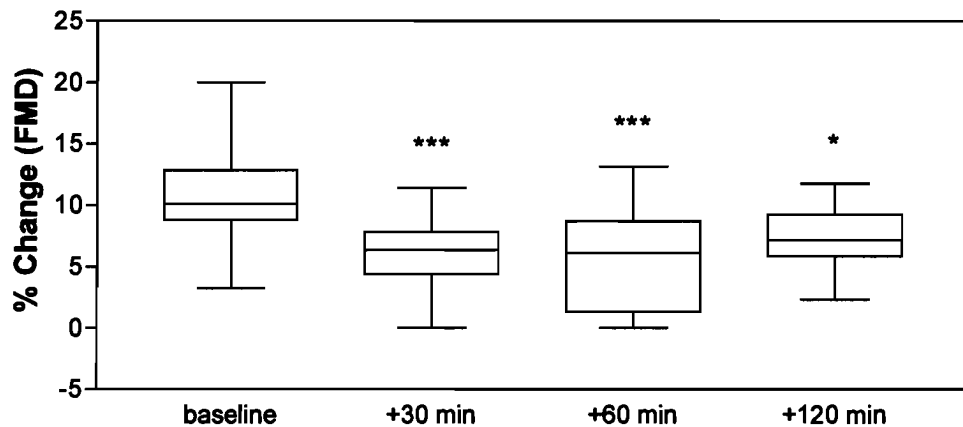


Figure 5.5. The FMD percent change at baseline and over 2 hours after wine consumption. * denotes a significance of $P < 0.0001$, and * denotes a significance of $P < 0.05$, relative to the baseline.**

The diameter of the artery increased significantly from baseline to 120 minutes after consumption of wine ($P < 0.0001$). The mean baseline diameter of 3.8 increased to 4.4, 4.4 and 4.5 mm respectively at 30, 60 and 120 minutes. At the same time, the FMD decreased significantly: 10.8% at baseline, 6.1% at 30 minutes, 5.6% at 60 minutes and 7.5% at 120 minutes after wine consumption ($P < 0.0001$). The blood flow in mL/min was calculated from the measured diameter of the artery and the measured blood velocity (Figure 5.2), and did not change significantly from baseline ($P = 0.6$).

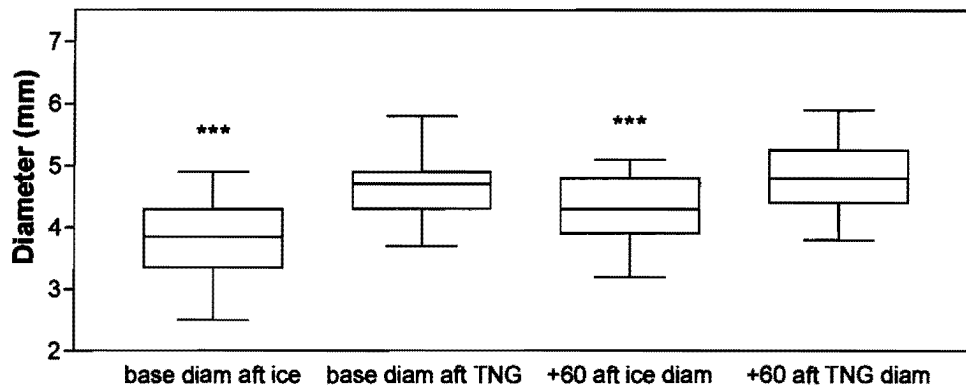


Figure 5.6. The effects of ice and TNG on the brachial artery diameter at baseline and 60 minutes. * denotes a significance of $P < 0.0001$.**

There was a statistically significant difference between the ice response before and after wine consumption, but no significant difference between the TNG responses ($P < 0.0001$ and $P = 0.07$ respectively).

The physiological range of brachial artery diameter was taken as that between the diameter on ice immersion (vasoconstriction) and that after TNG absorbance (vasodilatation). Out of interest, the baseline diameter was compared with this excursion, which was expressed as a percentage calculation as:

(baseline diameter - minimum diameter (ice)) / (maximum diameter (TNG) - minimum diameter (ice)) expressed as a percent

The mean brachial artery diameter for the group was 3.8 ± 0.6 mm. The mean baseline excursion for the group was $91.3 \pm 25.3\%$. Investigating whether males and females differed in their percent excursion showed there was no gender effect (84.6 ± 27.3 and $106.0 \pm 11.4\%$ respectively, $P = 0.12$). Similar analyses revealed no difference in the percent excursion between the younger and older halves of the subjects studied (87.5 ± 29.6 and $95.0 \pm 21.4\%$ respectively, $P = 0.57$). The baseline upper and lower half of the excursion percent was not associated with different FMD responses (9.9 ± 3.2 and $11.8 \pm 5.8\%$ FMD change respectively, $P = 0.43$).

5.3.3. ORAC analysis

The red wines that were analysed for their total antioxidant potential showed a range of results with a mean of 10.25 ± 9.33 mmol/L TE.

5.4. Discussion and conclusions

Brachial artery FMD is a non-invasive technique for assessing endothelial (vascular) function and dysfunction. Research studies have shown that abnormal endothelial function may be associated with atherosclerosis. This study found that a representative sample of red wine mediated vascular dilatation but did not improve FMD. The diameter of the brachial artery increased significantly within 30 minutes and lasted at least 2 hours after consumption of red wine. The systolic and diastolic blood pressure, heart rate and blood flow did not change significantly. FMD decreased significantly over 2 hours.

The subjects investigated in this small study were all healthy. It is possible that a larger number of subjects might have experienced an increase in FMD response. It is also possible that if subjects with CHD were studied or if the effects of chronic consumption of wine were studied, they might have revealed an increase in FMD status.

Previous studies have found that a number of factors may interfere with FMD, for example some studies found that FMD is inversely related to baseline arterial diameter (431,432), as did the present study. Ageing is associated with decreased FMD (433), as is sleep apnea (434), hypertension (435), circadian variations (436) and mental stress (437). In the present study, FMD was not affected by age. Other factors that may impede FMD are hypercholesteraemia, diabetes mellitus, smoking (active and passive) and obesity (428), as well as high-fat meals (194,438), although some have found that the latter did not influence endothelial function (193). Meals rich in thermally-stressed fats have also been shown to reduce FMD (325). Levenson *et al* found significant gender differences in shear-mediated vasoconstriction and vasodilatation, with women constricting more than men during arterial occlusion, and also undergoing greater changes in FMD than men (439). FMD was also found to be significantly different between men and women after a high-fat meal, with men showing a greater reduction in FMD than women (440). This study did not show significant gender differences.

Burns *et al* found that the capacity of different red wines to act as vasodilators varied widely, and this capacity was associated with concentrations of specific phenolic compounds in the wine, in particular resveratrol and catechins (441). Red wine has been found to inhibit endothelin-1 synthesis, possibly by modifying tyrosine kinase signalling in the endothelial cells (442). The antioxidant activity of the same cultivar and vintage of red wine in this study showed wide variation by ORAC, as did the South African red wines described by de Beer *et al*, by inhibition of microsomal lipid peroxidation (410). The activity of the red wines in this study was also lower than that described in the chylomicron study (chapter 4): 10.3 ± 9.3 compared with 24.8 ± 10.1 mmol/L TE. It is possible that wine with higher ORAC values might have resulted in an improved FMD response in this study. A study described by Hashimoto *et al* found no significant change in systolic blood pressure, a significant increase in heart rate and resting brachial artery diameter and, contrary to this study, a significant improvement in FMD by 120 minutes after red wine consumption (192). They, however, employed more than double the amount of wine compared with this study: 0.8g/kg body mass compared with 0.35g/kg body mass, for alcohol, in this study.

It is possible that a dose-related response would reveal a volume of ethanol that would result in improved FMD. It is also possible that beneficial effects on FMD relate only to specific time points beyond those studied in the volunteers. As shown in Chapter 4, the catechin concentration is increased at 3 hours postprandially. It is, however, likely that the catechins being water-soluble compounds like alcohol, could achieve increased concentration within the time course of the present FMD studies.

As the arterial diameter approaches its physiological limit upon dilating in response to the wine, its capacity to dilate becomes impaired. The baseline diameter relative to the physiological excursion did not seem to influence the response in FMD. There was a trend for the brachial artery to increase in diameter upon nitrite absorbance from baseline compared with 60 minutes after wine consumption. The decrease in response to ice immersion was significant. The duration of this effect is not known, but is likely to be the same as the vasodilatory effect.

This study did not attempt to separate the different effects of the antioxidant phenols and the ethanol on vascular function. Wine is a complex beverage made up of a large

number of different polyphenols as well as ethanol. Some studies have found that phenols, particularly the flavonoids in wine, improve endothelial function (195) and that the ethanol in the wine is not implicated (69,192). Red grape juice has been found to improve FMD (443), suggesting that ethanol is not essential. However, it may still provide additional improvement in endothelial function. Future studies will include assessment of the effects of water and ethanol consumption on endothelial function parameters.

In conclusion, this study found that arterial diameter increased after acute consumption of red wine, although FMD did not. Although this may appear to be a negative finding for health benefits of wine, this study considered only a small number of healthy rather than diseased subjects and only viewed the impact over 2 hours. Extending the duration of study and including larger numbers of subjects may result in an increase in FMD status.

CHAPTER 6. FINAL DISCUSSION AND CONCLUSIONS

Atherosclerosis is a major cause of morbidity and mortality in developed countries, and is rising rapidly in developing countries (263). In the Western Cape region of South Africa, despite it being predominantly a population regarded as similar to a developing country, CVD is the leading cause of mortality. The significantly lower rate of CVD in France compared with other developed countries, in spite of their similar dietary intake, prompted the French Paradox hypothesis, in which it was claimed that the increased consumption of red wine was responsible for the decreased CHD.

The social and economic implications of diseases such as CHD and cancer are huge. These common disorders have prompted extensive research into risk factors associated with influencing their prevalence and outcome. Amongst these factors is the detrimental effect of free radicals, and more specifically the free radicals associated with lipid peroxidation. Lipid peroxidation is a biochemical process that involves the potentially harmful constellation of products such as CD, LOOH and TBARS. These products have been implicated in deleterious health effects. Alcoholic beverages, in particular red wine, contain antioxidant phenolic compounds that may reduce lipid peroxidation. Many studies have been undertaken over the last several decades to prove this, and there is much support for the hypothesis that the moderate consumption of red wine is antithrombotic, antimicrobial and anticarcinogenic. Alcohol abuse, however, is a very real problem worldwide, and is responsible for many murders and road accidents. The incidence of fetal alcohol syndrome in South Africa is the highest in the world (33,35). In order that even moderate use can be advised, it is important to establish conclusively from epidemiological and experimental studies, as well as outcome studies, whether or not real benefits can be attained.

In this thesis, novel approaches were set up to determine the antioxidant effects of wine and some potentially beneficial effects on health, namely:

- A study to determine the protective effect of wine in an emulsion with PUFA-containing oil undergoing heating;

- A study involving volunteers who consumed a PUFA milkshake with and without wine, to determine the effects of the wine on the postprandial TG-containing lipoproteins;
- A study in which marinating red meat in red wine prior to cooking, was used to determine whether the wine could alter the progression of lipid peroxidation and prevent heat-induced production of lipid peroxidation products and
- A study which determined the effect of moderate consumption of wine on vascular function.

The studies described in this thesis present some interesting findings. There is a large variation in the inherent lipid peroxidation in MUFA and PUFA oils, as well as in red meat, both freshly-bought and after cooking. Wines have antioxidant potential demonstrable by the oil-boil assays as well as ORAC.

Research into lipid peroxidation is relatively new. A broad range of products needs to be examined and simpler, more affordable techniques for research into CHD in the developing world is desirable. In the studies described in this thesis, the methods used are applicable to most general laboratories. Recourse to more sophisticated analytical techniques were limited. Refined investigations to identify reactive intermediates and their products as well as to determine detailed compositional analysis of the substrates, could promote better targeted analyses.

Foodstuffs need care for their production and storage. Preparation techniques need consideration for avoidance of oxidative stress. The lipid peroxide contents in foodstuffs need systematic description and if confirmed to be noxious, may need to be declared with other nutrients on the label, along with fatty acid composition. Meats other than red meat may be more susceptible to lipid peroxidation although their PUFA reserves may be larger, but only future systematic studies can elucidate this.

While meat may be regarded simply as a protein source by the public, oils have already assumed importance in health-conscious members of the public. The wide variation in lipid peroxidation products even within the same class of oils, indicates the need for studies to improve the qualities of oil. This may need legislation and deliberate quality control should further studies prove these lipid peroxidation products to be important.

The impact of food preparation (temperature and cooking environment for example) needs to be investigated in detail for its influence on lipid peroxidation and health as heated edible oils are forming a large part of the diet of convenience foods.

Wine appears to be a good generic antioxidant which may not be amenable to much improvement, except that white wines have the potential to approach red wines in their antioxidant capabilities. Although it is not the objective of this thesis to compare the antioxidant effects of vitamins with the antioxidant effects of phenolic compounds in wine, it must be noted that the results of 2 large meta-analyses published in 2003 (444) and 2004 (445), showed that supplementation with various doses of antioxidant vitamins (A, C, E and β -carotene) had no beneficial effect on the risk of CVD or gastrointestinal cancers. Individual compounds such as resveratrol may provide future avenues of beneficial products with scope to be pharmaceutical agents. Application of wine as a marinade may preserve meat, but appears to confer little benefit in moderate cooking. More intense cooking may perhaps show benefit, especially in a baste to protect the exterior during grilling. Experiments from this thesis indicate scope for wine as an antioxidant in preparation of food where water and oil are heated in a mixture. This, as well as using wine in copper or iron pots, needs to be explored.

Studies on vascular function need to be interpreted with caution. The variability of brachial artery diameter along with its excursion from minimum to maximum is an interesting observation that may be useful to explore in understanding vascular behavior, and may provide insight into disease. The impact on FMD was apparently not positive, but has to be extended to chronic exposure or longer follow-up and contrasted with FMD on similar diameters achieved with other conditions.

Ultimately, beneficial effects of alcoholic beverages are best evaluated with an outcome study on healthy individuals with a healthy lifestyle but at moderate to high risk for CHD, by conducting a controlled, randomised, long-term, single-blind study. The studies in this thesis, where healthy chiefly Caucasian individuals were used, did not show any obviously different results for the different ethnic groups, but detailed studies of large numbers may reveal differences. In the meantime, epidemiological studies as well as experimental studies are suggestive of health benefits from moderate consumption. In this thesis, additional experimental evidence has been found for use of wine in food

preparation after demonstrating susceptibility and variability in foods, with prevention of oxidation under conditions simulating cooking. These findings contribute to the body of knowledge and clearly indicate no deleterious effects; they may even be interpreted as supportive of a beneficial effect.

University of Cape Town

APPENDIX
GENERAL METHODS

1.1 PREPARATION OF PLASMA AND SERUM FROM BLOOD SAMPLES

Blood samples were taken by venepuncture into either EDTA-containing (1g/L) vacutainer tubes (for plasma) or SST vacutainer tubes (for serum) and placed on ice. Plasma and serum were separated by centrifuging at 2000 x g for 15 minutes at 4°C in a Beckman GS-6R centrifuge. If storage was necessary, samples were stored under nitrogen gas at -20°C in the dark.

1.2 LIPID EXTRACTION

The Bligh and Dyer method (353), a modification of the Folch method (354) was used.

1.2.1 Lipid extraction of meat samples:

To 3.2mL homogenised meat was added 4mL chloroform and 8mL methanol. The mixture was vortexed for 30 seconds and then centrifuged at 2000 x g for 15min at 4°C in a Beckman GS-6R centrifuge. The monophasic supernatant was transferred to a separate tube on ice. Lipid extraction was performed twice more on the meat pellet, adding 2.4mL phosphate buffer, 3mL chloroform and 6mL methanol and again transferring the supernatant to the same separate tube. After this, 10mL chloroform and 10mL phosphate buffer were added to the monophasic supernatant, effectively separating the phases into an upper aqueous phase containing the non-lipid compounds and an organic phase containing the purified lipids. After centrifugation at 2000 x g for 15min at 4°C to ensure complete phase separation, the top phase was removed by aspiration and the bottom phase was dried in a Pierce Reacti-therm III (Reacti-Vap III) module (Rockford, IL) under nitrogen gas. The dried lipid extract was then re-suspended in 200µL chloroform and aliquoted into 10 x 20µL tubes. The extracts were again dried under nitrogen as above. Samples were stored under nitrogen gas at -20°C in the dark.

1.3 LIPID ANALYSES

1.3.1 TG and PL assays on lipid extracts:

The GPO-PAP kit was used for the TG assay and the MPR2 kit was used for PL assay. The inter-assay CV for TG and PL was <3%.

Standard curves using the supplied calibrators were prepared, from 0–100µg in a total volume of 500µL with physiological saline for TG and 0–15µg choline in a total volume of 30µL for PL. To each standard was added 50µL ethanol and 15µL 1% v/v Triton X-100, and the mixtures were vortexed for 20 seconds. Likewise, to each dried lipid extract sample was added 50 µL ethanol and 15 µL Triton, followed by vortexing, and the addition of 500 µL physiological saline. The samples were vortexed again. Precinorm® U was used as a commercial quality control calibrator, at a volume of 10µL, and assayed in a similar manner to the standards and samples. Into transparent Greiner microtitre plate wells were added 125µL of each of the standards and samples and 125µL assay reagent, followed by mixing. The TG and PL reactions were complete after 30 minutes at room temperature, after which the absorbance was read in a Labsystems spectrophotometer at 500nm. The mass of TG and PL in each sample was derived from linear regression of the set of standards. Since the standard was a choline solution, a conversion from choline to PL was made. The conversion factor was supplied in the kit insert.

1.3.2 Plasma and chylomicron TG assay:

The GPO-PAP kit was used for the TG assay. The inter-assay CV was 2.4%.

The standard curve was prepared as above but to a total volume of 50µL with 9g/L saline in the microtitre well. Plasma samples and chylomicron samples were likewise prepared by the addition of 10µL plasma/chylomicron sample to a final volume of 50µL with saline. After mixing, 250µL of the reagent was added and after a final shaking to mix, the reaction was complete after 20 minutes at room temperature. The absorbance was read in a Labsystems spectrophotometer at 500nm. The mass of TG in each sample was derived from linear regression of the set of standards as in 1.3.1. Precinorm U was used as a calibrator.

1.3.3 Serum and chylomicron PL assay:

The MPR2 kit was used for the PL assay. The inter-assay CV was 2.1%.

The standard curve was prepared as in 1.3.1, but to a total volume of 30 μ L with saline.

Serum samples were prepared by the addition of 10 μ L to a total volume of 30 μ L, while chylomicron samples were prepared by adding 15 μ L sample to 15 μ L saline. After mixing, 220 μ L of the reagent was added. The reaction was complete after 20 minutes at room temperature, the absorbance was read at 500nm and the mass of PL in each sample was derived as in 1.3.1.

1.3.4 Plasma T.Chol assay:

The MPR1 kit was used for the assay. The inter-assay CV was 2.0%.

The standard curve was prepared with the use of a known human cholesterol plasma sample, in 9g/L saline, to a total volume of 50 μ L, at 0–50 μ g. Plasma samples were prepared by adding 10 μ L of the sample to a total volume of 50 μ L in saline. To each of the standards and the samples was added 250 μ L of kit reagent in a microtitre plate well. These were mixed by shaking and allowed to react for 10 minutes at room temperature, after which the absorbance was read at 500nm. Precinorm U was used as the calibrator.

1.4 LIPID PEROXIDATION ANALYSES

1.4.1 CD measurement:

The method as described by Esterbauer *et al* and Pryor *et al* was used (387,388).

Dried extracted lipid samples, chylomicron samples and oil samples were dissolved in cyclohexane at appropriate dilutions, vortexed for 30 seconds and the absorbance of each sample was read at 234nm using the GBC spectrophotometer. To each lipid extract was added 2mL cyclohexane, to 100 μ L of each chylomicron sample was added 1mL cyclohexane and the dilution for oil to be in the range for the assay was 0.5-2mg/mL cyclohexane. The concentration of the CD in the samples was calculated by molar extinction. The extinction coefficient is $2.95 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$. The inter-assay CV was < 2%.

1.4.2 LOOH measurement:

The method as described by Jiang *et al* was used (389,390).

Dried extracted lipid samples were dissolved in 100 μ L chloroform. 100 μ L chylomicron samples and oil samples at 0.5–2mg/mL chloroform were mixed with 350 μ L methanol and then vortexed for 30 seconds to generate a monophasic. To each tube was added 50 μ L deionised water and 600 μ L FOX 2 Mix (1 part FOX 2A (2.5mM ferrous ammonium sulphate, 2mM Xylenol Orange (3, 3'-bis[N, N-di (Carboxymethyl)-aminomethyl]-o-cresolsulfonephthalein) (sodium salt) (Sigma Chemical Co, St Louis, USA) in 0.25M H₂SO₄) to 4.5 parts FOX 2B (0.88mM BHT in methanol)), prepared fresh for each assay. FeCl₃ and BHP standard curves were prepared as below in tables 1 and 2. Standard reactions were prepared by adding 50 μ L of each standard solution to 100 μ L chloroform and 350 μ L methanol, and then vortexing. FOX 2 Mix was added as above. Reactions were complete after 20 minutes at room temperature, after which the absorbance was read spectrophotometrically at 560nm. The consistency of each assay was assessed by calculating the ratio of BHP to FeCl₃ when absorbance was 1. The ratio was 5-6. The concentration of LOOH in each sample was determined by interpolation of non-linear regression of the t-BHP standards. The inter-assay CV was < 5%.

Table 1. FeCl₃ standard curve for the LOOH assay (stock FeCl₃ solution is 4mM).

| Volume FeCl₃ stock solution (μL) | Volume H₂O (μL) | Concentration of working solution (mM) | Mass of FeCl₃ in assay (nmol) |
|---|--|---|---|
| 100 | 900 | 0.4 | 20 |
| 200 | 800 | 0.8 | 40 |
| 400 | 600 | 1.6 | 80 |
| 500 | 500 | 2.0 | 100 |
| 800 | 200 | 3.2 | 160 |

Table 2. t-BHP standard curve for the LOOH assay (stock BHP solution is 6.96mM and working solution is 0.696 mM =1mL stock solution + 9mL deionised water).

| Volume working solution (μL) | Volume H_2O (μL) | Concentration of working solution (mM) | Mass of BHP in assay (nmol) |
|---|---|--|-----------------------------|
| 40 | 960 | 0.03 | 1.5 |
| 200 | 800 | 0.14 | 7 |
| 400 | 600 | 0.28 | 14 |
| 800 | 200 | 0.56 | 28 |
| 120 μL stock | 880 | 0.84 | 42 |



Figure 1. The LOOH reaction.

1.4.3 TBARS measurement:

TBARS were measured according to the method of Asakawa *et al* (391), with minor modifications.

Dried extracted lipid samples were dissolved in 50 μ L chloroform. Fifty μ L chylomicron samples and 50 μ L oil samples at 1–10mg/mL chloroform were mixed with 50 μ L 0.27g% FeCl₃. Fifty μ L ethanol were added to the tubes measuring total TBARS (tTBARS) (or those present at the time of testing as well as those generated during testing) and 50 μ L 0.22g% BHT (an antioxidant) (in ethanol) were added to the tubes measuring free TBARS (fTBARS) (or those present at the time of testing). Water and chloroform blanks were prepared by adding 50 μ L deionised water and 50 μ L chloroform to the same reagents as above. All samples were vortexed for 20 seconds, mixed with 750 μ L glycine buffer (buffers below), and 750 μ L TBA reagent (0.5g% 2-TBA, 0.3g% SDS) and vortexed again. Samples were heated at 100°C for 20 minutes. Five hundred μ L glacial acetic acid and 1mL chloroform were added to each sample. All samples were vortexed and then centrifuged at 2000 x g for 15 minutes. The absorbance of the aqueous supernatant was determined spectrophotometrically at 540nm. The maximum absorption is normally at 532nm, but only a 540nm filter was available. The inter-assay CV was < 12%.



Figure 2. The TBARS reaction.

1.5 CATECHIN ASSAY

The method of Kivits *et al* (392) was used.

One mL samples of EDTA plasma, prepared as described in 1.1 above, were mixed with 3mL 1g/L BHT in methanol to precipitate the proteins. The mixture was centrifuged at 2000 x g for 15 minutes at 4°C and the supernatant was reacted with 0.15g aluminium oxide (solid-phase alumina) for 4–6 hours in the dark at 4°C under nitrogen gas for adsorption onto the solid-phase, preventing the interference of turbidity in the assay. After centrifugation at 2000 x g for 15 minutes at 4°C, the alumina was washed with 3mL ether, dried under nitrogen and then reacted by vortexing for 1 minute with 1mL p-dimethyl amino cinnamaldehyde (DMACA) reagent (6mmol/L in methanol:perchloric acid:water 8:1:1 v/v). The alumina was centrifuged at 2000 x g at 4°C for 2 minutes and the absorbance of the supernatant was measured at 637nm at 7 minutes in a Labsystems spectrophotometer. A standard curve of (+)-catechin (0 – 50µM) dissolved in BHT/methanol and diluted in de-catechinised plasma that had been pre-reacted with alumina to remove any catechins, was similarly treated to determine the unknown catechin concentrations. The inter-assay CV was <5%.

1.6 ORAC ASSAY

The method as described by Cao *et al* (298,393) and modified by Ou *et al* (299) was used.

All ORAC analyses on wine and plasma were performed on a fluorescence spectrophotometer in white flat-bottomed 96-well microtitre plates. Red wine was diluted to 1: 1600 in 75mmol/L phosphate buffer, pH =7.4. One hundred µL plasma was mixed with 200µL ethanol to precipitate proteins and after centrifugation at 2000 x g for 15min at 4°C, the deproteinised plasma sample was diluted 400-fold in 75mmol/L phosphate buffer, pH =7.4. Fifty µL samples of wine and plasma were added to 50 µL buffer, 100 µL fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9h]-xanthen]-3-one)(disodium) (96nmol/L) and 100 µL AAPH (2,2'-azo-bis, (2-amidinopropane dihydrochloride) (107mmol/L). The reaction was followed from the addition of AAPH until completion, with fluorescence readings being taken at time 0, 2min, 5min and every

| | |
|--|---|
| Sigma Chemical Company, St Louis, USA/Sigma-Aldrich Chemie, Germany | AAPH, aluminium oxide, t-BHP, BHT, (+)-catechin, DMACA, fluorescein, perchloric acid, TBA, Trolox |
| SMM Instruments, Set Point Technology, South Africa | Varian Cary Eclipse fluorescence spectrophotometer |
| Sony Corporation, South Africa. | VHS Videorecorder SVO 9500 MDP |
| South African Scientific Products, South Africa. | Jouan MR 1812 microcentrifuge |
| The VirTis Company, Gardiner, NY | VirTis handishear |
| Varian Analytical Instruments, Walton- on-Thames, UK | Gas Chromatograph Varian CP - 3800 |
| Vitalcare Technologies, South Africa | Electronic BP apparatus Acutorr Plus (Datascope) |
| Wirsam Scientific and Precision Equipment, South Africa | GBC UV/VIS spectrophotometer |

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