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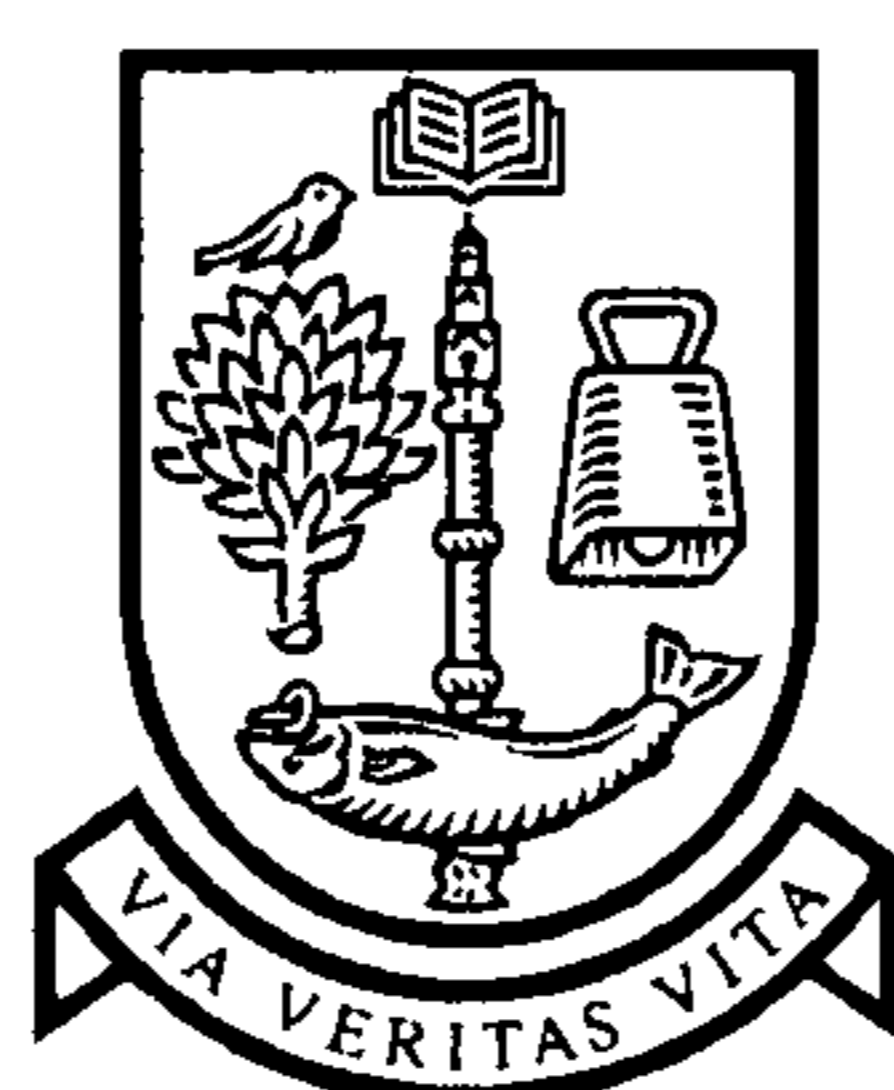
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Antioxidant Activity, Protective Effects and Absorption of Polyphenolic Compounds



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**A thesis submitted to the Faculty of Biomedical & Life Sciences,
University of Glasgow for the degree of Doctor of Philosophy (Ph D)**

Abstract

There is a growing awareness of the potential health benefit of a diet rich in fruits and vegetables and nutritional guidelines indicate that an increase in the consumption of these foods may decrease the risk of developing coronary heart disease (CHD) and certain cancers. These properties may be ascribed to the presence of antioxidants and recent attention in this regard has focused on phenolic and polyphenolic compounds. These compounds are present in a wide variety of commonly consumed foods and beverages in the diet and red wines are an especially abundant source derived from grape seeds, skins and vine stems. The objectives of the studies presented in this thesis were to identify and quantify the major phenolic components of red wine and to assess the contribution of individual compounds to the total antioxidant activity. Further aims were to investigate the influence of a moderate and regular consumption of red wine on the antioxidant status and on indices of oxidative stress associated with CHD in healthy volunteers, and to investigate the absorption, metabolism, sequestration in body tissues and excretion of the monomeric and polymeric flavan-3-ols in rats following ingestion of a grape seed extract (GSE).

Red wines were analysed for their phenolic content and antioxidant activity using a range of complementary techniques including HPLC-tandem mass spectrometry, preparative HPLC and HPLC with an on-line antioxidant detection system. HPLC-MS² revealed the presence of a number of flavonoids and phenolic compounds of which 19 were identified, with gallic acid, the flavan-3-ols and anthocyanins being the most abundant. Preparative HPLC was used in an effort to isolate the antioxidant components in red wine and 60 aliquots were collected. Each wine fraction was analysed for total phenolics, catechins and anthocyanins, while antioxidant activity was determined by electron spin resonance spectroscopy (ESR). The preparative HPLC step did not completely separate the compounds in red wine, nonetheless increasing antioxidant activity was highly and significantly associated with total phenolics ($r = 0.816$, $P < 0.001$) and total catechins ($r = 0.591$, $P < 0.01$); but there was no correlation with total anthocyanins ($r = 0.188$, $p=0.151$). HPLC with an on-line antioxidant detection system was subsequently used to separate and identify red wine phenolics. The findings from this study indicate that gallic acid, (+)-catechin, (-)-epicatechin, and procyanidin dimers

B1 and B2 were the major *in vitro* antioxidants identified in red wine. Collectively the flavan-3-ols contributed > 50 % of the total antioxidant capacity of each wine, while gallic acid contributed between 23-44 %. The flavonols and anthocyanins were minor antioxidant components in red wines. By combining HPLC, MS² and on-line assessment of antioxidant activity, the major phenolic compounds present in red wine were identified, together with their direct contribution to the total antioxidant activity.

A randomised, controlled study was performed with 20 free-living healthy volunteers in an effort to determine the effects of a daily moderate consumption of red wine on the antioxidant status and indices of lipid peroxidation and oxidative stress associated with CHD. Subjects in the red wine group consumed 375 mL/d of an antioxidant-rich red wine for two weeks. The total concentrations of phenolics were measured and individual phenolics in the wine and plasma were analysed by HPLC-MS². The antioxidant capacity of plasma was assessed with ESR and homocysteine and fasting plasma lipids were also determined. The production of conjugated dienes and thiobarbituric acid reactive substances (TBARS) were measured in copper oxidised low-density lipoprotein (LDL). Plasma total phenolic concentrations were highly and significantly increased by 4.4 μ M GAE after 2-weeks of daily red wine consumption ($P \leq 0.001$) that was associated with a non-significant increase in the plasma antioxidant activity. Trace levels of four metabolites, namely (+)-catechin glucuronide, (-)-epicatechin glucuronide, methyl-catechin glucuronide and methyl-epicatechin glucuronide were detected in plasma of the red wine group. These flavan-3-ol metabolites were not detected in plasma of the control group. Moreover, the maximum concentration of conjugated dienes and TBARS in copper-oxidised LDL were significantly reduced ($P \leq 0.05$) and HDL cholesterol concentrations were significantly higher ($P \leq 0.05$) following red wine consumption. None of these parameters were observed in the control group. The findings from this investigation suggest that some phenolics appear to be directly absorbed into the bloodstream from the gastrointestinal (GI) tract following a daily moderate consumption of red wine, and are able to exert some protective effects such as raising HDL cholesterol concentrations and enhancing the resistance of LDL to withstand oxidative modification.

The absorption, metabolism and excretion of flavan-3-ols and procyanidins were investigated in rats following the ingestion of a single acute dose of a tannin-rich grape seed extract (GSE). The liver, kidney, brain and GI tract together with plasma, urine and faeces were collected at several time points up to 24 h after ingestion of the GSE and the flavan-3-ol contents were analysed by reversed phased HPLC with tandem mass spectrometry and diode array detection. In this study, small amounts of the GSE flavan-3-ols moved out of the stomach and into the duodenum/jejunum, and to a greater extent the ileum 1 h after ingestion and in the caecum after 2 h with relatively small amounts being detected in the colon after 3 h. The GI tract contained principally the parent GSE flavan-3-ols and procyanidins, however trace amounts of four metabolites, namely (+)-catechin glucuronide, (-)-epicatechin glucuronide, methyl-(+)-catechin glucuronide and methyl(-)-epicatechin glucuronide, were detected in the duodenum/jejunum and ileum. In contrast to the GI tract, plasma contained exclusively flavan-3-ol metabolites in detectable quantities which reached a peak plasma concentration 3 h after GSE ingestion; (+)-catechin glucuronide ($36.0 \pm 2.5 \mu\text{moles/L}$) and (-)-epicatechin glucuronide ($34.4 \pm 0.2 \mu\text{moles/L}$) and in small amounts methyl-(+)-catechin glucuronide ($19 \pm 0.3 \mu\text{moles/L}$) and methyl(-)-epicatechin glucuronide ($11.4 \pm 0.7 \mu\text{moles/L}$). All four metabolites were also detected in the liver and kidneys. They were also detected in urine along with (+)-catechin sulphate, (-)-epicatechin sulphate, methyl-(+)-catechin sulphate and methyl(-)-epicatechin sulphate. In addition, urine also contained in low amounts the procyanidin dimers B₁, B₂, B₃ and B₄ as well as the trimer C₂ and an unknown GSE trimer. The levels of (+)-catechin and (-)-epicatechin metabolites excreted in urine relative to the quantity of the monomers ingested were 27 and 36 %, respectively, after 24 h. The findings from this investigation indicate that monomeric together with smaller amounts of oligomeric and polymeric flavan-3-ols can be directly absorbed into the bloodstream. The monomers are metabolised forming glucuronidated, sulphated and methylated derivatives. Moreover, this study provides further, albeit indirect, evidence that the procyanidin oligomers in the GSE were not depolymerised in the GI tract releasing monomeric flavan-3-ols to any extent after ingestion.

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Acknowledgements

I gratefully acknowledge my supervisor Professor Alan Crozier for his continual support, advice and expertise throughout this study. Special thanks to Bill Mullen for his technical knowledge and expertise on LC-MS, and to the rest of the Crozier lab, past and present, especially Alison, Alex, Bill, Gina, Indu, Mandie and Roslee. Thank you for your friendship, support and fun times over the past years.

I would like to express my sincere gratitude to Dr Siobhan Higgins for all her help over the years; especially her knowledge and expertise, critical reading and always having time for me.

Dr Pierre-Louis Teissedre and Cyril Auger are gratefully acknowledged for their collaborative work at the Faculty of Pharmacy and Unité of Nutrition, University of Montpellier, France.

Dr Muriel Caslake and fellow research staff at the Department of Pathological Biochemistry, Glasgow Royal Infirmary for use of laboratory facilities.

Financial support from the BBSRC and Safeway Stores plc. is appreciatively recognised, with special thanks to Moira Howie and the Wine, Beer & Spirits Team at Safeways.

A special mention to my family for their love and support, and finally, and most importantly to David for his continual support, encouragement and patience over the years.

Abbreviations

ACN	Acetonitrile
a.m.u.	Atomic mass unit
CHD	Coronary heart disease
DNA	Deoxyribonucleic acid
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ESR	Electron-spin resonance
FRAP	Ferric reducing ability of plasma
GAE	Gallic acid equivalents
GSE	Grape seed extract
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
LDL	Low density lipoprotein
LC-MS	Liquid chromatography-mass spectrometry
n.d.	Not detected
PDA	Photo diode-array
PUFA	Polyunsaturated fatty acid
SEM	Standard error of the mean
TBARS	Thiobarbituric reactive substances
TEAC	Trolox equivalent antioxidant capacity
UV	ultra-violet
v/v	volume/volume
w/v	weight/volume
λ max	Wavelength of maximum absorption

Aims of Study

Red wine (*Vitis vinifera*) provides a rich source of phenolic and polyphenolic compounds derived from grape seeds, skins and vine stems. Recent evidence has suggested that a moderate consumption of red wine may protect against the development of CHD. Phenolics act as potent antioxidants *in vitro* however the contribution of individual components in red wine, their protective effects *in vivo* and their absorption and metabolism have yet to be addressed.

This study set out to achieve the following aims:

1. To identify and quantify the major phenolic contributors to the *in vitro* antioxidant capacity of red wine.
2. To investigate the influence of a moderate daily consumption of red wine on the antioxidant status and on indices of oxidative stress associated with CHD in healthy volunteers.
3. To determine the absorption, metabolism, excretion and distribution of a grape seed extract rich in (+)-catechin and procyanidins in a rat model system.

Chapter 1 Introduction

1.1 Diet and Health

A diet rich in fruits and vegetables has long been recognised to protect against chronic diseases including cardiovascular disease (CHD and stroke) and certain cancers (WHO, 2003), and current dietary guidelines recommend a daily intake of at least 5 portions (400 g). Although the protection afforded by fruits and vegetables can be partly explained by associated lifestyle factors including abstinence from smoking and increased physical activity (Lampe, 1999), specific dietary constituents are considered to be important to health.

Phenolic and polyphenolic compounds are secondary metabolites widespread in the plant kingdom and form an integral part of the human diet with fruits, vegetables, tea and red wine providing an especially abundant source. During the past decade, interest has arisen in these compounds as there is some evidence to suggest that an increased consumption of phenolic rich foods/beverages may help prevent disease. Polyphenols are reducing agents and their potential health-related properties have been ascribed to their powerful antioxidant abilities, which may protect the body from damaging oxidation reactions, caused by 'free radicals' (Kanner *et al.*, 1994).

1.2 Free radicals, Oxidative stress & chronic disease

It is becoming increasingly evident that wide ranges of chronic diseases have oxidation events as a major component of their pathophysiology and has been implicated in the aetiology of a number of degenerative diseases including cancer, atherosclerosis and chronic inflammatory diseases (Beckman & Ames, 1998). Harman first proposed the 'free radical theory of ageing', which stated that free radicals produced during metabolism and other biological reactions cause cumulative cell damage leading to ageing and eventual death (Harman, 1956). This theory has since been transformed into a more general premise which highlights that an over-production of reactive oxygen species (ROS) during normal metabolic processes, or a loss of the

protective mechanisms that reduce the ability to withstand oxidative challenge is intricately connected to ageing and lifespan (Beckman & Ames, 1998).

1.2.1 Source of free radicals

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life. They are characterised as having one or more unpaired electrons and are therefore capable of reacting with electron donors to equilibrate its charge (Rice-Evans, 2001). Endogenously free radicals are generated as by-products through electron transport chains during normal aerobic metabolism (Halliwell, 1996), peroxisomes and the cytochrome P-450 system (Wiseman *et al.*, 1997). They are essential for the regulation of normal metabolic processes and immune function including cell growth, energy production, phagocytosis and the synthesis of nucleic acids, hormones and proteins. Free radicals are also generated exogenously through cigarette smoke, products of ionising UV radiation, environmental pollutants, lipid oxidation products in foods and excessive intakes of iron (Halliwell *et al.*, 1992; Kubow, 1993) (Table 1.1). Free radicals can be classified into oxygen, nitrogen, carbon and sulphur based molecules and include hydroxyl radical ($\text{OH}\cdot$), superoxide anion ($\text{O}_2^{\cdot-}$), singlet oxygen ($^1\text{O}_2$) and hydrogen peroxide (H_2O_2). The hydroxyl radical is the most reactive of the oxygen centred radicals and will react with the first available bio-molecule they encounter. Other free radicals include the 2 gaseous radicals nitric oxide ($\text{NO}\cdot$) and nitrogen dioxide ($\text{NO}_2\cdot$), and the carbon centred radicals ($\text{R}\cdot$) of organic compounds; alkoxy ($\text{RO}\cdot$) and peroxy ($\text{ROO}\cdot$) formed during the peroxidation of lipids. While the sulphur based radicals ($\text{RS}\cdot$) and ($\text{RS-SR}^{\cdot-}$) are involved in cellular function.

Table 1.1 Endogenous and exogenous sources of free radicals.

Source	Mechanism
Endogenous	
Mitochondrial electron transport	Leakage of superoxide due to inefficient reduction of oxygen
Transition metal ions	Copper and iron facilitate hydroxyl radical formation
Inflammation	Free radicals released by activated phagocytes
Enzymes <i>e.g.</i> Xanthine Oxidase	Release superoxide during reperfusion of ischemic tissues
Exogenous	
Drug metabolism	Free radical intermediates created during metabolism
Cigarette smoke	Gas phase rich in free radicals
Radiation	X-rays, UV light

1.2.2 Free radical reactions in biological systems

All of the major macromolecules found within the body are susceptible to oxidative modification by free radicals, including lipids, DNA and protein. Lipid peroxidation is probably the most extensively investigated free-radical induced process (Moore & Roberts, 1998). The lipid component of biological membranes is highly susceptible to oxidation and may undergo a rapid and destructive chain peroxidation process (Karten *et al.*, 1988). Lipid peroxidation is initiated by free radical attack on a double bond associated with a PUFA resulting in the abstraction of a H^+ atom from a methylene (CH_2) group, the rate of which determines the rate of initiation. The $OH\cdot$ radical is particularly effective in initiating lipid peroxidation (Morrisey *et al.*, 1994). The formation of the lipid radical is then accompanied by molecular re-arrangement that results in stabilisation of the unstable carbon radical into a conjugated diene. The conjugated diene reacts very quickly with molecular oxygen, and the peroxy radical thus formed is a crucial intermediate. Peroxy radicals can combine with each other or they can attack membrane proteins, however they are also capable of abstracting H^+ from adjacent fatty acid side chains in membranes and so propagate the chain reaction of lipid peroxidation. Lipid hydroperoxides can undergo further oxidation via its interaction with transition metals including copper and iron, yielding a complex mixture of secondary degradation products including hydrocarbon gases (ethane and pentane) and aldehydes (malonaldehyde and 4-hydroxynonenal) (Fig. 1.1). Proteins and nucleic acids seem to be less susceptible than lipids to free radical attack, in that there seems to be less possibility in the formation of rapidly progressing chain reactions. Nonetheless, DNA can undergo a series of reactions with ROS leading to DNA strand breaks (double and single) and base modifications which may result in genetic mutations, cell death, damage to DNA repair enzymes and chromosomal re-arrangements (Halliwell & Arouma, 1991). Moreover, amino acyl constituents crucial for the function of proteins are especially vulnerable to radical damage. The consequences of such damage may be aggregation, cross-linking or protein degradation and fragmentation, depending on the nature of the protein component and the attacking free radical species involved leading to altered enzyme activity, membrane and cellular function (Wolff *et al.*, 1986).

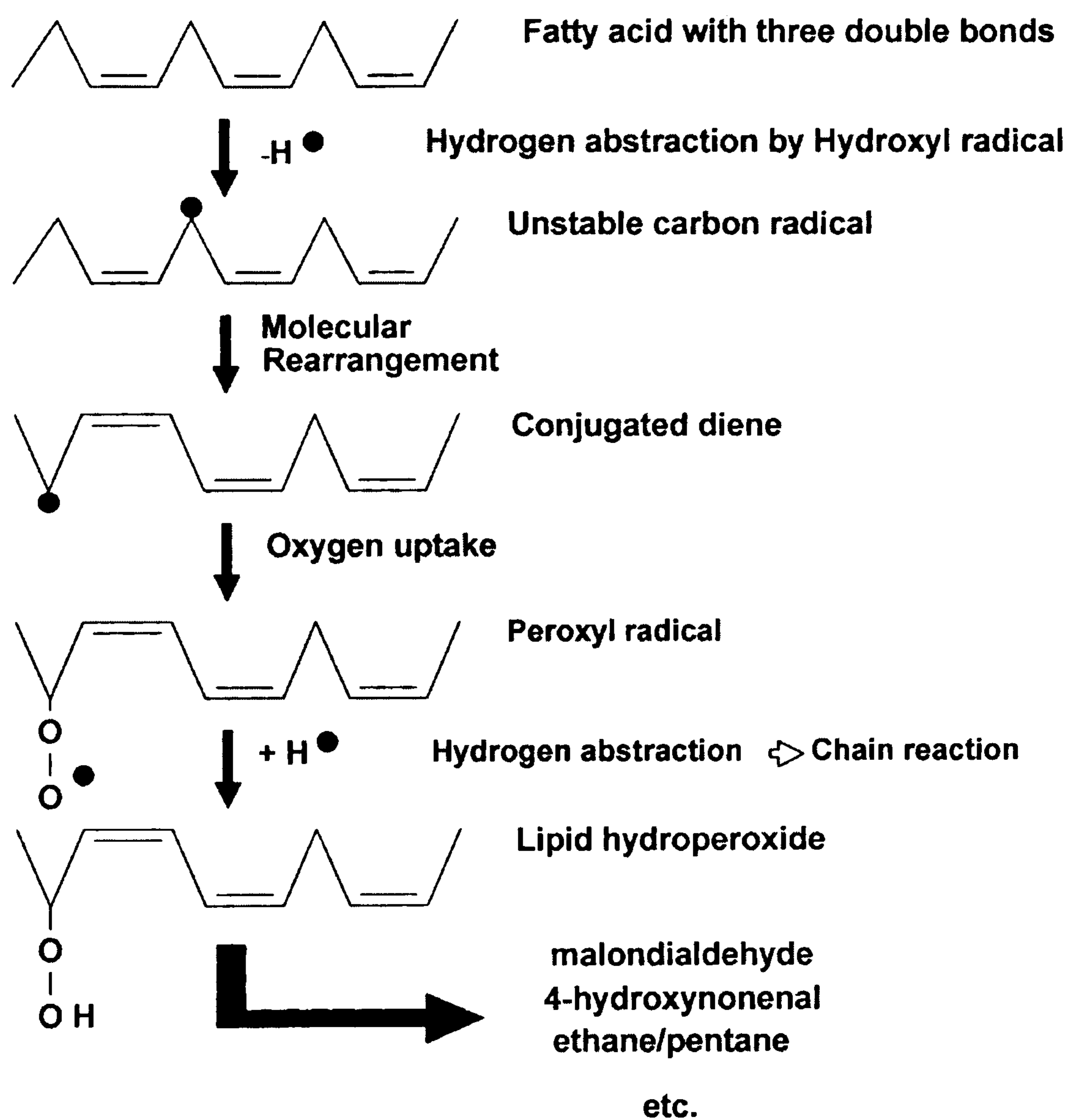


Figure 1.1 Basic reaction sequence of lipid peroxidation (Young & McEneny, 2001).

1.2.3 Antioxidant defence mechanisms

A number of antioxidant defence mechanisms and repair systems exist in the body to protect against the damage and reduce the adverse effects of free radicals (Table 1.2). This complex network involves mechanisms that reduce the initial formation of oxygen centred radicals of organic compounds (peroxyl and alkoxy radicals); scavenge free radicals, break chain-reactions and repair systems for damage (Yu, 1994). Intracellular antioxidant enzymes include superoxide dismutase, which removes superoxide radicals by accelerating the formation of hydrogen peroxide; glutathione peroxidase, converts hydrogen peroxide to water, and catalase, which breaks down hydrogen peroxide (Morton *et al.*, 2000). Non-enzymatic antioxidants act mainly as scavengers in oxidative chain reactions. They can be divided into lipid phase chain breaking antioxidants such as tocopherols and aqueous phase chain breaking antioxidants such as ascorbate. Several extracellular antioxidants such as proteins (transferrin, lactoferrin, albumin and caeruloplasmin) and urate prevent free radical reactions in the body by sequestering transition metals, thus reducing the occurrence of fenton-like reactions (Khan & Kasha, 1994).

Table 1.2 Enzymatic and non-enzymatic physiological antioxidants.

Antioxidant	Properties
<i>Enzymatic</i>	
SOD	removes $O_2^{\cdot-}$ by accelerating formation of H_2O_2 .
GSH	removes H_2O_2 and organic hydroperoxide
CAT	removes H_2O_2
<i>Non-enzymatic</i>	
vitamin C	free radical scavenger; recycles vitamin E
vitamin E	major chain-breaking antioxidant in cell membrane
glutathione	multiple roles in cellular antioxidant defence
uric acid	scavenger of OH radicals
α -lipoic acid	recycles vitamin C; effective glutathione substitute
carotenoids	scavenger of ROS; singlet oxygen quencher
bilirubin	extracellular antioxidant
ubiquinones	reduced form are efficient antioxidants
metal ions: <i>e.g.</i> transferrin	chelate metals ions responsible for fenton reactions
NO	free radical scavenger; inhibitor of lipid peroxidation

Adapted from Fang *et al.* (2002). SOD-superoxide dismutase; GSH-glutathione peroxidase; CAT-catalase; NO-nitric oxide; ROS-reactive oxygen species.

1.2.4 Antioxidant activity of polyphenols

Although the body has a number of endogenous defences, antioxidants from dietary sources also have an important role to play in quenching free radicals and in preventing damage. In addition to the common dietary antioxidants; vitamins C and E, phenolics and polyphenolics are purported to act as potent antioxidants in biological systems *in vitro*. The protective effects have been ascribed to their capacity to transfer electrons to free radicals, chelate metal catalysts (Ferrali *et al.*, 1997), activate antioxidant enzymes (Elliot *et al.*, 1992) and inhibit oxidases (Cos *et al.*, 1998). Scavenging free radicals can prevent the oxidation of biological matrices, and chelating metal ions such as copper and iron, can reduce the initiation of chain reactions in fatty acids present in LDL. Phenolics are also involved in the regeneration of vitamin C and E when they accept/donate an unpaired electron or H^+ from a free radical. The antioxidant activities of polyphenols are closely related to their structure and a number of important structural determinants have been identified. The common flavonoids, myricetin, quercetin, rutin and gallic acid have been shown to exert greater antioxidant capacities than the conventional antioxidant vitamin α -tocopherol. This is due to the number of hydroxyl groups which enable the compound to donate H^+ and delocalise the resulting free electron. In general the greater the number of hydroxyl groups on a structure, the greater the antioxidant activity (Salah *et al.*, 1995). Studies have shown that quercetin is a potent antioxidant due to the number and distribution of its hydroxyl groups (Rice-Evans *et al.*, 1995), in addition to (+)-catechin (Teissedre *et al.*, 1996). Particular combinations are found to offer greater activity. Bors *et al.* (1990) proposed the following structural determinants for effective radical scavenging properties of flavonoids: (1) The *O*-dihydroxy (catechol) structure in the B-ring. This is an obvious radical target site for all flavonoids with a saturated 2,3-double bond. (2) The 2,3-double bond in conjugation with a 4-oxo function, which is responsible for electron delocalisation from the B-ring. (3) The additional presence of both 3' and 5' hydroxyl groups for maximum radical scavenging potential and strong radical absorption. Moreover, the ability of flavonoids to donate electrons and stop chain reactions is attributed to the phenolic hydroxyls with activity increasing with the number of $-OH$ groups in the A and B rings, particularly the 3',4'-OH of the ring B and the *meta*

arrangement of –OH groups at the 5' and 7' positions of ring A are reported to further increase the antioxidant activity of a compound (Frankel, 1999).

1.2.5 Methods to determine antioxidant activity

A number of techniques have been developed to measure the activity of an antioxidant. Some techniques are based on a chemical reaction, while others make use of a biological response. Some of the most common methods used to assess the antioxidant capacity of phenolic rich foods and beverages are electron spin resonance (ESR), trolox equivalent antioxidant activity (TEAC), chemiluminescence, 2,2 azobis (2 amionopropane) hydrochloride (AAPH) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), while the ferric reducing antioxidant potential (FRAP) assay is most commonly used for the assessment of plasma antioxidant activity.

1.2.5.1 Electron spin resonance spectroscopy

ESR spectroscopy is unique in that it is only sensitive to transitions involving unpaired electrons, whereby the decay of radical resonance of either a water-soluble or organic-soluble radical is examined over time. This enables the kinetics and the stoichiometry of the donation of the H⁺ from the antioxidant to the radical to be determined (Duthie, 1999). Unlike many other antioxidant assays this method can be used with turbid or coloured samples such as red wine (Gardner *et al.*, 1999). The application of this approach has been tested in parallel with the common FRAP assay in the analysis of red wines (Burns *et al.*, 2000). Although two different methods were used to assess the antioxidant capacity the results obtained were highly correlated.

1.2.5.2 TEAC – trolox equivalent antioxidant activity

The TEAC assay is based on the oxidation-induced decolourisation of radical cation ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), and determines the ability of hydrophilic H⁺ donating antioxidants to scavenge the ABTS⁺ radical compared with Trolox (a water soluble analogue of vitamin E) (Miller *et al.*, 1993). The ABTS⁺ radical is not found naturally in the body and is strong compared with other

oxidising species. As the activity of an antioxidant compound depends on the free radical used this technique offers little information about the biological reactions of the antioxidants. This method overcomes some of the difficulties associated with other inhibition assays; although stringent control of reaction conditions and time is critical in obtaining reproducible results. This method gives a non-linear dose response, is insensitive to small changes in individual antioxidants when used in complex samples such as plasma, and antioxidant stoichiometric factors vary with test conditions (Schofield *et al.*, 1996).

1.2.5.3 AAPH method

The ability of compounds to trap peroxy radicals is assessed using the 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) assay. A steady stream of peroxy radicals is initiated by the addition of the azo compound. The decrease in absorbance/fluorescence of a reactive compound is quantified in the presence of a radical scavenger (Ghiselli *et al.*, 1998).

1.2.5.4 DPPH method

The radical scavenging effect of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) involves the quenching of the absorbance of a compound due to the scavenging of radicals by an antioxidant. This method can be extended to examine the kinetics of the radical scavenging by following the reaction over time rather than just at a fixed time point. This method has been used to determine the antioxidant activity of wines and phenolic compounds (Fauconneau *et al.*, 1997).

1.2.5.5 Chemiluminescence

The principal of this method is based on the oxidation of luminol by hydrogen peroxide in a reaction catalysed by horseradish peroxidase. Under normal circumstances this reaction produces low intensity light emission however in the presence of antioxidants light emission is diminished. The length of time of light quenching is related to the

amount of the antioxidant present. This approach has been used to determine the antioxidant capacity of plasma and phenolic compounds (Whitehead *et al.*, 1992).

1.2.5.6 FRAP assay

The FRAP assay is simple, inexpensive and robust and provides an index of antioxidant activity (Benzie & Strain, 1996). It describes the ability of a compound to reduce Fe^{3+} to Fe^{2+} . This reduction is accompanied by the formation of a blue colour in the presence of a ferric-tripyridyltriazine (Fe^{3+} -TPTZ) complex. The dose-response is linear over a wide range, stoichiometric factors of antioxidants are constant in pure solution, aqueous mixtures and in complex mixtures such as plasma, and therefore is suitable for the analysis of a range of biological fluids and extracts of foods/beverages. FRAP values are similar to those obtained using other types of antioxidant techniques including chemiluminescence (Whitehead *et al.*, 1992), TRAP (Lissi *et al.*, 1995) and ESR, as described previously.

1.3 Chemistry and Classification of Phenolics

Plants synthesise a vast and diverse range of secondary metabolites, the majority of which do not appear to be involved in essential processes of plant physiology. Their production in plant tissues is influenced by a wide range of environmental factors in response to stress, including exposure to UV radiation, attack by fungi or bacteria and disease (Landry *et al.*, 1995). As a consequence a number of physiological and ecological functions have been described in plants including plant defense, chemical signalling, protection against UV radiation, regulation of hormones, organogenesis, in addition to contributing to their colour and flavour (Vogt *et al.*, 1994; Bravo, 1998). In humans they have been reported to exhibit a wide range of biological effects including anti-bacterial, anti-viral, anti-inflammatory and anti-allergic properties (Cook & Samman, 1996). Phenolics are characterised by having an aromatic ring bearing one or more hydroxyl (OH) substituents, and range from simple low molecular weight, single aromatic compounds to the large highly polymerised and complex tannins. They occur predominantly as conjugates with sugars, glucuronic or galacturonic acids, other phenols or, less frequently, to aromatic carbon atoms (Bravo, 1998). Modifications in

their basic structure such as oxidation, hydroxylation, glycosylation and methylation has led to a wide range of phenolic substances with over 8000 structures reported to date (Strack, 1997). Phenolics can be classified according to the number and arrangement of their carbon atoms and can be classified into two major groups, the flavonoids and the non-flavonoids. The majority of phenolics are synthesised from carbohydrates via the shikimate and phenylpropanoid pathways (Fig. 1.2).

1.3.1 Flavonoids

Flavonoids comprise one of the largest and most widely dispersed groups of secondary plant metabolites (Kuhnau, 1976) with over 6000 flavonoids indentified to date (Harbone & Williams, 2000). Flavonoids mainly exist in plants as glycosides, while aglycones (lacking sugar moieties) occur less frequently. Their common structure is that of diphenylpropanes which has 15 carbon atoms arranged in three rings (C₆-C₃-C₆) consisting of two benzene rings (A & B), which are connected by an oxygen containing pyrane ring (C) (Figure 1.3).

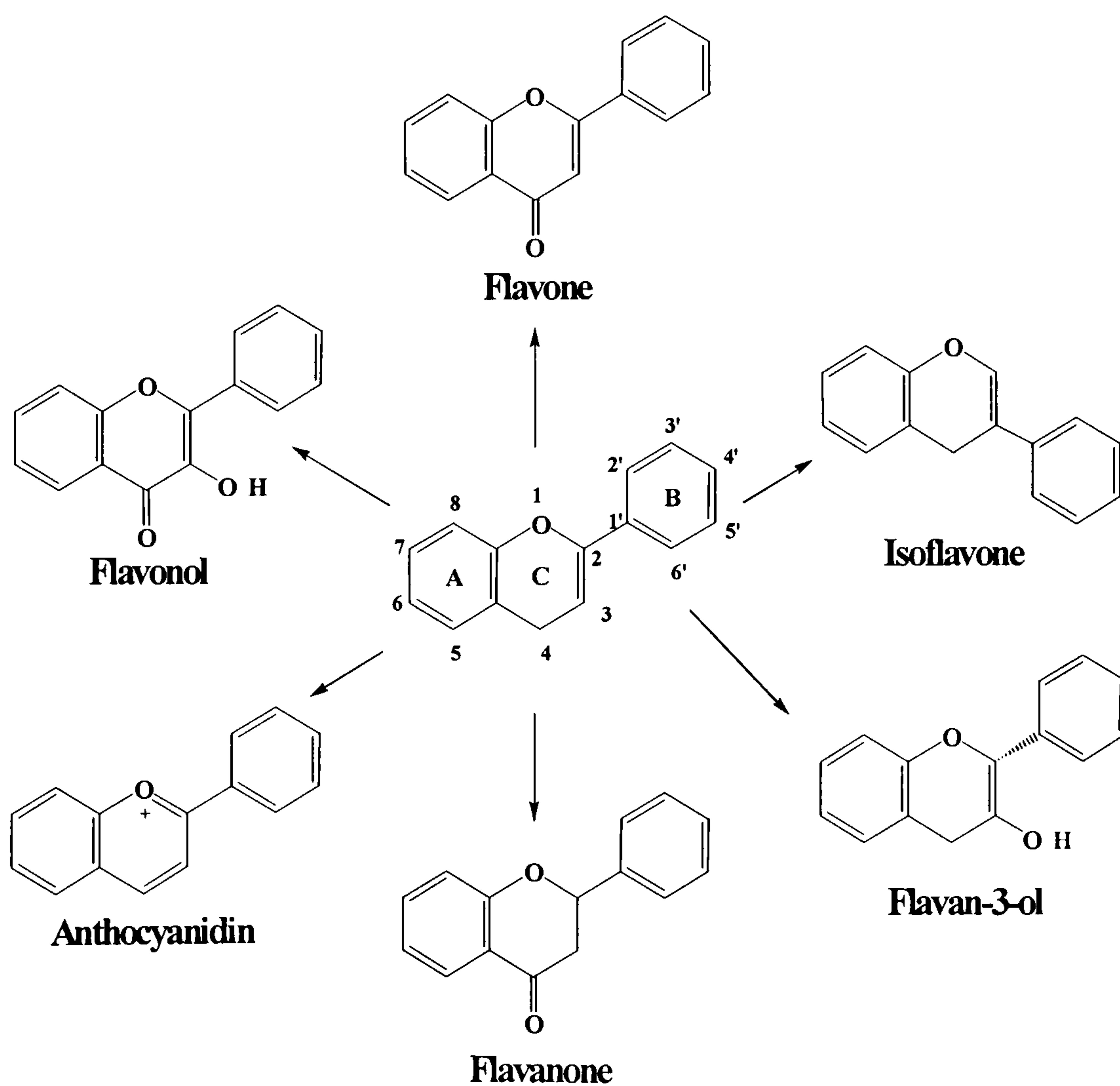


Figure 1.3 Generic structures of the major flavonoids.

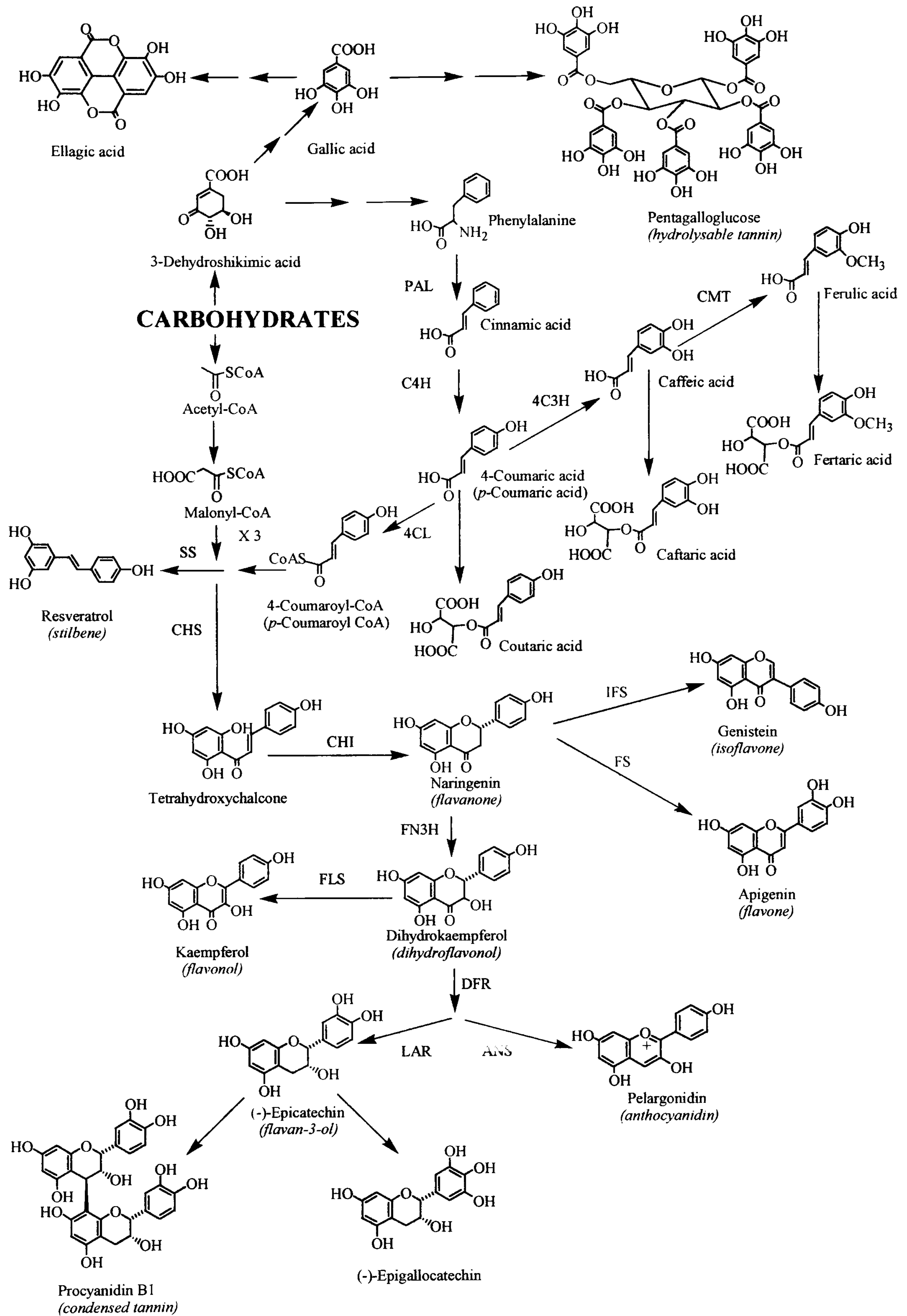


Figure 1.2 The Shikimic acid, phenylpropanoid & flavonoid biosynthetic pathways. Enzyme abbreviations: PAL-phenylalanine ammonia-lyase; C4H-cinnamate 4-hydroxylase; 4C3H-4-coumarate 3-hydroxylase; CMT-cafeate methyl transferase; 4CL-4-coumarate: CoA ligase; SS-stilbene synthase; CHS-chalcone synthase; CHI-chalcone isomerase; IFS-2-hydroxyisoflavone synthase; IFD-2-hydroxyisoflavanone dehydratase; FNS-flavone synthase; FHT-flavanone 3-hydroxylase; FLS-flavonol synthase; DFR-dihydroflavonol 4-reductase; ANS-anthocyanidin 4-reductase; LAR- leucoanthocyanidin 4-reductase (Goldberg, 2003a).

Flavonoids are formed from the combination of derivatives synthesized from phenylalanine (via the shikimic acid pathway) and acetic acid; the 'A' ring is formed from three acetate units (via the malonic acid pathway) and the 'B' ring with the 3-carbon bridge is constructed of a phenylpropane unit via the shikimic acid pathway. The first step involves the formation of phenylalanine from phenylpyruvate. Phenylalanine is transformed to trans-cinnamic acid, which is then hydrolyzed to *p*-coumaric acid (C-9). The C-9 acids condense with three C-2 (malonyl-coA) units to form a C-15 chalcone. Subsequent ring closure and hydration give rise to such compounds as the 3-hydroxyflavonoids (catechins) and 3, 4-diolflavonoids (flavonols) (Dixon & Steele, 1999). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring and as such are subdivided according to their chemical structures into six classes: flavanones, flavones, flavonols, isoflavonoids, anthocyanins, and flavan-3-ols. Flavonoids are present in most edible fruits and vegetables, and are found in a wide range of beverages including tea and red wine. The main dietary flavonoids and their sources are shown in Table 1.3.

1.3.1.1 Flavonols

Flavonols are mainly represented by myricetin, quercetin, kaempferol and isorhamnetin. Conjugation occurs most commonly at the 3-position of the C ring although 5, 7, 4', 3' and 5' substitutions also occur (Herrman, 1976). Flavonols are commonly found in a wide range of fruits, vegetables and beverages with the most common flavonol in the diet being quercetin.

Quercetin is present in various fruits and vegetables, including tea and red wine but the highest concentrations are found in onions (Hertog *et al.*, 1992). Quercetin is present in plants in many different glycosidic forms (Kühnau 1976) with quercetin-3-rutinoside, also called quercetin-3-rhamnoglucoside or rutin, being one of the most widespread forms. Other flavonols in the diet include kaempferol (broccoli), myricetin (berries) and isorhamnetin (onion).

1.3.1.2 Anthocyanidins

Anthocyanidins, an extended conjugation made up of the aglycone of the glycoside anthocyanins, are a group of water-soluble compounds responsible for most floral, fruit and leaf pigmentation in nature (Clifford, 2000a). The most common naturally occurring anthocyanins are 3-*O*-glycosides. Structurally anthocyanins consist of an anthocyanidin bound to one or more sugar moieties, and with a sugar always present at the C3 position and frequently on carbons 7, 3' and 5'. They can also form conjugates with hydroxycinnamates and organic acids such as malic acid and acetic acid. Anthocyanins are responsible for the red, blue or violet colour of edible fruits including grapes, plums and berries, with levels increasing during fruit maturation (Peterson & Dwyer, 1998). The most common anthocyanidins include pelargonidin, cyanidin, delphinidin and malvidin (Kühnau, 1976).

1.3.1.3 Flavan-3-ols

Flavan-3-ols range from the simple monomers (+)-catechin and its isomer (-)-epicatechin, to the oligomeric and polymeric proanthocyanidins, also known as condensed tannins. In addition to forming complexes with other flavan-3-ols, they are hydroxylated to form the gallo catechins, and also undergo esterification with gallic acid. Furthermore, methylation, prenylation and *O*-glycosylation reactions have all been reported (Porter, 1992). (+)-Catechin and (-)-epicatechin are found in various fruits and vegetables such as apples, pears, grapes and peaches (Arts *et al.*, 2000a), with the highest concentrations of catechins found in tea and red wine (Arts *et al.*, 2000b).

1.3.1.4 Flavones

Flavones are structurally similar to the flavonols, however they lack oxygenation at position C3. A variety of substitutions is possible providing a wide array of natural and synthetic compounds; these include hydroxylation, methylation, *O*- and *C*-alkylation and *O* and *C*-glycosylation. Most flavones occur as 7-*O*-glycosides (Bohm, 1998).

Flavone distribution appears to be limited to only a few plant families, with the main flavones in the diet being apigenin and luteolin. They have been identified in celery (apigenin), sweet red pepper (luteolin), parsley and other herbs (Hertog *et al.*, 1992).

1.3.1.5 Flavanones

Flavanones are mainly represented by taxifolin, naringenin and hesperitin. Flavanones are characterized by the absence of the C2-C3 double bond and the presence of a chiral center at C2. The flavanone structure is highly reactive and they have been reported to undergo hydroxylation, glycosylation and *O*-methylation reactions. The main dietary source of flavonones is citrus fruit and the most commonly consumed is hesperitin from oranges (Rousseff *et al.*, 1987). Naringenin is found in tomatoes and tomato-based products. Fresh tomatoes, especially tomato skin, also contain naringenin chalcone, which is converted to naringenin during processing to tomato ketchup (Krause & Galensa, 1992).

1.3.1.6 Isoflavanones

Isoflavonoids are characterised by having the B ring attached at the C3 of the phenylchromane structure. Isoflavonoids are derived from the biosynthetic pathway and can be converted into a wide range of different isoflavonoids including isoflavones, isoflavanones and isoflavonols (Harbone, 1993). Isoflavones are mainly represented by daidzen and genistein. The main dietary source is soybeans and soy products, with soy containing ~ 1 mg of genistein and daidzen per gram of dry bean and much lower concentrations are present in other legumes (Mazur, 1998; Liggins *et al.*, 2000). These compounds have received much attention due to their putative role in the prevention of breast cancer and osteoporosis (Tapiero *et al.*, 2002).

Table 1.3 The main dietary sources of flavonoids

Flavonoid¹	Food source	Content of aglycone (mg/kg)
<i>Flavonol</i>		
Quercetin-3, 4'-glucoside	onion	280-490 ¹
Quercetin-3-rhamnoglucoside (rutin)	black tea	10-25 ²
	blackcurrant	44 ³
Quercetin-3-galactoside	apple	21-72 ¹
Myricetin	green beans	30 ¹
Kaempferol	kale	210-470 ¹
<i>Flavone</i>		
Luteolin-7-apiosylglucoside	sweet red pepper	15-39 ¹
Apigenin	celery	15-60 ¹
<i>Flavanone</i>		
Hesperitin-7-rhamnoglucoside (hesperidin)	orange	116-201 ⁴
Naringenin-7-rhamnoglucoside (naringin)	grapefruit	68-302 ⁴
<i>Flavan-3-ols</i>		
(+)-catechin	apple; red wine	4-16 ⁵ ; 16-53 ⁶
(-)-epicatechin	apple; red wine	67-103 ⁵ ; 9-42 ⁶
procyanidins	chocolate; red wine	165; 22 ⁹
<i>Anthocyanins</i>		
Cyanidin-3-glucoside	blackcurrant	760 ⁷
Cyanidin-3-rutinoside		
Delphinidin-3-glucoside		590 ⁷
Delphinidin-3-rutinoside		
<i>Isoflavanones</i>		
Genistein-7-glycoside	soy beans	480 ⁸
Daidzein-7-glycoside		330 ⁸

Adapted from: ¹ Hollman & Arts (2000); ² Hertog *et al.* (1993); ³ Häkkinen *et al.* (1999); ⁴ Mouley *et al.* (1994); ⁵ Arts *et al.* (2000a); ⁶ Arts *et al.* (2000b); ⁷ Nyman & Kumpulainen (2001); ⁸ Mazur *et al.* (1998); ⁹ Hammerstone *et al.* (2000).

1.3.2 Non-flavonoids

The main non-flavonoids found in the diet are the C₆-C₁ hydroxybenzoates, C₆-C₃ hydroxycinnamates and polyphenolic C₆-C₂-C₆ stilbenes. Phenolic acids exist primarily as conjugates and are rarely found in their acidic forms, often found bound to alcohols, sugars, polysaccharides, or organic acids through ester bonds.

1.3.2.1 Hydroxybenzoates

Hydroxybenzoates also referred to as phenolic acids include benzoic acid and derivatives including salicylic acid. Gallic acid is the major hydroxybenzoate and is synthesised from phenylalanine via 3-dehydroshikimic acid. It is converted to ellagic acid and a range of gallotannins, with the formation of hydrolysable tannins (polymers of gallic and ellagic acids). Black tea and red wine provide rich dietary sources of gallic acid (Dunfresne & Fransworth, 2001; Soleas *et al.* 1997).

1.3.2.2 Hydroxycinnamates

Hydroxycinnamic acids: *p*-coumaric, ferulic, sinapic, caffeic acids and their derivatives are the most important subclass of phenolic acids. Cinnamic acid is produced by the deamination of the amino acid phenylalanine by ammonia lyase (PAL), with *p*-Coumaric acid being produced by hydroxylation of cinnamic acid. In addition to being found in their free form hydroxycinnamates are also found esterified to sugars, organic acids and choline (Strack, 1997). Caffeic acid occurs in foods mainly as an ester with quinic acid or chlorogenic acid (5-caffeoylquinic acid). Coffee is a major dietary source of chlorogenic acid in the diet with dietary intakes estimated at 0.5-1 g/day (Clifford, 2000b).

1.3.2.3 Stilbenes

The stilbene family has a C₆-C₂-C₆ structure and are known to be phytoalexins, a class of antibiotic compounds produced as a part of a plant's defense system against disease. Trans-resveratrol (trans-3,5,4'-trihydroxystilbene) is synthesised by condensation of 4-

coumaroyl CoA with three units of malonyl CoA, each of which donates two carbon atoms, in a reaction catalysed by stilbene synthase. Resveratrol exists as two isomers; *cis* and *trans* with *trans-resveratrol-3-O-glucoside* commonly found in various families of plant tissues such as eucalyptus, spruce, and lily. Grapes, peanuts and their products are considered the most important dietary sources of the resveratrol, with levels of 0.02-1.79 $\mu\text{g/g}$ and 0.6-8 $\mu\text{g/mL}$ reported in peanuts and red wine (Sanders *et al.*, 2000).

1.4 Dietary Sources & Levels of Intake

Dietary intake values for phenolic and polyphenolic compounds is limited and often difficult to interpret largely due to the limited food composition data available. There are also large differences in the phenolic content of foods depending on variety, storage conditions, methods of preparation and degree of ripeness (Crozier *et al.*, 1997). Differences in dietary habits and food preferences further complicate dietary intake estimations for example it is estimated that phenolic acids account for approximately one third of total phenol intake and flavonoids account for two thirds. Coffee is a major dietary source of chlorogenic acid and intakes have been estimated to be around 0.5-1 g/day (Clifford, 2000b), therefore heavy coffee drinkers will likely consume more phenolic acids than flavonoids. Conversely, tea drinkers may have a much greater intake of flavonoids since the average flavonoid intake from tea alone in the UK has been estimated to be 430 mg/d. This is based on a population average consumption of 3.3 cups of tea per day (Wiseman *et al.*, 2001). In Japan, isoflavone intake is estimated to be around 30-40 mg/day (Kimira *et al.*, 1998), considerably higher than those in western populations (Kirk *et al.*, 1999). The bioavailability of certain flavonoids differs greatly depending on the food source. Hollman *et al.* (1997) reported that the absorption of quercetin from onions is four-fold greater than from apples or tea. The limited food composition data available to date do not take these factors into account and therefore their use may introduce significant measurement error in dietary studies. However, there is now an increasing number of comprehensive databases providing information on the flavonoid content for a wide range of foods/beverages in a range of populations including the USA (www.nal.usda.gov/fnic/foodcomp) and The Netherlands (Hollman & Arts, 2000).

1.4.1 Flavonols and Flavones

Kuhnau, (1976) estimated total flavonoid intake at over 1000 mg per day (expressed as glycosides) in the USA, consisting mainly of the flavonols, flavanones and flavones. However, the analytical methods used at the time were not standardised or specific and the validity of the data remains questionable. HPLC methods were developed and validated for the analysis of three major flavonols (quercetin, myricetin and kaempferol), and two flavones (apigenin and luteolin) in commonly consumed fruits, vegetables and beverages in The Netherlands (Hertog *et al.*, 1992). The findings from this study were used to calculate the intake of flavonols and flavones in over 4000 free-living adults in the Dutch National Food Consumption Survey. The average intake of flavonols and flavones was estimated to be 23 mg/day, with quercetin contributing 16 %; kaempferol, 3.9 mg/day; myricetin, 1.4 mg/day and flavones contributing only 7 % (Hertog *et al.*, 1993). In this population tea provided the major dietary source of flavonol intake (48 %), followed by onions (29 %) and apples (7 %).

Consistent results have been reported in the USA with overall intake of flavonoids estimated at 20 mg/day (Rimm *et al.*, 1996a), and in the UK total intake of flavonoids (quercetin, kaempferol, apigenin and luteolin) was estimated to be 30 mg/day, with quercetin contributing 64 % of the total intake (Wearne, 2000). In the Seven Countries Study Hertog *et al.* (1995) calculated flavonol intakes and reported that tea was the predominant source of quercetin in The Netherlands and Japan, and wine was the major source in Italy. Onions and apples contributed most in the US, Finland, Greece and former Yugoslavia. Similar findings have been reported by Sampson *et al.* (2002), whereby onions, tea and apples provided the major dietary sources of flavonoid intake in the USA. It is important to note that the reported dietary flavonoid intakes and food sources are largely based on the content of three flavonols (quercetin, myricetin and kaempferol) and two flavones (apigenin and luteolin) therefore total flavonoid intakes and content of foods can be assumed to be greater than those reported (Table 1.4).

1.4.2 Flavan-3-ols

Information on the dietary intakes of total flavan-3-ols has been reported in a national survey of 6200 Dutch males and females aged 1-97 years (Arts *et al.*, 2000a). Average intake was estimated to be 50 mg/day with tea providing a major dietary source in all age groups, followed by chocolate, apples and pears. Similar estimates have been reported in a previous study with levels of intake at 20-50 mg/day (Dragsted *et al.*, 1997). However, data on the dietary sources and levels of intakes of individual flavan-3-ols is less well documented. De Pascual-Teresa *et al.* (2000a) quantified individual flavan-3-ols in a range of foods and beverages, with chocolate, tea and red wine providing the major dietary sources. (-)-Epicatechin, (+)-catechin and procyanidin B₂ were the most abundant flavan-3-ols identified in these foods. Similar findings were documented by Carando and Teissedre, (1999) whereby catechins (as the sum of monomers, procyanidin dimers and trimers) were the most abundant in red wine (56 mg/100 mL) and green tea (42 mg/100 mL). Ruidavets *et al.* (2000) created a catechin food composition table giving information on the total catechin average supplied by 100 g of food. Green tea (169 mg); strawberries (56 mg); red wine (25 mg) and apricots (20 mg) provided the richest food sources. The proanthocyanidin content of grape, apple, hawthorn, elderberry, chokeberry, sour cherry and blackcurrant has been reported to be between 0.3 and 0.9 g/kg (Wilska-Jeszka, 1996).

1.5.3 Anthocyanins

Anthocyanins are present in red fruits and berries as well as red wines and the intake of anthocyanins may exceed 200 mg/day (Kuhnau, 1979). The content in fruits varies considerably between 0.25 to 700 mg/100 g fresh weight, with some berries containing in excess of 100 mg per 100 g (McGhie *et al.*, 2003). Blackcurrants contain delphinidin conjugates (1150-2500 mg/kg), raspberries and redcurrants contain cyanidin conjugates (100-600 mg/kg and 177 mg/kg respectively) and pelargonidin conjugates are found in strawberries (150-350 mg/kg) (Clifford, 2000a; Maatta *et al.*, 2001). Levels in red wines have been reported to be around 12 mg/100 mL (Timberlake, 1998).

Table 1.4 Reported dietary sources and intakes of flavonols and flavones

Country	Dietary intake (mg/d)	Main dietary sources
Denmark	26	tea, onions, apples
Finland	0-41	fruit, vegetables, apples, onions
Greece	15	fruits and vegetables
Italy	23-35	red wine, fruits and vegetables, soups
Japan	17-68;	green tea
The Netherlands	23-33	tea, onions, apples
United States	20	onions, black tea

Adapted from Aherne & O'Brien, (2002).

1.5 Epidemiology and Flavonoids

1.5.1 Flavonoids and Cardiovascular disease

Several epidemiological studies have investigated the association between flavonoid intake and CHD. Flavonoid consumption has been linked to a lower risk of heart disease in some, but not all, studies. In the Zutphen Elderly Study (Hertog *et al.*, 1993) the flavonoid (flavonol and flavone) intake of 805 Dutch men aged 65-84 years was assessed and a significant inverse association between dietary flavonoid intake and mortality from CHD was reported. An inverse but weaker association with the incidence of myocardial infarction was further reported. Those in the highest tertile of flavonoid intake had a risk of heart disease that was about 58 % lower than that of counterparts in the lowest tertile of intake. Those in the lowest tertile consumed 19 mg or less of flavonoids per day, whereas those in the highest tertile consumed approximately 30 mg per day or more. The 10-year follow up of the Zutphen Elderly Study reported a clear-dose response relationship between flavonol intake and CHD mortality (Hertog *et al.*, 1997). In a prospective study of 34,492 postmenopausal women in Iowa total flavonoid intake was associated with a decreased risk (RR=0.62) in the group with the highest flavonoid intake (Yochum *et al.*, 1999). Mortality from CHD was weakly associated with flavonol and flavone intake in a Finnish population with those in the highest quartile of flavonoid intake having a risk of mortality that was about 27 % (for women) and 33 % (for men) lower than that of those in the lowest quartile (Knekt *et al.*, 1996). In a recent study Knekt *et al.* (2002) examined the flavonoid intake of 10 054 men and

women and found that a high dietary intake of quercetin was associated with a reduced mortality from ischaemic heart disease. In contrast the protective effect of flavonoids could not be confirmed in other studies. For Welsh men, flavonol intake did not predict a lower rate of ischemic heart disease; on the contrary disease outcome was higher in all quartiles of high flavonol intake (Hertog *et al.*, 1997). For U.S. male health professionals, data did not support a strong link between flavonol and flavone intake and mortality was found only in men with a previous history of CHD (Rimm *et al.*, 1996a). In a recent prospective study Sesso *et al.* (2003) reported that flavonoid intake was not strongly associated with a reduced risk of CVD, and there was no evidence of any effect from individual flavonols or flavones. Similarly, there was no evidence for a protective effect from any food source although there was an inverse but non-significant association with broccoli, tea and apple consumption. A recent study by Arts *et al.* (2001) investigated the association of (+)-catechin intake and incidence of and mortality from ischemic heart disease and stroke using data from The Zutphen Elderly Study. While the authors concluded that catechin intake, mainly from tea, apples and chocolate, reduced the incidence of heart disease there was no association between catechin intake and stroke.

1.5.2 Flavonoids and cancer

Observational evidence for a protective effect of flavonoids against cancer remains contradictory. Analysis of the data from the Seven Countries Study failed to detect an association between intake of flavonoids (flavonols and flavones) and mortality from total cancer, lung cancer, stomach cancer and colon cancer (Hertog *et al.* 1995) or with the incidence of all-case cancer in the Zutphen Elderly Study (Hertog *et al.* 1994). Consistent findings were reported in a large cohort study of 120 850 Dutch men and women between intake of flavonols and flavones with stomach, lung or colon cancer (Goldbohm *et al.* 1995). Conversely, in a cohort study of nearly 10 000 Finnish men and women an inverse relationship was observed between dietary flavonol intake and the development of all-case cancer, particularly against lung cancer (Knekt *et al.*, 1997). Similar findings were reported in a case-control study by Garcia-Closas *et al.* (1998), whereby the flavonols quercetin and kaempferol were found to be the most protective against the incidence of gastric cancer. In a recent case-control study Peterson *et al.*

(2003) reported an inverse relationship between consumption of vegetables and incidence of breast cancer. Moreover, they reported a highly significant inverse association of flavone intake with breast cancer, whereas this association became non significant with flavanones, flavan-3-ols, flavonols, anthocyanidins or isoflavones.

1.6 Grape and wine phenolics

Red wine is a product of the natural fermentation of grape juice/must and is produced from a variety of different grape cultivars including Cabernet Sauvignon, Merlot, Pinot Noir, Rondinella, Sangiovese, Grenache, Tempranillo and Carignan. The commercial production of wine is a long and rigorous process leading from the initial crushing or pressing of the grape to the final bottling of the finished wine. The methods used for red wine vinification are highly variable depending on the country, region, winery, winemaker and vintage. Generally, once the grapes are harvested they are destemmed and pressed and the juices (must) together with the crushed grapes are fermented for ~ 5-10 days at 25 °C. Phenolics contained within the skins, seeds and flesh of black grapes are extracted during the process of vinification and with prolonged extraction the fermented must can contain up to 40 % of the phenolics originally present in the grape. Once alcoholic fermentation is complete the solids are removed and the young wine is subjected to malolactic fermentation. This secondary fermentation process is responsible for the conversion of the strong malic acid found in wine to the weaker lactic acid. This softens the acidity of the wine and adds to its complexity and stability. The wine is then matured in stainless steel vats or in the case of higher quality wines in oak barrels for varying periods of time prior to filtration and bottling (Soleas *et al.*, 1997).

The production of white wine is slightly different from the processes used during the making of red wine. White wine is produced from both black and white grape varieties and is subject to limited maceration and slight juice oxidation prior to fermentation. Solids are removed and the clarified juice is fermented for 5 days at 16 °C. The resultant must which is low in phenolics, due to the gentle maceration of the grapes and the early removal of the seeds and skin, then undergo malolactic fermentation prior to maturation,

filtration and bottling. Red wines therefore contain large amounts of phenolic compounds derived from the skin, seeds and flesh of black grapes. Phenolics are of particular importance to the characteristics and the quality of red wine, influencing the appearance, taste, aroma, mouth-feel and anti-microbial properties (Soleas *et al.*, 1997).

1.6.1 Phenolic composition of red wine

Red wine is a rich and concentrated source of phenolic compounds containing grape, yeast and even oak derived products (German and Walzem, 2000). However, the major compounds are grape derived; from the seeds, flesh and skins of black grapes (Singleton, 1982). Wines are extremely heterogenous in terms of their colour, flavour, appearance, taste and composition as a consequence of the differences in viticulture and vinification methods (Table 1.5). Vineyard factors such as grape variety, quality, and level of maturity, climate, geographical origin and disease pressure affect the phenolic compounds which accumulate in the grape. During vinification the length of skin contact, temperature and presence of seeds, vine stems and enzymes have all been reported to influence the extraction of phenolics into the fermenting juice (McDonald *et al.*, 1998; Price *et al.*, 1995; Kovac *et al.*, 1992). The most common phenolics present in significant quantities include free and conjugated flavonols, hydroxycinnamates, gallic acid, ellagitannins, stilbenes, flavan-3-ols, free and polymeric anthocyanins (Fig. 1.4).

Table 1.5 Range of concentrations of phenolics in red wines

Phenolics	Range (mg/L)
Total flavonols	5-55
Total stilbenes	1-18
Gallic acid	8-71
Total hydroxycinnamates	66-124
(+)-Catechin and (-)-epicatechin	8-60
Free and polymeric anthocyanins	41-150
Total phenolics	824-4059

Adapted by Burns *et al.* (2000).

1.6.1.1 Flavonols

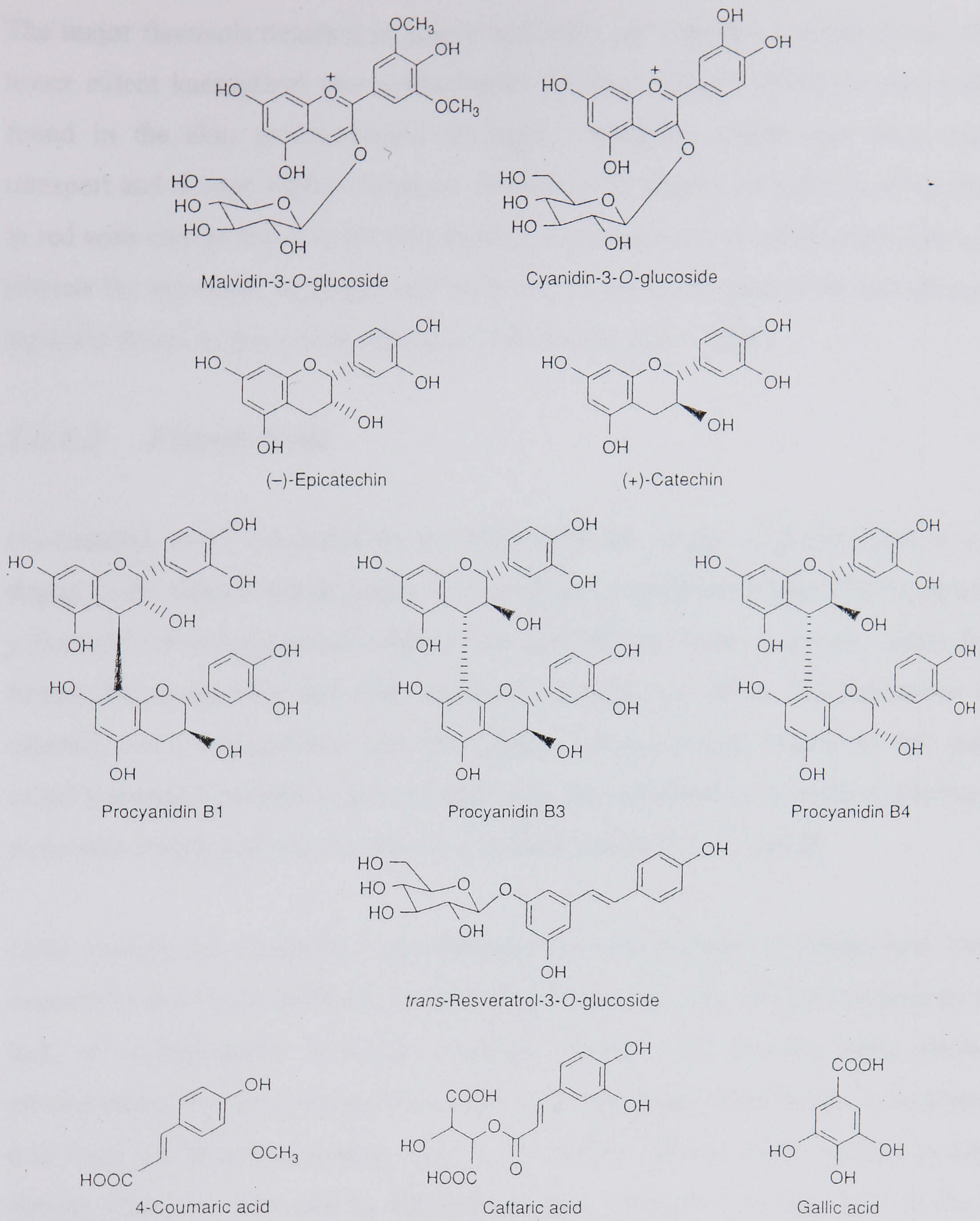


Figure 1.4 Structure of the major phenolics present in red wine

1.6.1.1 Flavonols

The major flavonols detected in grapes and wine are quercetin and myricetin, and to a lesser extent kaempferol and isorhamnetin. In black grapes flavonols are exclusively found in the skin predominantly as sugar conjugates which aids their solubility, transport and storage within the tissue. In contrast to grapes, up to 50 % of the flavonols in red wine can be found in the free form as a consequence of vinification which acts to liberate the aglycone. In grapes and wine they occur as *O*- glucosides and glucuronides, typically bound to glucose or rhamnose (McDonald *et al.*, 1998).

1.6.1.2 Flavan-3-ols

(+)-Catechin and (-)-epicatechin are located in the seeds of grapes, and to a lesser degree in the skins of black grapes. Lower levels of epigallocatechin (ECG), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) are found in grapes, which decrease further during ripening and wine maturation (Singleton, 1982). Polymerization of (+)-catechin and (-)-epicatechin and their gallate esters produce oligomers and polymers called proanthocyanidins (often referred to as procyanidins or condensed tannins), high molecular weight polymers of flavan-3-ol units linked by C-C bonds.

Until recently the complete characterisation of procyanidins in grapes and wine was impossible due to the difficulty in analysing high molecular weight compounds and the lack of commercially available standards. Grape seed contains high amounts of procyanidins. Dimeric procyanidins are the simplest and most common in grapes, and they have 4-8 linked monomers. B₁, B₂, B₃ and B₄ are the most common procyanidin dimers. These are followed by the less common 4-6 linked isomers such as B₅, B₆, B₇ and B₈ (Sun *et al.*, 1998). Young red wines contain (+)-catechin, (-)-epicatechin and oligomeric procyanidins composed mainly of soluble dimers, trimers and tetramers. However, during wine maturation the levels of these compounds decrease and there is a concomitant increase in higher oligomers which are less soluble (Haslam, 1998).

1.6.1.3 *Anthocyanins*

Anthocyanins are responsible for the red-purple tones of grapes and their corresponding wines. They are not found in white wine due to a synthetic blockage in the shikimate pathway and also due to the removal of the skins during the process of white wine making (Soleas *et al.*, 1997). They are found exclusively in the skins of black grapes and are efficiently extracted into the wine during maceration. The term anthocyanidin refers to the aglycone structures such as malvidin. Anthocyanins may be acylated on the sugar moiety with aromatic and aliphatic acids. The 3-*O*-glucosides of malvidin, peonidin, petunidin, petunidin and delphinidin are the most abundant anthocyanins in *V. Vinifera* and red wines (Burns *et al.*, 2000; Baldi *et al.*, 1995). During storage and ageing of red wine anthocyanins react with procyanidins to produce complex pigments which may precipitate. This is evident by the colour change in a wine from the original sharp bright blue-red tone to red-orange giving the wine a deeper hue.

1.6.1.4 *Non-flavonoids*

The primary non-flavonoids in wines are derivatives of hydroxycinnamic and hydroxybenzoic acids, especially gallic acid and its ellagic acid dimer and stilbenes (Soleas *et al.*, 1997). They are structurally simpler and are stored primarily in cell vacuoles of grape cells and are easily extracted on crushing. Gallic acid is present in the flesh of black and white grapes and their respective wines; however they found in much higher levels in red wines and may be 5-10-fold higher (Frankel *et al.*, 1995). Substantial amounts of caffeic acid are converted to caftaric acid and lower levels of coutaric acid, fertaric acid also accumulate in the skin and flesh of black and white grapes. In wine the stilbene, *trans*-resveratrol (3,5,4' -trihydroxystilbene) is found in the free form and also as the conjugate *trans*-resveratrol-3-*O*- β -glucoside (also known as polydatin or piceid). Piceid is known to play a role in disease resistance acting as a phytoalexin in grapes where it accumulates in the skin in response to fungal infection. The presence of the *cis* isomer of both free and conjugated resveratrol has also been reported in red wine. However, the levels of *trans*-resveratrol in red wine are found in much lower concentrations compared with other phenolics (Soleas *et al.*, 1997).

1.7 Wine and CHD

Since ancient times wine has closely been associated with diet particularly in Mediterranean countries, and is thought to contribute to the good health of the Mediterranean population (Willett, 1994). The association between a moderate and regular consumption of red wine in the protection against CHD has been well publicised (Puddley & Croft, 1999) an association popularised as the “French paradox” (Renaud and de Logeril, 1992). The paradox describes the seemingly low mortality rates in certain populations of France despite high levels of associated risk factors; serum cholesterol, systolic blood pressure, dietary saturated fat intakes and prevalent smoking rates. Red wines do not contain significant amounts of vitamins or selenium however provides an especially abundant and palatable source of polyphenols and one explanation for this anomaly is that the Mediterranean diet typically found in France, along with the regular consumption of red wine offered a form of protection.

1.7.1 The French paradox

A high dietary intake of saturated fat is a well known risk factor for CHD (St. Leger *et al.*, 1979). In most countries the level of dietary saturated fat is positively associated with mortality from CHD, however in some regions of France this relationship is less obvious (Renaud and de logeril, 1992). Evidence from the Monica program, one of the world’s largest CHD surveillance systems, confirmed that death rates from CHD were much lower in France, particularly in Toulouse, than in any other industrialised country (WHO, 1989). In fact the incidence of CHD in France was found to be the lowest in a study of several countries, second to Japan where the intake of dietary saturated fat is exceptionally low (Criqui and Ringel, 1994). This finding is paradoxical since saturated fat intake in France represents 14-15 % of energy intake, higher than the recommended 10 % of total energy. St. Leger *et al.* (1979) observed that among all countries France had the highest per capita intake of wine and the lowest rate of heart disease. This observation was further supported by data from the US that showed a negative correlation between death from acute myocardial infarction and wine consumption across all states (Werth, 1980). In a prospective study in men from Eastern France Renaud *et al.* (1998) observed that a moderate intake of alcohol, mostly in the form of

wine, was negatively associated with CHD mortality in those consuming two or more glasses of wine per day. Similarly, in a large-scale Danish prospective study intake of beer or spirits was not associated with a reduction in the risk of mortality from CHD, but rather a moderate consumption of red wine was most beneficial (Groenbaek *et al.*, 1995). It is well established that a moderate consumption of alcohol (~10-30 g/day) is associated with a reduced risk of CHD and overall total mortality (Rimm *et al.*, 1991; Klatsky *et al.*, 1992). Many studies have observed a U- or J-shaped correlation between alcohol consumption and risk of death from all cause mortality, with those consuming a moderate intake (1-2 drinks per day) having a lower incidence than abstainers and heavy drinkers (Marmont *et al.*, 1981; Doll *et al.*, 1994). The protective effect of alcohol has been shown to be independent of sex, age, ethnicity, smoking habits and physical activity (Kittner *et al.*, 1983). However, red wine is thought to provide additional health benefits beyond those of alcohol alone (Rosenberg *et al.*, 1981; Rimm *et al.*, 1996b). Polyphenols in red wine act as antioxidants and can reduce the free-radical mediated oxidation of LDL and the resulting atherogenicity (Furham *et al.*, 1995; Teissedre *et al.*, 1996; Nigidkar *et al.*, 1998), inhibit platelet aggregation (Pace-Asiak *et al.*, 1995) and endothelium-dependent relaxation of blood vessels, mediated by the NO-cGMP pathway (Fitzpatrick *et al.*, 1993).

1.7.2 Red wine and HDL cholesterol

The cardio-protective effects of alcohol have largely been attributed to its ability to raise the concentration of HDL cholesterol, a well defined negative risk factor for CHD (Gaziano *et al.*, 1999). This lipoprotein is central to the removal of excess cholesterol from the peripheral cells to the liver for excretion via reverse cholesterol transport. Although there is conflicting evidence some studies have reported that red wine can increase the concentration of HDL cholesterol and apolipoprotein A1, an important anti-atherogenic factor. Lavy *et al.* (1994) reported a significant increase in plasma HDL cholesterol concentrations following the consumption of 400 mL of red wine in human subjects. These effects were not observed following the consumption of white wine. In a recent clinical trial the effects of red wine and alcohol alone were assessed for their ability to increase HDL cholesterol concentrations. Serum HDL cholesterol significantly

increased following alcohol and red wine intake, as well as raising apolipoprotein A1 concentrations (Estruch, 2000).

To determine the possible beneficial effects of alcohol and non-alcoholic components of red wine Senault *et al.* (2000) compared the effect of red wine, de-alcoholised red wine and alcohol consumption on HDL cholesterol concentrations in humans. De-alcoholised red wine had no effect while red wine increased the serum levels of HDL cholesterol and apolipoprotein A1. Alcohol also increased apolipoprotein A1, however total cholesterol and triacylglycerides, which are known to be atherogenic, were also raised with alcohol alone. The authors concluded that the increase in HDL cholesterol with red wine consumption may have been due to the antioxidant properties which may protect lipoproteins against the pro-oxidant effects of alcohol.

1.7.3 Red wine and Platelet aggregation

Platelets contribute to the development of CHD through a number of mechanisms including alterations in platelet function, coagulation and fibrinolysis, resulting in atherosclerotic plaques or plugs which ultimately result in thrombus formation and vessel occlusion. An inhibitory effect of alcohol on platelet aggregation was first reported by Haut *et al.* (1974), and since then a number of *in vivo* and *in vitro* studies have shown an inverse association between platelet aggregation and alcohol consumption.

Hendriks *et al.* (1994) investigated the effect of moderate alcohol consumption on the fibrinolytic factors involved in clot formation. An increase in the level of tissue plasminogen activator (tPA), a clot dissolving enzyme, was observed. Other studies have shown that alcohol consumption inhibits thromboxane A2 production; a factor formed in platelets via the cyclo-oxygenase (COX) pathway and is a powerful pro-aggregatory agent favouring platelet aggregation (Seignuer *et al.*, 1990; Pace Asciak *et al.*, 1996).

A number of animal studies have demonstrated the ability of grape and red wine derived phenolic compounds to inhibit platelet aggregation (Osman *et al.*, 1998). Demrow *et al.* (1995) investigated the effect of red wine, white wine and grape juice on platelet

activity and thrombus formation in an *in vivo* dog model. Red wine and grape juice was effective anti-platelet and anti-thrombotic compounds when administered intravenously (1.6 mg/mL, 13 % alcohol in 200 mL saline) and intragastrically (4 mL/kg) indicating that the active compounds are absorbed and transported to the bloodstream. Administration of white wine had no effect suggesting that specific compounds in grape juice and red wine contain anti-thrombotic and platelet inhibitors in addition to ethanol.

There is evidence that specific phenolics exert anti-thrombotic properties *in vitro*. Russo *et al.* (2000) reported a significant anti-aggregatory effect with de-alcoholised red wine fraction rich in procyanidins, (+)-catechin and anthocyanins. No activity was associated with other phenolic constituents. Additional studies have shown *trans*-resveratrol to be a strong inhibitor of the COX pathway in reducing thromboxane A₂ production (Pace-Asciak *et al.*, 1995), and consumption of grape juice enriched with *trans*-resveratrol decreased thrombin induced platelet aggregation in human subjects (Pace-Asciak *et al.*, 1996). However, other studies have not shown the same effect following consumption of de-alcoholised red wine in human subjects (Lavy *et al.*, 1994; Pellegrini *et al.*, 1996; Rein *et al.*, 2000).

1.7.4 Red wine and Vasorelaxation

The vascular endothelium forms a barrier between the vascular smooth muscle and the flow of blood and controls the contraction and relaxation of the vascular muscle by responding to vaso-active stimuli, blood flow and stress (Stoclet *et al.*, 1997). The inability of blood vessels to contract and relax efficiently has been implicated in the development of atherosclerosis.

A number of studies have investigated the vasodilatory activity of red wine, grape juice and grape skin extract (Fitzpatrick *et al.*, 1993; 1995; Andriambelosen *et al.*, 1998). In the presence of three red wines, pre-contracted rat aortic rings were relaxed by 86, 89 and 53 %, compared with 0 and 20 % for the two white wines analysed (Fitzpatrick *et al.*, 1993). The skin extracts resulted in relaxations of 100 and 96 % for red and white grapes respectively. Red wine and grape juice exhibited endothelium-dependent

relaxation of blood vessels via enhanced generation and/or increased biological activity of NO leading to increased levels of cGMP. Ethanol at concentrations comparable to a typical wine showed no relaxation capacity. Diebolt *et al.* (2001) demonstrated a reduction in blood pressure in normal and hypertensive rats following the administration of red wine. While consumption of purple grape juice improved endothelium dependent flow mediated vasodilation in coronary artery patients with impaired endothelial function (Stein *et al.*, 1999).

The identification of the active compounds in grape skins and red wines remains under investigation. However, Andriambelosen *et al.* (1998) investigated the ability of wine fractions to relax pre-contracted aorta. Fractions containing oligomeric tannins and anthocyanins exhibited similar vasodilation activity as the original wine extract, however the anthocyanin containing fraction was more potent than the oligomeric tannin fraction. Similarly, a correlation between the anthocyanin content of red wines with vasodilation effect was reported by Burns *et al.* (2000). Further studies have shown that monomeric catechins and gallic acid have no effect on vasodilation, but anthocyanins and procyanidins enriched wine fractions were the most active. The threshold for relaxation by procyanidin oligomers was between 0.5 and 4 $\mu\text{g/mL}$ (Fitzpatrick *et al.*, 2000), with much higher concentrations $> 0.1 \text{ mg/L}$ required for anthocyanins (Andriambelosen *et al.*, 1998).

Besides NO, red wine has also been shown to have an effect on the formation of other mediators of vascular tone including prostacyclin (Derek *et al.*, 1997). In addition the synthesis of a potent vasoconstrictor, endothelin-1 has recently been shown to be reduced by red wine in bovine aortic endothelial cells. The decreased synthesis of endothelin-1 was associated with the inhibition of tyrosine kinase family of phosphorylating enzymes. This effect was associated with the phenolic content of red wine (Corder *et al.*, 2001).

1.8 Role of oxidised LDL in atherosclerosis

The exact chemical nature of the pathogenesis of atherosclerosis remains unknown however oxidative modification of LDL, a major cholesterol carrying lipoprotein in

human plasma, has been implicated as a key factor in its development (Steinberg *et al.*, 1989). Endothelial cell damage is the initial event leading to atherosclerosis and increases the permeability of the blood vessel allowing LDL to pass into the intima layer where they become internalised and begin to accumulate in the vessel cell wall (Fig. 1.5). At this stage LDL is oxidised through peroxidation by free radicals. The oxidatively modified LDL no longer binds to the native LDL receptor but to a scavenger receptor of macrophages (which bind toxic or foreign particles) and is accumulated internally. This leads to the production of cytokines which stimulate the influx of monocytes into the intima leading to further uptake of oxidised LDL. The monocytes gradually become lipid laden and transform into foam cells. These cells build up leading to the formation of a fatty streak and to vascular occlusion. Oxidised LDL have other attributes conducive to atheromatous changes including blocking of reverse cholesterol transport uptake by HDL and chemotactic properties promoting the adhesion of platelets to the superficial endothelium (Witztum, 1994).

1.8.1 In vitro studies on the inhibition of LDL

The inhibition of LDL oxidation by wines, grape juice and grapes have been demonstrated *in vitro* and have been attributed to their total phenolic content. Red and white wines have been reported to inhibit LDL oxidation *in vitro* (Frankel *et al.*, 1993, 1995; Kanner *et al.*, 1994; Teissedre *et al.*, 1996). An investigation of 20 Californian wines, standardised to 10 μ M GAE found that red wines inhibited LDL oxidation by between 37 and 65 % compared with between 27 and 46 % with white wines (Frankel *et al.*, 1995). The correlation between the antioxidant activity of the wines with LDL oxidation was further investigated. Gallic acid ($r=0.92$, $p<0.001$), (+)-catechin ($r=0.76$, $p<0.001$), myricetin ($r=0.70$, $p<0.001$) and quercetin ($r=0.08$, $p<0.001$) were all found to be highly correlated with the prevention of LDL oxidation. Moreover, Teissedre *et al.* (1996) demonstrated that a wine fraction containing (+)-catechin, (-)-epicatechin and procyanidin dimers and trimers, specifically B₂, B₈ and C₁, were the most active in inhibiting LDL oxidation by 70.6 % by determining hexane, a specific volatile oxidation product of *n*-6 PUFA lipids (Frankel *et al.*, 1992).

In a recent study Serafini *et al.* (2000) failed to show any protective effects from white wine. In this study the ability of red wine, alcohol free red wine and white wine to inhibit LDL oxidation was investigated. The presence of a high concentration of red wine in the medium protected LDL from oxidation in a dose-dependent manner (0.3, 0.5, 0.7 μ l) and was the most efficient in protecting against oxidation. The ability of grapes and grape juices to inhibit LDL oxidation has also been reported. Meyer *et al.* (1997) investigated the ability of *V. Vinifera* grapes to inhibit LDL oxidation. At a concentration of 10 μ M GAE grapes were able to inhibit LDL oxidation by between 22 and 60 %, compared with 62 and 91 % at concentrations of 20 μ M GAE. The inhibition of oxidation by grapes was strongly correlated with levels of total phenols ($r=0.89$, $p<0.01$) and also with anthocyanins ($r=0.56$, $p<0.05$) and flavonols ($r=0.56$, $p<0.05$). Similar results were obtained by Frankel *et al.* (1998) whereby commercial grape juice (10 μ M GAE) were able to inhibit LDL oxidation by between 62 and 75 %.

1.8.2 In vivo studies on the inhibition of LDL

Although *in vitro* studies have consistently shown that wine phenolics have significant abilities to inhibit LDL oxidation, *ex vivo* and *in vivo* studies have produced contrasting results to date. This suggests that there are other determining factors *in vivo* which may limit their ability to produce protective effects, for example nutrient interactions and gut microflora may limit the bioavailability of phenolics. Fuhman *et al.* (1995) investigated the effect of red and white wine consumption on the susceptibility of LDL to oxidative modification in human subjects following the consumption of 400 mL/ day of wine for 2 weeks. Red wine consumption decreased LDL lipid peroxidation in response to copper induced oxidation by 46 %, 72 % and 54 % as determined by TBARS, lipid peroxides and conjugated dienes. In contrast, white wine consumption resulted in an increase in the propensity of LDL to undergo oxidation. The pro-oxidant effect of white wine may be due to the combination of alcohol and the absence of protective polyphenols.

The effect of alcohol was eliminated in a later study by de Rijke *et al.* (1996). In this study the alcohol content of red and white wine was reduced, however no effect on the oxidizability of LDL was observed after consumption of either red or white wine. It was

speculated that the removal of alcohol might have impeded the absorption of the phenolic compounds. Interactions between phenolics and other nutrients contained in beverages such as red wine can lead to the formation of insoluble complexes such as protein-tannin interactions that can affect the bioavailability. Reducing the alcohol content of wine has been shown to produce a linear increase in protein-tannin interactions and a decrease in the antioxidant capacity (Serafini *et al.*, 1997).

Nigdikar *et al.* (1998) investigated the effect of consuming different forms of polyphenols on the inhibition of LDL oxidation. Subjects consumed either red wine, a capsule of powdered red wine phenolics, the same extract dissolved in either white wine or an alcoholic drink, for two weeks. After this period the levels of phenolics in plasma increased by 38 % for red wine, and 27 and 28 % for white wine containing the extract, and the extract respectively. The lag time for copper-induced LDL oxidation increased by 17.8 min after red wine; 14.2 min after the capsule and 11.7 min after the white wine and the extract. The results from this study are in agreement with a previous study in which subjects consumed 400-500 mL red wine daily for 2 weeks (Kondo *et al.*, 1994). Moreover, the effect of consuming a red grape juice concentrate on the oxidizability of LDL was demonstrated in a study by Day *et al.* (1997). 7 subjects consumed 125 mL of red grape juice concentrate for 7 days. An increase in the serum total antioxidant capacity from 441 to 478 $\mu\text{mol/L}$ was observed 1 h after consumption and LDL showed an increased resistance to oxidation by UV light.

The protective effects of consuming red wine with a high fat meal was demonstrated in a recent study by Natella *et al.* (2001). Six subjects consumed a high fat meal with either 400 mL of red wine or an isocaloric hydroalcoholic solution. Following red wine consumption an increase in the antioxidant capacity of the plasma was observed and LDL obtained after the meal was more resistant to lipid peroxidation than fasting LDL. The findings from this study suggest that phenolic antioxidants in red wine protect LDL from the susceptibility of oxidative modification and might offer a plausible mechanism for the French paradox.

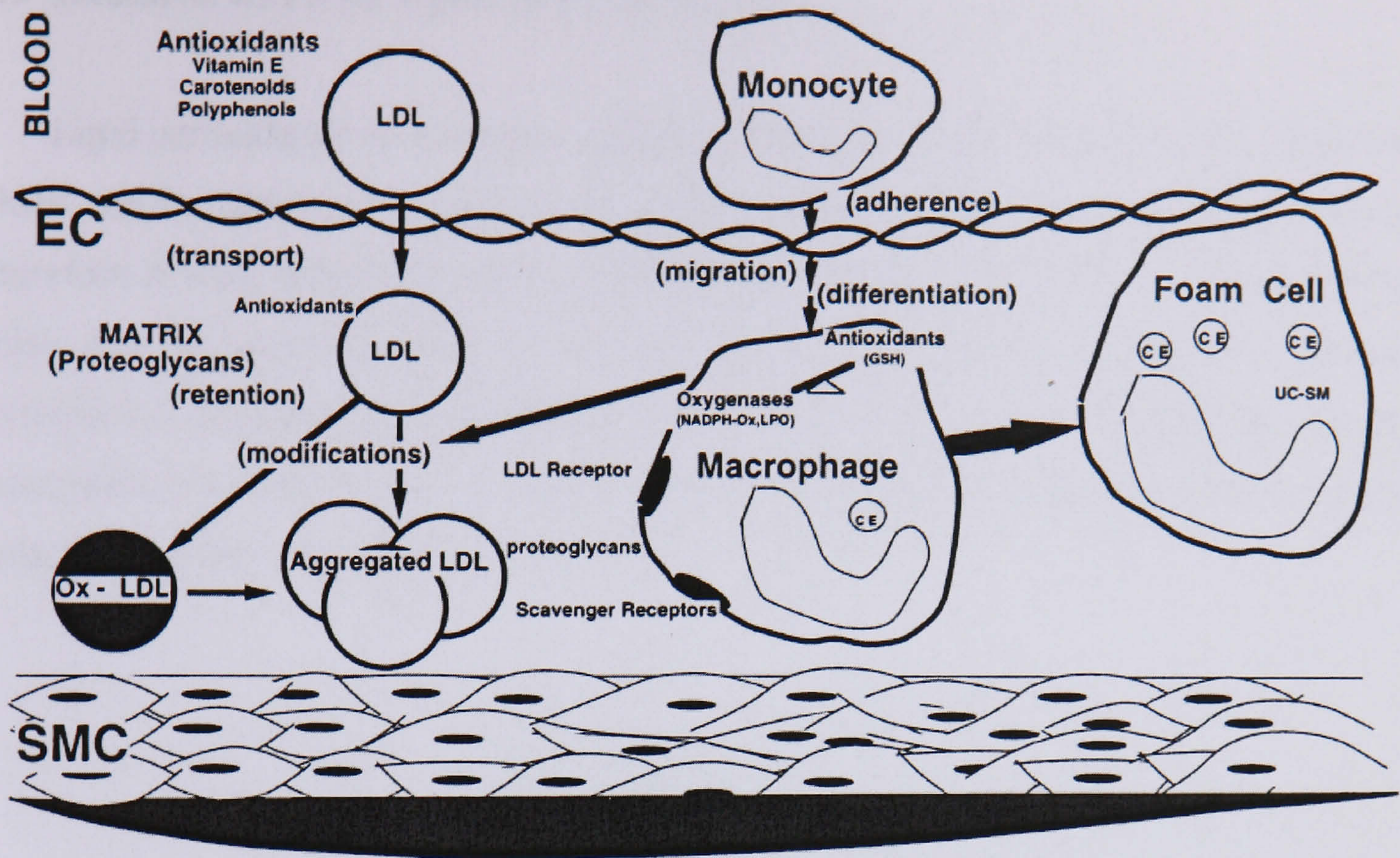


Figure 1.5 Macrophage-mediated oxidation and aggregation of LDL and foam cell formation. NADPH-Ox: NADPH oxidase; LPO: lipoxygenase; GSH: reduced glutathione; CE: cholesteryl ester; UC-SM: unesterified cholesterol-sphingomyelin. Adapted from Avriam & Fuhrman, (1998).

1.9 Biomarkers of lipid peroxidation

Lipid peroxidation is a complex process resulting in a number of potential products which can be measured as indicators of free-radical mediated degradation of lipids. Therefore a wide range of markers can be used for their evaluation. Peroxidation of lipids can be assessed either by determination of the concentrations of primary peroxidation products or secondary degradation products in body fluids or on the susceptibility of lipids to oxidation induced *ex vivo* either by transition metals or by the generation of free radicals (Fig. 1.6).

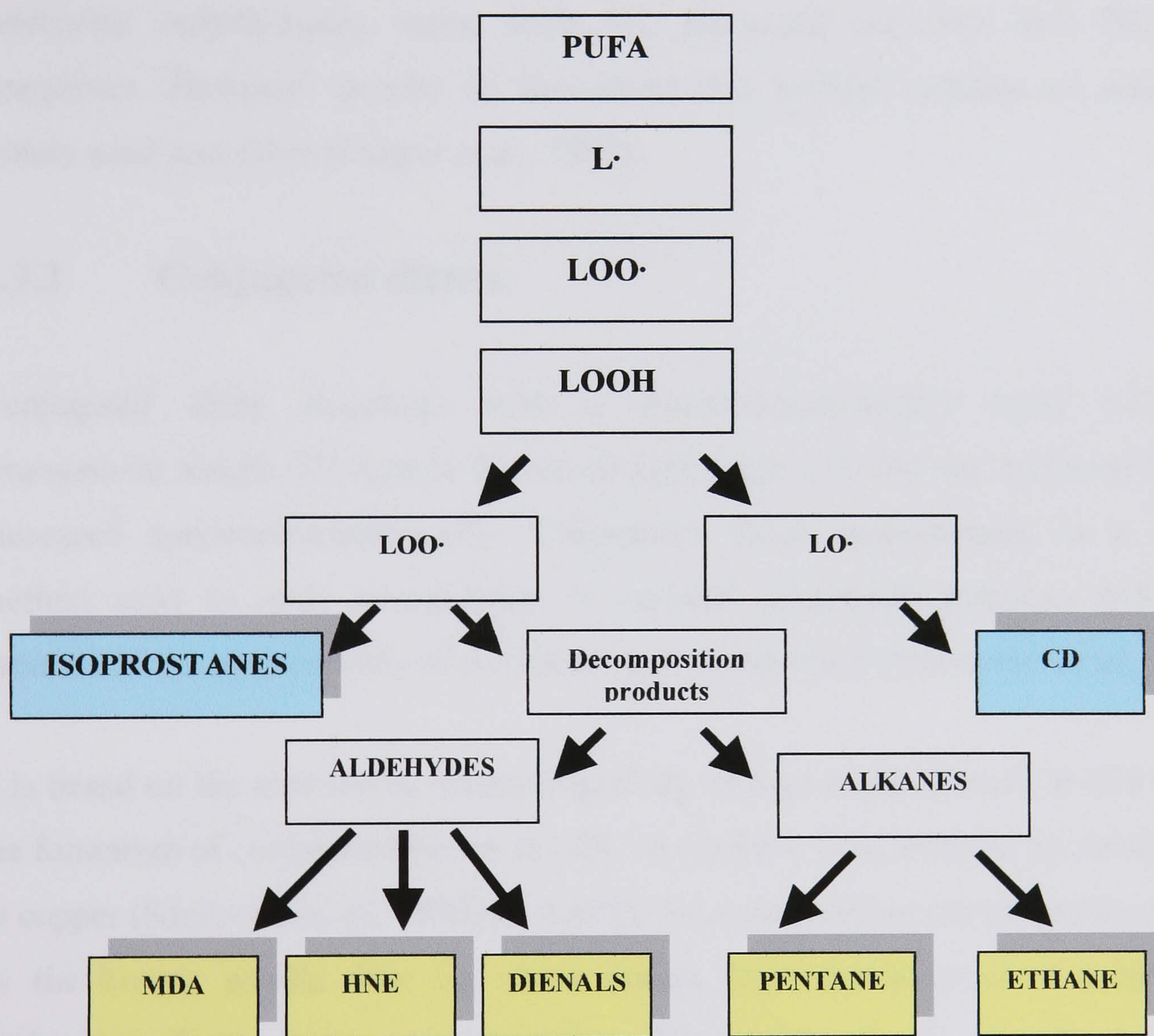


Figure 1.6 The products and pathways relating to lipid peroxidation. PUFA-polyunsaturated fatty acids; L•-lipid radical; LOO•-peroxyl radical; LO•-alkoxyl radical; LOOH-lipid hydroperoxides; CD-conjugated dienes; MDA-malonaldehyde; HNE-4-hydroxynonenal. Adapted from Dotan *et al.* (2004).

1.9.1 TBARS

The TBARS method is the most common for determining plasma lipid peroxidation. Malonaldehyde (MDA) is generated as a degradation product from peroxidised lipids, and as a side product of enzymatic metabolism of thromboxanes and prostaglandins (McMillan *et al.*, 1978), and is the major TBARS formed primarily by the decomposition of peroxides of fatty acids with 3 or more double bonds (Esterbauer *et al.*, 1990). The basis of the TBA method is the reaction of MDA with TBA at low pH and high temperatures to form a coloured complex (MDA-TBA complex) which can be quantified based on intensity of fluorescence between 532-535 nm. A major disadvantage of this method is that it is non-specific and other compounds (other aldehydes, carbohydrates, amino acids, bile pigments) can react with TBA to form complexes. However, despite its limitations this method remains an accepted and widely used procedure (Draper *et al.*, 1993).

1.9.2 Conjugated dienes

Conjugated diene structures with a double-single-double bond (-C=C-C=C-) arrangement absorb UV light in the wavelength range 230-235 nm, and therefore can be measured spectrophotometrically. Conjugated diene measurement is a successful method used to study peroxidation of isolated lipoprotein fractions and gives an estimate of the susceptibility of the lipoprotein to oxidation (Esterbauer *et al.*, 1989)

It is based on the continuous monitoring of the change in absorbance at 234 nm due to the formation of conjugated dienes in LDL which have been initiated by metal ions such as copper (Kleinveld *et al.*, 1992) or AAPH. Two consecutive phases can be determined by the kinetic profile. The lag phase occurs from the initiation of oxidation until conjugated dienes begin to accumulate. The length of the lag phase is directly proportional to the concentration of antioxidants. As the reaction proceeds antioxidants are consumed linearly with time and the rate completing reaction slows down. As a result, the propagation phase, the period during which conjugated dienes accumulate, increases until a maximum rate of the uninhibited autoxidation is reached for steady state conditions (Fig. 1.7).

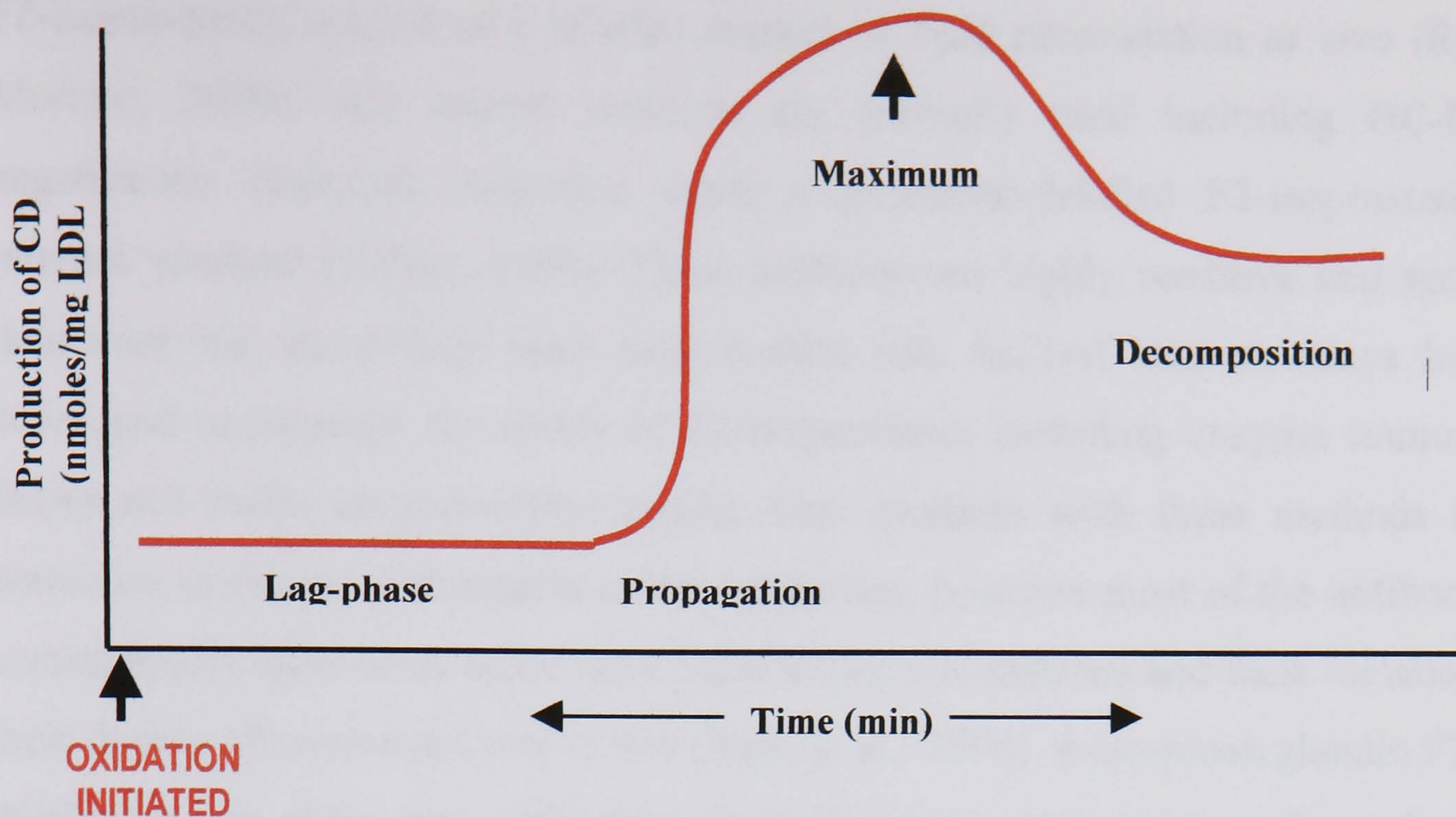


Figure 1.7 Kinetic profile of conjugated diene formation

1.9.3 Measurement of exhaled alkanes

Volatile hydrocarbons mainly ethane and pentane have been used and validated as a measure of lipid peroxidation *in vitro* and *in vivo* studies (Frank *et al.*, 1980). *n*-3 PUFA oxidation has been shown to result in an increase in ethane excretion while *n*- 4, 6 and 7 PUFA oxidation yield increased excretion of propane, pentane and hexane. Significant background levels resulting from hydrocarbon contamination of inhaled air are a major limitation of this technique (Springfield *et al.*, 1994).

1.9.4 F2-Isoprostanes

The discovery of the isoprostanes as products of lipid peroxidation has been a major advance in the ability to assess lipid peroxidation *in vivo* (Lawson *et al.*, 1999). Isoprostanes are a complex family of compounds produced from arachidonic acid via a free-radical catalyzed mechanism. *In vitro* generation of auto-oxidation products derived from PUFA was described more than 30 years ago. However, the first demonstration that these compounds were produced in humans was shown by Morrow *et al.* (1990), whereby prostaglandin-F2-like compounds, termed F2-isoprostanes, were generated by free-radical-induced peroxidation of arachidonic acid. Quantification of

F2-isoprostanes is used as a reliable marker of lipid peroxidation *in vivo* (Roberts & Morrow, 2000), and several methods are currently used including GC-MS with negative-ion chemical ionization using a deuterium-labeled F2-isoprostane as an internal standard (Tsikas, 1998). These methods are highly sensitive and specific but their cost and technology limit their routine use. Several immunoassays have been developed to measure the levels of F2-isoprostanes including enzyme immunoassays (EIA) and radio immunoassays (RIA). One problem with these methods is cross-reactivity of related compounds to the antibodies, however most of the antibodies used commercially have been tested with other major isoprostanes and their metabolites and their degree of cross-reactivity is low (Basu *et al.*, 1998). 8-iso-prostaglandin F2 α (8-iso PGF2 α) is one of the major F2 isoprostanes formed *in vivo* and have been shown to be present in increased amounts in human atherosclerotic lesions. Plasma, serum or urinary levels have been shown to be increased in subjects with hypercholesterolemia, liver cirrhosis and diabetes mellitus (Morrow *et al.*, 1995) and in smokers (Reilly *et al.*, 1996).

1.10 Absorption and metabolism of polyphenols

The fate of ingested polyphenols in the digestive tract, including the absorption and metabolism, remains largely unknown. Research in this area has produced conflicting results; however current knowledge in this regard is briefly summarized below and illustrated in Fig. 1.8.

Ingested polyphenols enter the digestive system primarily in the form of glycosides (although some aglycones may be present). The glycosides may then be de-conjugated by the action of non-specific β -glucosidases, present in the food itself or on the surface of the mucosal cells (Aherne & O'Brien, 2002). Both aglycones and glycosides have been reported to be absorbed, however conjugates are more hydrophilic than the aglycones and the removal of the hydrophilic moiety appears to be important for the passive diffusion across the intestinal mucosa (Scalbert & Williamson, 2000). It has been suggested that the intestinal sodium glucose transporter might be involved in carrying phenolic glucosides through the intestinal cell wall. However, this has not been proven *in vivo* (Aherne & O'Brien, 2002).

Polyphenols undergo extensive metabolism, mainly involving conjugation reactions including *O*-methylation and/or conjugation with glucuronides and/or sulphates, during their passage through the enterocytes (Kuhnau, 1976). Certain transporter proteins in the enterocytes may actively transfer the glycoisides back into the intestinal lumen. Absorbed polyphenols are bound to albumin and transported in the circulation to reach the liver via the portal vein (Manach *et al.*, 1996). In the liver, they are metabolized or secreted into the bile (Aherne & O'Brien, 2002). Although the liver seems to be the main organ involved in the metabolism of polyphenols there is evidence of metabolism occurring in the intestinal mucosa and kidney. Administration of 2 g of (+)-catechin in rats resulted in free catechin in the plasma after 30 min, while a low dose of 2 mg/kg resulted in the formation of conjugated metabolites. The results from this study suggest that the intestine is an important site for the metabolism of polyphenols, with the liver playing a secondary role to further modify the conjugated phenolic (Piskula *et al.*, 1998).

Un-absorbed or re-excreted polyphenols reach the large intestine where they may undergo metabolism to more simple compounds by the colonic microflora and the degradation products (*e.g.* phenolic acids in the case of flavonoid metabolism) may be absorbed by passive diffusion. The polar, and therefore water soluble, polyphenol glucuronides and sulfate that escaped biliary excretion and enterohepatic circulation are eliminated from the body by urinary excretion

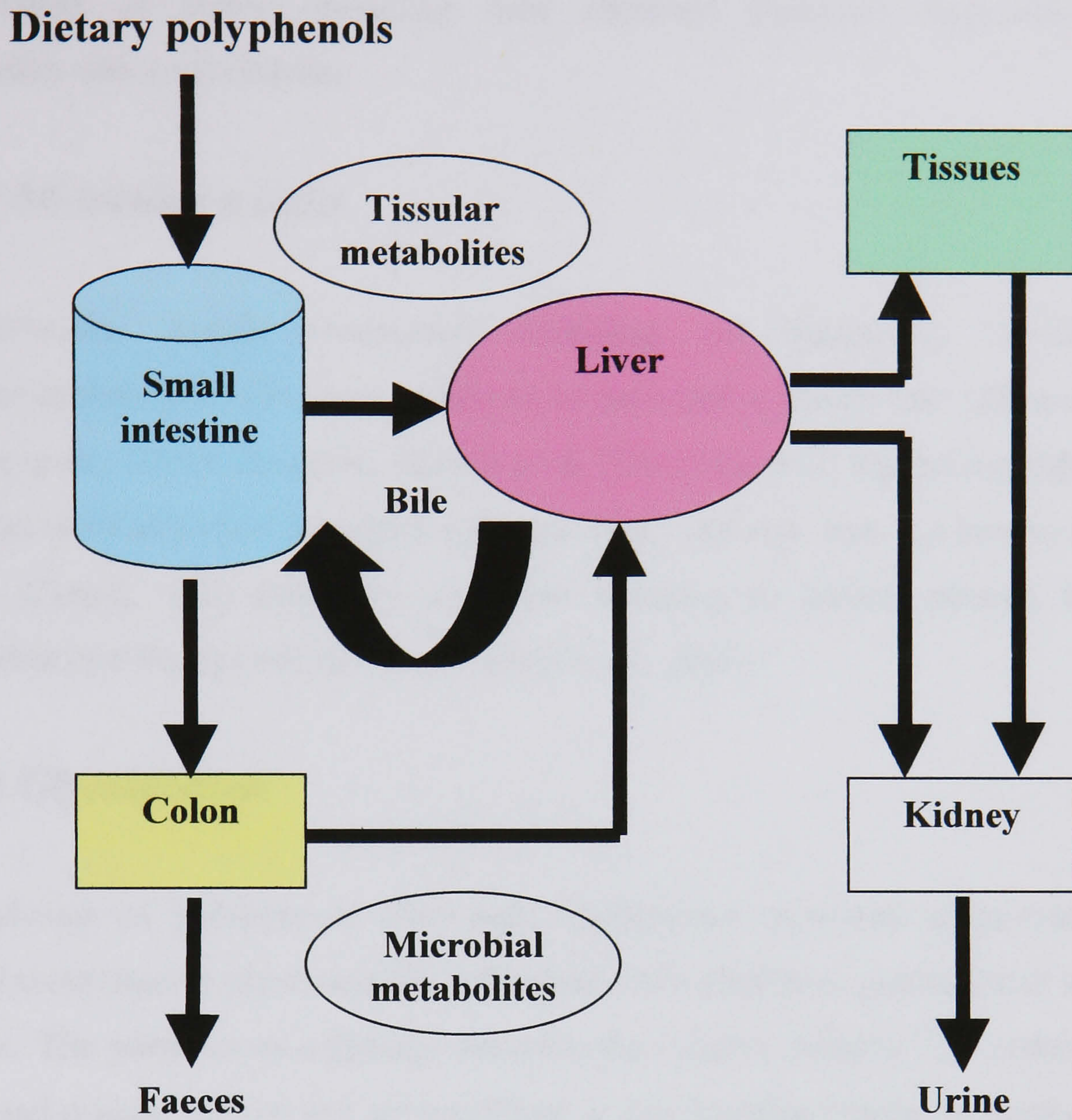


Figure 1.8 Possible routes for consumed polyphenols in humans. Adapted from Scalbert & Williamson, (2002).

1.10.1 Factors affecting bioavailability of phenolics

The rate and extent of intestinal absorption and metabolism of phenolics are influenced by a number of factors including their chemical structure, molecular weight, glycosylation and esterification.

1.10.1.1 Molecular weight

High molecular weight compounds including tea theaflavins ($M=568$) and proanthocyanidins ($M=577+$) are unlikely to be absorbed in the GI tract (Donovan *et al.*, 2002; Lee *et al.*, 1995). However, Deprez *et al.* (2001) reported that procyanidin dimers and trimers were absorbed through a cell monolayer derived from the human intestinal cell line Caco-2, and dimer B₂ has been detected in human plasma following consumption of a flavan-3-ol rich cocoa (Holt *et al.*, 2002).

1.10.1.2 Glycosylation

Certain classes of polyphenols (flavonols, isoflavones, flavones, anthocyanins) are usually glycosylated in plants and this influences their chemical, physical and biological properties. The partition co-efficients measure the relative affinity of a compound for aqueous and organic phases and are important in determining whether a compound will passively diffuse across a biological membrane and how they might partition in a cell. The flavonol quercetin has a partition co-efficient of 1.2 ± 0.1 however quercetin-3-*O*-rhamnoglucoside has a value that is considerably lower (0.37 ± 0.1) showing greater hydrophilicity (Brown *et al.*, 1998).

1.10.1.3 Esterification

Ester linked substitutions have marked effects on absorption and subsequent bioavailability. Hydroxycinnamates such as caffeic acid are commonly esterified to sugars, organic acids and lipids. It has been reported that caffeic acid is better absorbed than chlorogenic acid; it's ester with quinic acid. Olthof *et al.* (2001) reported 95 % of intestinal absorption for caffeic compared with only 35 % for chlorogenic acid in

ileostomy subjects. Similarly, in an isolated rat intestine model absorption of caffeic acid was higher (0.695 nmol) than its quinic ester (0.115 nmol) (Spencer *et al.*, 1999).

1.10.2 Absorption of flavonols

There is now a considerable body of evidence supporting the view that the flavonol conjugates are preferentially absorbed, and that the nature of the conjugation may be important. Hollman *et al.* (1995) investigated the absorption of quercetin in ileostomy subjects following the consumption of an onion test meal or capsules containing quercetin or quercetin rutinoid. 52 % of quercetin glucosides, 24 % quercetin and 17 % quercetin rutinoid were absorbed. Although the authors demonstrated the absorption of quercetin they emphasized the importance of glycosylation as a means of enhancing absorption and considered quercetin glucosides to be the predominant form of the flavonol in human plasma.

Consistent findings were observed by Graefe *et al.* (2001), whereby quercetin glucuronides were found in the plasma of human subjects reaching a peak plasma concentration at 0.7 h after the ingestion of an onion supplement containing 100 mg of quercetin, and following the consumption of red wine (Crozier *et al.*, 2000). It has been proposed that flavonol glucosides, such as quercetin-4'-glucoside could be absorbed intact into the small intestine using the sodium-dependent glucose transporter (SGLT1) (Hollman *et al.*, 1995). However, studies with human intestinal Caco-2 cell monolayers have shown that quercetin-4'-glucoside and quercetin-3,4'-diglucoside were not absorbed despite the operation of SGLT1 which was demonstrated by the active transport of glucose (Walgen *et al.*, 1998).

1.10.3 Absorption of flavan-3-ols

Generally, flavan-3-ols are found in plasma methylated and/or conjugated to sulphate or glucuronic acid. Recent work by Okushio *et al.* (1999) reported that both the *O*-methylated and glucuronidated conjugates were detected in rat urine following oral administration of (-)-epicatechin. Moreover, epicatechin-5-*O*- β -glucuronide and catechin-5-*O*- β -glucuronide have been detected in plasma, bile and urine of rats following oral administration of (-)-epicatechin and (+)-catechin respectively (Harada *et al.*, 1999).

In humans, 3-*O*-methylcatechin, sulphated and glucuronidated metabolites have been detected in plasma following consumption of either red wine or de-alcoholised red wine (Donovan *et al.*, 1999). Maximum levels of 50-170 nmol/L of total (+)-catechin metabolites were detected 1 h after consumption. Although free (+)-catechin and 3-*O*-methylcatechin were detected, they were comparatively lower than those of (+)-catechin sulphate and/or glucuronide conjugates. More recently, Bell *et al.* (2000) investigated the absorption of (+)-catechin in human subjects following the consumption of red wine. Free and conjugated forms attained peak plasma concentrations at 60 min with 7.9 % of the administered dose being absorbed.

Low urinary recoveries of (+)-catechin were reported by Golberg *et al.* (2003b). Twelve healthy volunteers were randomly assigned to consume 25 mg/kg of (+)-catechin in 3 different matrices including white wine, grape juice and vegetable juice. Only 1.2-3 % of the dose of (+)-catechin administered was excreted in the urine over 24 h. To explain the low absorption of (+)-catechin the authors concluded that some re-absorption of (+)-catechin in the renal tubules might have occurred, and there is some evidence that (+)-catechin may be preferentially excreted in the bile (Das *et al.*, 1971). Piskula & Terao (1998) proposed that the mechanism of flavan-3-ol metabolism involved glucuronidation in the small intestine as the first detoxification step followed by *O*-methylation in the liver and kidney. Kuhnle *et al.* (2000) demonstrated in an isolated intestinal model that (-)-epicatechin and (+)-catechin are extensively *O*-methylated during transfer across the jejunum. The major metabolites transferred across the intestinal epithelium of the jejunum are glucuronidated and *O*-methylated metabolites of

the parent flavanol. Up to 30% of compounds detected in the jejunal serosal fluid were *O*-methylated and an additional 20% were *O*-methylated and glucuronidated. Current knowledge on the absorption and metabolism of the oligomeric and polymeric flavan-3-ols, or procyanidins in humans and animals is limited and contradictory.

Nonetheless, dimer B₂ has been detected in human plasma following ingestion of a cocoa supplement, with concentrations reaching a maximum of 41 nmol/L by 2 h (Holt *et al.*, 2002). Moreover, Baba *et al.* (2002) reported on a study with rats on the absorption of dimer B₂. 50mg/Kg of a cocoa powder was fed to rats and analysis by HPLC-MS confirmed the presence of B₂ (0.5 μM), epicatechin (0.2 μM) and 3'-*O*-methyl-epicatechin (0.15 μM) 30 min post-ingestion of the cocoa in plasma. B₂ (83 nmol/L), epicatechin (free 12.5 nmol/L or conjugated 29.6 nmol/L) and 3'-*O*-methyl-epicatechin (free 12.8 nmol/L or conjugated 13.9 nmol/L) were also detected in the urine within 18 h.

1.10.4 Absorption of anthocyanins

One of the first studies to provide evidence for the uptake of anthocyanins in humans *in vivo* was reported by Paganga & Rice-Evans (1997). HPLC analysis confirmed the identification of anthocyanins in plasma in their glycosylated form. Following the consumption of 300 mL of red wine containing ~218 mg of anthocyanins, 1.5-5.1 % of the anthocyanins were recovered in the urine within 12 h (Lapidot *et al.*, 1998). Two of the identified compounds were unchanged, whereas other compounds appeared to have undergone molecular modifications. More recently, Miyazawa *et al.* (1999) investigated the absorption and metabolism of anthocyanins in human subjects following the ingestion of elderberry and black currant juice concentrates. Subjects ingested 2.7 mg of cyanidin-3-glucoside and 0.25 mg of cyanidin 2,5-diglycoside/kg of body weight. Only intact anthocyanins were detected in plasma. After 1 h plasma levels of cyanidin-3-glucoside reached 13 μg/L with only trace levels of cyanidin-2, 5-diglycoside detected. Anthocyanins have also been detected in plasma in very low concentrations 0.5-1 h after consumption, falling to near baseline levels within 6-8 h (Cao *et al.*, 2001).

In a recent study, Wu *et al.* (2002) demonstrated that methylation of the 3'-hydroxyl group in addition to glucuronidation of anthocyanins is possible. Cyanidin-3-*O*-glucoside and cyanidin-3-sambubioside were detected in the urine of elderly women following the ingestion of an elderberry extract. Four metabolites (peonidin-3-glucoside, peonidin-3-sambuboside, peonidin monoglucuronide and cyanidin-3-*O*-glucoside monoglucuronide) were further detected in the urine, however were present in much lower levels. Similarly, Felgines *et al.* (2003) reported the presence of pelargonidin glucuronides, a pelargonidin sulphate and the aglycone pelargonidin following the consumption of strawberries in human subjects. However, the levels of anthocyanins recovered are exceptionally low, with 0.1 %, or less, of the ingested dose being detected in the urine (McGhie *et al.*, 2003).

1.10.5 Absorption of non-flavonoids

1.10.5.1 Gallic acid

The absorption and metabolism of gallic acid has been widely investigated in a number of animal studies (Booth *et al.*, 1959; Glick *et al.*, 1981; Murdiati *et al.*, 1992). Conjugation, 4-*O* methylation, decarboxylation and dehydroxylation reactions have been reported with 4-*O*-methylgallic acid, resorcinol glucuronide and 2-*O* methypyrogallol being the major urinary metabolites found in animals. Following the administration of 50 mg gallic acid in humans, 4-*O*-methylgallic acid and gallic acid were detected in the urine and plasma by HPLC-UV detection with recoveries for both reaching > 90 %. No further metabolites were detected in either plasma or urine despite a thorough search (Shahrzad and Bitsch, 1998). Similar findings were reported by Adu-Amsha Caccetta *et al.* (2000) whereby 4-*O*-methylgallic acid was detected in the urine following red wine consumption in human subjects. Additional metabolites have been identified in humans following prolonged consumption of black tea (Hodgson *et al.*, 2000), with 4-*O*-methylgallic acid, 3-*O*-methylgallic acid and 3,4-*O*-dimethylgallic acid being detected in urine. However, it remains unknown whether these metabolites are derived from gallic acid alone, as there is some evidence to suggest that gallate esters may contribute, together with breakdown products of the flavonoids (Zhu *et al.*, 1992).

1.10.5.2 *Hydroxycinnamates*

Caffeic, *p*-coumaric and ferulic acid have been detected in human urine following a high intake of fruit (Bourne & Rice-Evans, 1998), and there is evidence that caffeic acid is absorbed in the small intestine (Olthof *et al.*, 2001). Ferulic acid, isoferulic acid, caffeic acid and vanillic acid have been detected in urine after administration of 1g of caffeic acid (Jacobson *et al.*, 1983). The mechanism of absorption of hydroxycinnamates remains uncertain although it is thought to involve the sodium dicarboxylate co-transporter (SDCT1) (Clifford *et al.*, 2000b).

1.10.5.3 *Trans-resveratrol*

Trans-resveratrol has been detected intact in plasma after the administration of red wine (Bertelli *et al.*, 1996) and pure *trans*-resveratrol (Juan *et al.*, 1999) to rats. Moreover, free, glucuronidated and sulphated conjugates of *trans*-resveratrol were detected in human plasma and urine following administration of 25 mg/kg, with 16-17 % of the dose excreted in the urine by 24 h (Goldberg *et al.*, 2003b). Kuhnle *et al.* (2000) demonstrated the absorption and metabolism of *trans*-resveratrol using an isolated rat small intestine model. *Trans*-resveratrol was metabolised to its glucuronide conjugate. However, only small amounts of resveratrol were absorbed across the enterocytes of the jejunum and ileum unmetabolised. The major compound detected on the serosal side was the glucuronide conjugate of resveratrol (96.5 % \pm 4.6 of the amount absorbed) indicating the susceptibility of resveratrol to glucuronidation during transfer across the rat jejunum.

In summary, although there is some evidence indicating potential health benefits of dietary polyphenols, information concerning their bioavailability remains undetermined. This is largely due to a lack of data concerning flavonoid composition in food and the inconsistent use of current methodologies. The work presented in this thesis circumvents these problems by providing a quantitative analysis of grape and wine derived phenolics and their subsequent metabolites in biological samples.

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Chapter 2 Materials and Methods

2.1 Chemicals

(+)-Catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate were obtained from AABB Chemicals (Southampton, UK). Quercetin, gallic acid and epigallocatechin were obtained from Sigma (Poole, Dorset, UK). Malvidin-3-*O*-glucoside was purchased from Extrasynthase (Lyon, France). The grape seed extract used in this study was produced by Partoeno (Bordeaux, France). Standards of the procyanidin dimers B₁, B₂, B₃ and B₄ were generously provided by Dr Pierre-Louis Teissedre from the Faculty of Pharmacy, University of Montpellier 1, France. These were obtained from a grape seed extract using procedures described by Teissedre *et al.* (1995). Methanol (HPLC grade), ethanol and acetonitrile (HPLC grade) were purchased from Rathburn Chemicals (Walkerburn, Peebleshire, UK). Formic acid, trifluoroacetic acid (TFA), citric acid, 1,1,3,3-tetraethoxypropane (TEP) and Folin-Ciocalteu's phenol reagent were supplied by Sigma. Disodium carbonate (Na₂CO₃), butylated hydroxytoluene; di-sodium hydrogen orthophosphate and sodium dihydrogen orthophosphate dihydrate were obtained from BDH Chemicals Ltd (Poole, UK). Concentrated hydrochloric acid, acetic acid (glacial), disodium hydrogen phosphate (Na₂HPO₄) and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Loughborough, Leicestershire, UK). All other chemicals and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated.

2.2 Wines

2.2.1 Details of bottled wines analysed

All bottled wines were supplied by Safeway Stores plc. Details of origin and varietal composition are summarised in Table 2.1. Wines were selected to represent a variety of wines produced from various grape cultivars, geographical regions and those most commonly consumed. Aliquots of wine were removed from the bottle for analysis and the remaining wine stored under nitrogen and re-corked. Unless otherwise stated wine was untreated prior to analysis.

2.3 Colorimetric methods

2.3.1 Determination of total phenolics

The method of Singleton and Rossi (1965) was used to determine the total phenolic content of wine. This method determines phenols and oxidized substances by producing a blue colour from reducing yellow heteropoly phosphomolibdate-tungstate anions. In brief, 200 μL of a 1:5 dilution of sample was added to 10 mL of a 1:10 diluted Folin and Ciocalteu reagent and 1.8 mL of distilled water. After 5 min 7.0 mL of a Na_2CO_3 solution (115 g L^{-1}) was added and the reaction mixture was left at room temperature for 2 h. The absorbance of the solution was read at 765 nm against water blank on a Cecil 3000 series spectrophotometer (Cecil Instruments Ltd, Cambridge, UK). The optical density (O.D.) was compared to a standard curve prepared with 50 to 500 mg L^{-1} gallic acid and results are expressed as gallic acid equivalents (GAE).

Total phenolics in plasma were also estimated with Folin-Ciocalteu reagent using a modification of the method of Swain and Hillis (1959). This method avoids interference from proteins in biological samples (Serafini *et al* 1998). In brief, 500 μL of sample was added to 1 mL of 1.0 M HCl, and vigorously vortexed for 60 s. Following incubation at 37°C for 60 min, 1.0 mL of a 2.0 mol/L NaOH in 75 % methanol was added, and the resulting mixture vortexed for 3 min. 1.0 mL of 10 % (v/v) phosphoric acid was added and the sample was centrifuged at 1500 x g 10 min. The supernatant was removed and kept on ice in the dark and the pellet was extracted again by adding 1.0 mL of a solution 1:1 (v/v) acetone:water and centrifuged for 10 min at 2700 x g. The two supernatants were combined and filtered through a 0.45 μm filter (Millipore, Tyne and Wear, UK), and 200 μL of sample were assayed for total phenolics with Folin-Ciocalteu as described above and expressed as μM GAE.

2.3.2 Colorimetric analysis of anthocyanins

The anthocyanin content of red wines was estimated using a pH shift method adapted from Ribereau-Gayon and Stonestreet (1965). Two test tubes were set up each containing 140 μL of wine and 140 μL 0.1 % concentrated HCl in 95 % ethanol. 1.5 mL of 2 % concentrated HCl (pH 0.6) was added to one tube and 1.5 mL of pH 3.5 buffer

(300 mL 0.2 M Na₂HPO₄ and 700 mL 0.1M citric acid, adjusted to pH 3.5 with 0.1 M citric acid) to the other. Absorbance was read at 700 nm to allow for the correction of haze and then at 520 nm for anthocyanin determination. Anthocyanins were quantified as malvidin-3-glucoside equivalents, the major anthocyanin in red wine, using the extinction co-efficient 28000. At < pH 1 anthocyanins are found entirely in their red flavylum form allowing determination of the total anthocyanins. At pH 3.5 the flavylum form of the anthocyanin is primarily in equilibrium with the colourless carbinol, therefore absorbance is due to polymeric anthocyanins or interfering brown substances. The difference in absorbance between pH <1 and pH 3.5 is due to the free anthocyanin content.

2.3.3 Determination of total catechins

Total catechins were determined in wine fractions spectrophotometrically with 4-dimethylaminocinnamaldehyde (DMACA) as described by Kivitis *et al.* (1997). Samples were diluted 10-fold with 12.5 % ethanol prior to the addition of 3 mL of 6 mM DMACA (dimethylaminocinnamaldehyde) in a methanol/perchloric acid/water mix (8:1:1, v/v). A scan of absorbance between 500 nm and 750 nm was determined 6 min after the initiation of the reaction. Peak area was related to a standard curve obtained with (+)-catechin standard. Due to the interference from anthocyanins, the 604-684 nm region of the scan was used for catechin quantification.

Table 2.1 Details of red wines analysed for total phenolic content and antioxidant capacity.

Wine	Year	Origin	Grape variety
1. Claret non-vintage	2000	Bordeaux, France	Cabernet Sauvignon
2. Beaune	2000	France	Pinot Noir
3. Beaujolais	2001	Beaujolais, France	Gamy
4. Cotes du Rhone	2001	Rhone, France	Carignon, Cinsault
5. Vin de pays de L'Ardeche	2000	Ardeche, France	Syrah, Grenache
6. Etienne Barret Crozes Hermitage	2001	France	Syrah
7. Minervois	2000	Aude, France	Carignon
8. Corbieres	2001	France	Corbieres
9. Chianti Classico	1999	Tuscany, Italy	Chianti
10. Valpolicella	2000	Veneto, Italy	Valpolicella
11. Aged in Oak Valdepenas Reserva	1996	Valdepenas, Spain	Cencibel
12. Castillo de Sierra Rioja	1998	Rioja, Spain	Rioja
13. Young Vatted Tempranillo	2000	Spain	Tempranillo
14. Bairrada	1999	Portugal	unknown
15. Young Vatted Merlot	2001	Bulgaria	Merlot
16. Vin de Pays L'Ardeche Rose	2001	Ardeche, France	Grenache, Syrah
17. Australian Red	2001	Australia	Cabernet Sauvignon
18. Australian Oaked Cabernet Sauvignon	2000	Australia	Cabernet Sauvignon
19. Young Vatted Pinotage	2000	South Africa	Pinotage
20. Cinsault	2001	South Africa	Cinsault
21. Cabernet Sauvignon	2000	South Africa	Cabernet Sauvignon
22. Caballo de Plata	2000	Argentina	Bonarda-Barbera
23. Cabernet Sauvignon	2001	Argentina	Cabernet Sauvignon
24. Argentinian Shiraz	2001	Argentina	Shiraz
25. Inti Malbec	2001	Argentina	Malbec
26. Chilean Dry Red	2001	Chile	Cabernet Sauvignon
27. Chilean Cabernet Merlot	2001	Chile	Cabernet Sauvignon, Merlot
28. Chilean Cabernet Sauvignon	2001	Chile	Cabernet Sauvignon
29. Granverano Carmenere	2001	Chile	Carmanere
30. Pinot Noir Estancia	2000	California	Pinot Noir
31. Young Vatted Cabernet Sauvignon	2000	Bulgaria	Cabernet Sauvignon

2.3.4 Determination of the antioxidant power using ferric reducing ability of plasma

The FRAP assay described by Benzie and Strain (1996) was used to estimate the antioxidant power of wine samples. This method measures the ability of a solution to reduce a ferric-2,4,6-tri-2-pyridyl-s-triazine (TPTZ) complex (Fe^{3+} TPTZ) to the ferrous form Fe^{2+} , producing an intense blue colour with absorption at 593 nm. The reaction is non-specific and any half life reaction, which has a less positive redox potential, under reaction conditions, than the $\text{Fe}^{3+}/\text{Fe}^{2+}$ -TPTZ half-life reaction will drive the Fe^{3+} -TPTZ reduction. In the FRAP assay excess Fe^{3+} is used and the rate limiting factor of the Fe^{2+} -TPTZ, and hence the colour formation, is the reducing ability of the sample. The absorbance at 593 nm was measured 4 min after the addition of the FRAP reagent (acetate buffer, pH 3.6; FeCl_3 , TPTZ in 40 mM HCl) to the reaction mixture (0.025 % solution). The absorbance change of the aliquot diluted 1:40 in distilled water was due to the combined reductive activity of all the reacting antioxidants present within the sample. Optical density was compared to a standard curve prepared with 0-1.0 mM ferrous sulphate (FeSO_4) and results are expressed as the mean concentration of Fe^{2+} produced / mM.

2.3.5 ABTS+ determination of antioxidants

The antioxidant capacity of rat plasma was measured at the Unit of Nutrition, University of Montpellier II, France using a commercial kit (Randox Laboratories Ltd, Crumlin, UK). This method measures the ability of ABTS (2,2V-azino-di-(3-ethylbenzothiazoline sulfonate) to produce the radical cation ABTS^+ when incubated with peroxidase and hydrogen peroxide, producing a relatively stable blue-green color with absorption at 600 nm. Antioxidants present in plasma suppress the production of ABTS^+ to a degree proportional to their concentration. Trolox (1 mmol/L) was used as a standard and the plasma antioxidant power is expressed as Trolox equivalents (Miller *et al.*, 1993).

2.3.6 Protein estimation

An estimation of the protein concentration of dialysed LDL was carried out according to the method of Markwell *et al.* (1981). To each tube, 100 μL of a 1:10 dilution of LDL, 300 μL of distilled water and 2 mL of Biruet's reagent (100 mL Na_2CO_3 in 0.1 M NaOH; 1:1 ratio of Folin-Ciocalteu reagent; 1 mL NaK tartrate; 1 mL CuSO_4 ; 1 mL SDS [sodium dodecyl sulfate]) was added. Samples were mixed and left to stand at room temperature for 10 min. Two hundred μL of Folin-Ciocalteu reagent was added to each tube, vortexed and left to stand at room temperature for 30 min. A standard curve was prepared with 0 to 50 $\mu\text{g}^{-\text{L}}$ bovine serum albumin and samples were adjusted to a protein concentration of 50 μg LDL protein/mL and 1 μM EDTA in quartz cuvettes and the optical density was read at 750 nm.

2.3.7 Thiobarbituric reactive substances

Malondialdehyde is one of many aldehyde compounds produced by lipid peroxidation, and is the most commonly measured index of oxidative stress in human studies. A commonly used method for the assessment of oxidative stress is the TBARS assay, which aims to quantify the amount of malondialdehyde (MDA) formed as a result of lipid peroxidation. This assay involves reacting samples with 2-thiobarbituric acid (TBA) under high temperatures (90-100°C) and acidic conditions. TBA reacts with malondialdehyde to produce a stable adduct that can be quantified spectrophotometrically. LDL that was oxidised for measurement of conjugated dienes was subsequently used for the measurement of TBARS (Buege & Aust, 1978). Following 3 h oxidation, the reaction was stopped by addition of 200 μM EDTA and 40 μM butylated hydroxytoluene (BHT) and the samples were stored at 4°C for a maximum of 7 d. TBARS were determined by adding 1mL TCA-TBA-HCL reagent (15 % trichloroacetic acid: 0.375 % thiobarbituric acid: 25 mol HCL) to a 0.8 ml aliquot of the LDL solution (oxidised as above), mixing the sample, and heating it in boiling water at 100 °C for 15 min. After placing the tubes in a bath of cold water for 5 min samples were centrifuged at 2000 rpm for 10 min and absorbance was measured at 535 nm. Quantification of TBARS was performed by comparison with a standard curve of malonaldehyde equivalents generated by acid catalysed hydrolysis of 1,1,3,3 -tetra-ethoxypropane (TEP).

2.4 Spectral assays

2.4.1 Electron spin resonance

The ability of phenolics to reduce free radicals in the aqueous phase was estimated by electron spin resonance spectroscopy (ESR) as described by Gardner *et al.* (1998). This method is based on the ability of antioxidants in a sample to donate a hydrogen atom or electron to the synthetic free radical potassium nitro-disulphonate (Fremy's salt). A 300 μL aliquot of plasma was added to 2.7 mL phosphate buffered saline (PBS) and mixed with an equal volume of 50 μM of Fremy's radical in ethanol/water (12:88, v/v). After 20 min following completion of the reaction the ESR spectra of the low field resonance of the Fremy's radical was obtained. Signal intensity was obtained by double integration and the concentration was calculated by comparison with a control reaction using ethanol/water (12:88, v/v) without sample. Spectra were obtained at 21°C on a Bruker ECS 106 spectrophotometer equipped with a TM110 mode cavity operating at 9.5 GHz (x-band frequency). The microwave power and modulation amplitude was set at 2 mW and 0.01 mT.

2.4.2 Conjugated dienes assay

LDL was isolated from plasma by density gradient ultra-centrifugation at 35,000 rpm for 15 h at 15°C according to the method of Griffin *et al.* (1990). The LDL fraction was removed and samples were dialysed at 4°C against 40 L of PBS containing 10 μM Na_2EDTA (pH 7.4) for 42 h while shaking gently in a microdialysis unit (GibcoBRL, Life Technologies Ltd, Paisley, UK). The protein concentration of dialysed LDL was adjusted to a concentration of 50 μg LDL protein/mL and 1 μM EDTA in quartz cuvettes. Oxidation of LDL was initiated by addition of CuSO_4 (10 μL , 3.75 mM) added to 2.5 mL of LDL solution (50 μg protein/mL) to give a final concentration of 15 μM CuSO_4 (Kleinveld *et al.*, 1992). Absorbance was measured at 234 nm, every 2 min at 30°C. Maximal oxidation rate (nmol/mg LDL protein/min), maximal diene concentration (nmol/mg LDL protein) and lag phase (min) were estimated using exact co-ordinates provided by the kinetics software application (Beckman DU 600).

2.5 High performance liquid chromatography analyses

2.5.1 Measurement of carotenoids, retinol & tocopherols

The levels of six carotenoids (lutein/zeaxanthin, β -cryptoxanthin, all trans-lycopene, α -carotene and all trans β -carotene), retinol and α - and γ -tocopherol were measured in plasma by reversed phase HPLC with UV and fluorimetric detection according to the method of Hess *et al.* (1991). Two hundred μ L plasma was added to 200 μ L water and 400 μ L ethanol and mixed for 10 s. To this 700 μ L hexane and 100 μ L echinone were added, mixed and centrifuged for 5 min. The hexane layer was reduced to dryness and dissolved in 200 μ L of 1,4 dioxan; ethanol: acetonitrile (1:1:3, v/v). After shaking for 5-10 min, 150 μ L of sample was injected onto a 5 μ m Ultrasphere ODS, 25 cm x 4.6 mm (i.d.) column (Beckman Instruments Ltd, High Wycombe, Bucks, UK) and eluted at a flow rate of 1.3 mL/min. The detector wavelengths were changed during the run as follows: absorbance: 0-11.5 min at 450 nm; 11.6-18.0 min at 470 nm and 18.0-30 min at 450 nm. For fluorescence detection: 0-5.5 min at 330/470 nm (excitation/emission); 5.6-20.0 min at 298/328 nm and 20.1-30 min at 349/480 nm. The elution order and retention times of the compounds were as follows: retinol ($t_R = 2.7$ min); β -cryptoxanthin ($t_R = 6.0$ min); γ -tocopherol ($t_R = 6.4$ min); α -tocopherol ($t_R = 7.2$ min); lycopene ($t_R = 8.9$ min); α -carotene ($t_R = 14.9$ min); β -carotene ($t_R = 15.8$ min) and lutein/zeaxanthin ($t_R = 16.8$ min). The HPLC system comprised of a Waters 470 scanning fluorescence detector, a 600E system controller and a 486 tuneable absorbance detector (Waters Chromatography, Millford, MA, USA). The system was run using Millennium version 2.1 software. Extraction efficiency was determined by use of an echinone internal standard and quantified by reference to five point calibration curves.

2.5.2 Determination of ascorbic acid

Ascorbic acid ($t_R = 5.3$ min) was determined from 0.6 mL plasma and measured by ion-pair reversed phase HPLC with UV detection (Ross, 1994). After vortex mixing for 5 min samples were centrifuged (9000 x g, 4°C, 10 min) and the clear supernatant was injected onto a 5 μ m Nucleosil ODS, 25 cm x 4.6 mm (i.d.) column (Jones

Chromatography, Henygoed, Mid Glamorgan, UK) with a Supelco C₁₈ guard cartridge (Anachem, Beds, UK) at a flow rate of 0.6 mL/min monitored at 263 nm. The HPLC system comprised of two Gilson 305 pumps and a 20 µL loop volume automated sampler module connected to a Gilson 116 UV detector (Anachem, Beds, UK). The mobile phase comprised 25 mM myristyltrimethyl ammonium bromide, 0.05 M sodium hydroxide, 0.06 M acetic acid and 7.5 % acetonitrile adjusted to pH 5.5. Peak identification and purity was confirmed using ascorbate oxidase.

2.5.3 On-line analysis of antioxidants: ABTS⁺ decolourisation assay

The antioxidant activity of wine was determined using the ABTS⁺ on-line decolourisation assay based on the methods of Dapkevicius *et al.* (2001) and Koleva *et al.* (2001). On-line assessment allows complex mixtures to be separated by HPLC and the antioxidant contribution of individual components can be evaluated. The instrumental set-up is illustrated in Figure 2.1. A 2 mM ABTS⁺ stock solution containing 3.5 mM potassium persulfate was prepared and incubated at room temperature in darkness overnight. ABTS⁺ reagent was prepared by diluting the stock solution 8-fold in phosphate buffer at pH 8. Twenty µL of a 1:5 dilution of wine was injected into a HPLC system comprising a LC pump, a PDA detector and a UV-VIS detector (Surveyor HPLC, Thermo Finnigan). Separation was carried out using a 250 x 4.6 mm i.d. 4 µm Synergi RP-Max column (Phenomenex, Macclesfield, UK) maintained at 40°C and eluted at a flow rate of 1 mL/min with a 60 min gradient at 4-25 % acetonitrile and 0.1 % formic acid. Following separation of the compounds, the eluent was mixed with the ABTS⁺ reagent at a flow rate of 0.5 mL/min supplied by a Shimadzu LC-10 AP VP liquid chromatography pump. A Shimadzu GT-1543 vacuum degasser was used to remove any oxygen in the reagent prior to mixing. After mixing through a 3 m x 0.25 mm i.d. loop, the absorbance was measured by a UV detector at 720 nm (Nemphlar Bioscience, Lanark, UK). Data were analysed using Thermofinnigan Chromquest™ software version 4.0.

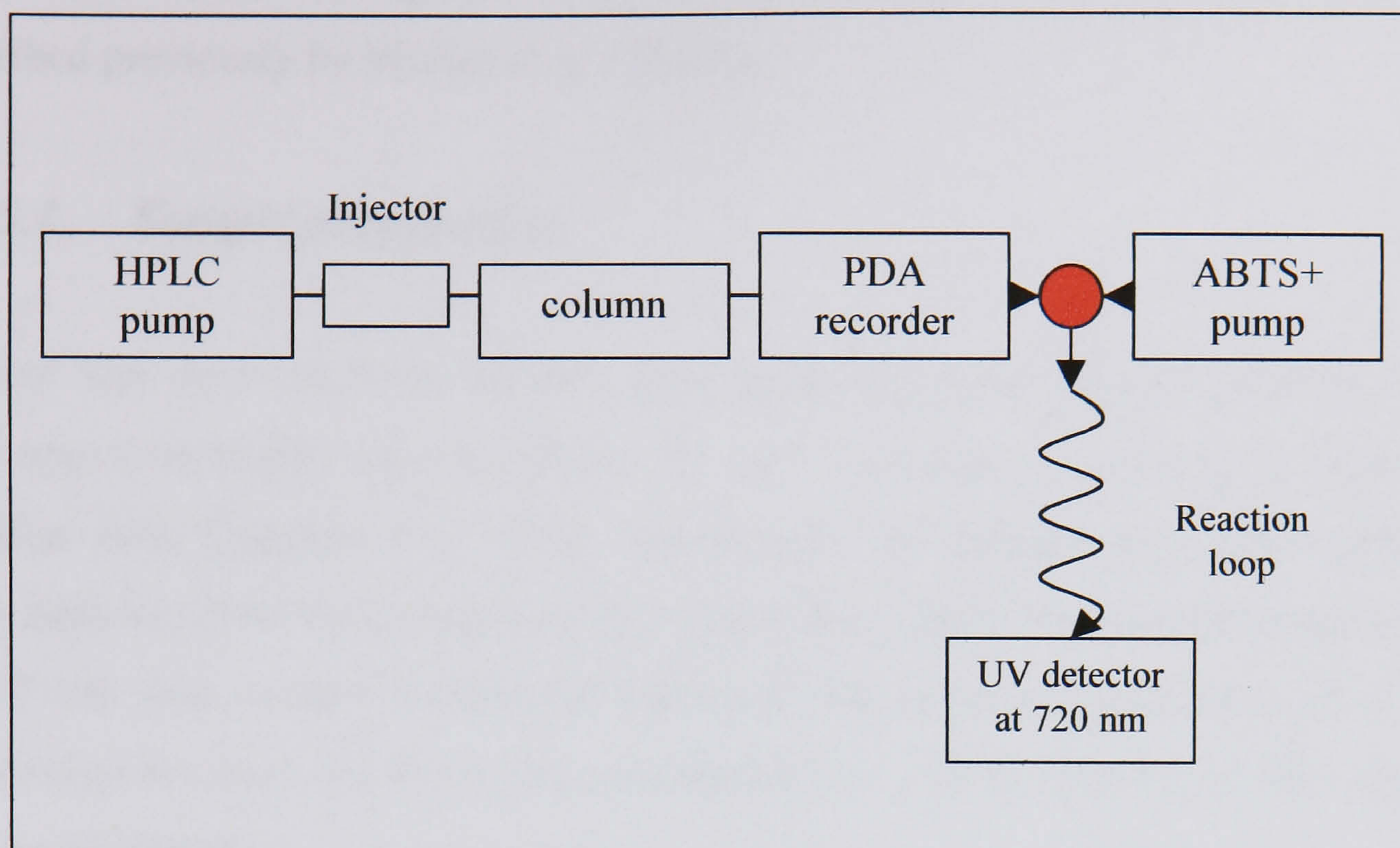


Figure 2.1 Instrument set-up for the HPLC analysis of radical scavenging compounds using an on-line reaction with ABTS+.

2.5.4 Plasma homocysteine concentrations

Homocysteine concentrations were measured in plasma using a DS30 homocysteine assay kit and a DS30 analyser (Drew Scientific Ltd, Barrow-in-Furness, Cumbria, UK) according to the method of Duthie *et al.* (2002). Following addition of internal standard (2-mercaptoethylamine) the disulphide bonds in the sample are reduced using tris (2-carboxyethyl) phosphine hydrochloride. Protein is precipitated from the solution and the thiol groups in the supernatant are derivitised with a fluorescent specific dye. The derivatives are separated and detected by their fluorescence ($\lambda_{\text{excitation}} = 385 \text{ nm}$, $\lambda_{\text{emission}} = 515 \text{ nm}$). Quantitative evaluation of the homocysteine concentration was achieved by comparison with a two-point calibration.

2.5.5 Preparative HPLC fractionation of wine

A Chilean Cabernet Sauvignon (wine 28) was fractionated using preparative HPLC as described previously by Mullen *et al.* (2002a).

2.5.5.1 *Sample preparation*

Alcohol was removed from 100 mL wine by rotary evaporation. The de-alcoholised wine was then loaded onto a 160.0 x 44 mm i.d. column packed with Diaion[®] Ion Exchange resin (Supelco, PA, USA). The column was washed with 10% methanol in 0.1% aqueous TFA which removed the sugars, after which the retained phenolics were eluted with four column volumes of methanol. The methanol was removed by rotary evaporation in vacuo and the residue resuspended in 5 % ACN in 0.5 % TFA, to a final volume of 100 mL.

2.5.5.2 *Fractionation of wine*

Wine was analysed using a preparative HPLC system. The system comprised a Hewlett Packard 85B gradient controller (CA, USA), two LC-10A pumps (Anachem, Luton, UK), a CTO-6A column oven set at 40°C, Rheodyne preparative injector model 3725 with a 10 mL injection loop (HPLC technology, Herts, UK), a 150 x 20 mm i.d., 5 µm ODS-H optimal[®] column (Capital HPLC, Broxburn, UK) and linked to a Dynamax UV absorbance detector monitoring at λ 520 nm and 371 nm (Rainin Instrument Co. Inc., MA, USA). Fractions were collected using a Gilson FC 203 fraction collector (WI, USA). Data was collected and processed via a Reeve Analytical (Glasgow, UK) 2700 data handling system. Ten mL of wine 28 was analysed using a 40 min gradient of 5 to 30 % ACN in 0.5 % TFA (with a 5 min hold at 30 %) eluted at a flow rate of 10 mL/min. Sixty fractions were collected 3 minutes after the injection of the wine collecting 7.5 mL/min of eluent.

2.6 HPLC-tandem mass spectrometry analysis

HPLC-tandem mass spectrometry (MS-MS) was the major analytical device used for the identification of phenolic compounds in wine and biological samples. Samples were analysed on a Surveyor HPLC system comprising a HPLC pump, diode array absorbance detector, scanning from 250 to 700 nm and an autosampler, cooled to 4°C (Thermo Finnigan, San Jose, USA). Separation was carried out using a 250 x 4.6 mm i.d. 4 µm Synergi RP-Max column (Phenomenex, Macclesfield, UK) eluted at a flow rate of 1 mL/min, with the column oven maintained at 40°C. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 mL was directed to a LCQ Deca XP ion trap mass spectrometer fitted with an electrospray interface as illustrated in Figure 2.2 (Thermo Finnigan, San Jose, USA). Analysis was carried out using both negative and positive ion mode for the detection of phenolic compounds in wine and biological samples.

Various gradients and conditions were used to detect phenolic compounds in wine and biological samples (Table 2.2). Full scan mode was used for the accurate determination of the parent ion and data-dependant MS² mode used to obtain fragmentation data. In this analysis the spectrum of ions is first scanned from m/z (mass to charge ratio) 100 to 2000 amu. The most significant ion in the scan is then collected in the ion trap, fragmented and the spectrum of the fragments recorded. The mass spectral information was used to identify the compounds. The m/z ratio of the molecular ion and the fragmentation pattern from these give unique fingerprints for each compound. Selective reaction monitoring was used where compounds could not be identified using the full scan mode. Selective reaction monitoring mode provides an extremely selective method for detecting target compounds. During chromatography the SRM mode removes all signals except from the characteristic process, MS¹ is set to transmit a precursor ion that is specific to the target compound and MS² is set to transmit a product ion of that precursor.

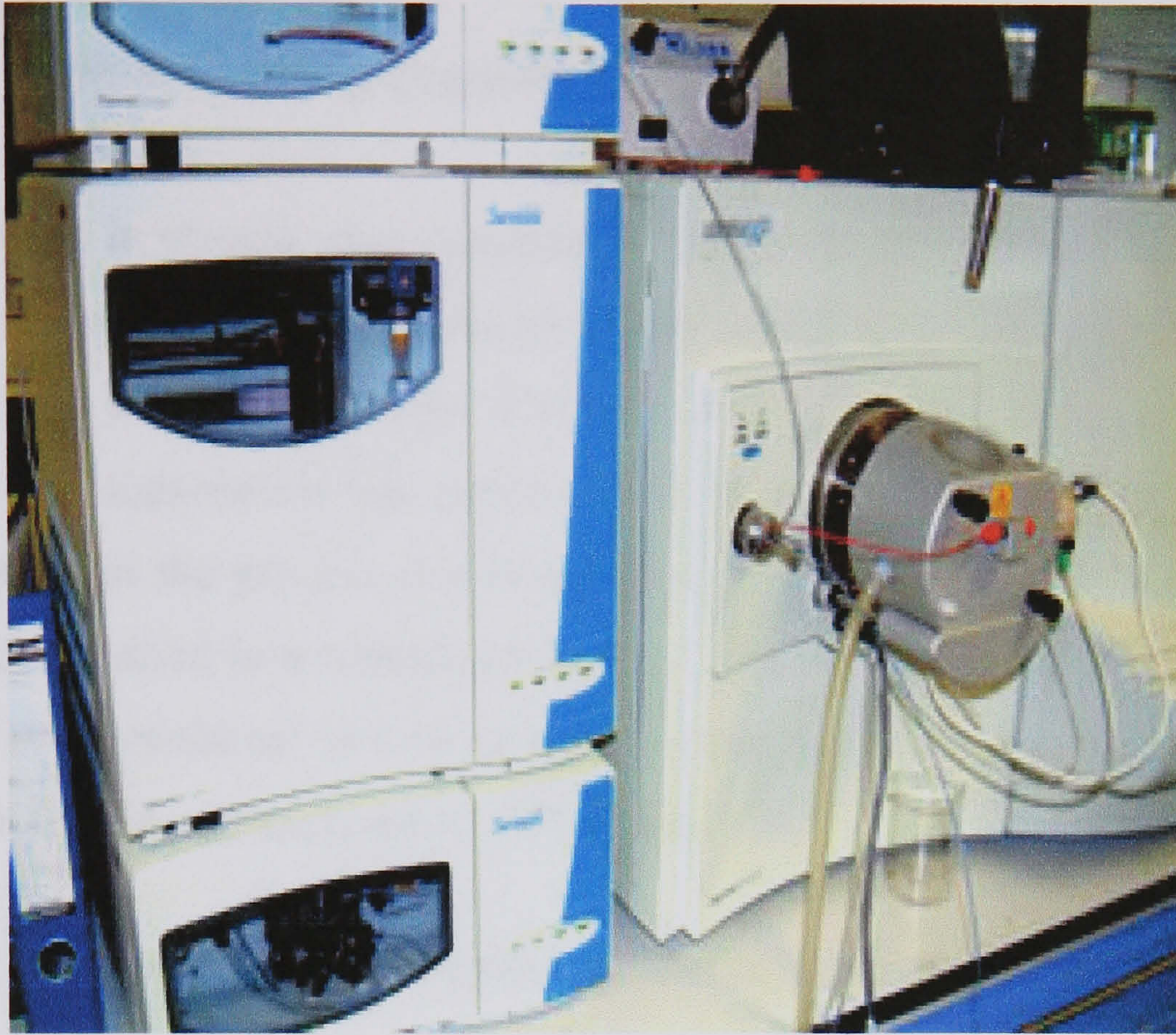


Figure 2.2 LCQ DECA XP ion trap mass spectrometer

Table 2.2 Gradient conditions and MS techniques used for the analysis of wine and biological fluids.

Sample	Gradient	Ion mode	Inj. Vol. (mL)	SRM
wine	5-40 % acetonitrile in 1% formic acid over 60 minutes	+/-	100	no
wine fractions	4-25 % acetonitrile in 1% formic acid over 60 minutes	+/-	100	no
plasma	5 to 40 % acetonitrile in 0.1% formic acid over 60 minutes	-	100	yes
urine	5 to 20 % acetonitrile in 0.1% formic acid over 60 minutes	-	100	yes
rat tissues	5 to 40 % acetonitrile in 0.1% formic acid over 60 minutes	-	100	yes

+: positive ion, -: negative ion, SRM:selective reaction monitoring

2.7 Analysis of phenolics in biological tissue

2.7.1 Extraction of phenolics from plasma

Proteins in plasma were precipitated according to a modified method of Day *et al.* (2002). In brief 1.5 mL of acetonitrile was added to 0.5 mL plasma. Samples were vortexed for 30 s every 2 min over a 10 min period, before centrifugation at 4000 rpm for 20 min. The supernatant was removed and the pellet was further extracted with 1.5 mL methanol and the process was repeated as described above. The two supernatants were mixed and dried in a centrifugal vacuum concentrator at 38°C and resuspended in 500 µL of 10 % methanol in 1 % aqueous formic acid. The sample was centrifuged at 25000 g for 10 min and analysed by HPLC-MS-MS.

2.7.2 Extraction of phenolics from rat tissues

Liver, kidney, brain, stomach, duodenum/jejunum, ileum, caecum, colon and faeces were extracted according to the method of Mullen *et al.* (2002b). In brief 4 g of liver, kidney and brain, and 2.5 g of gastrointestinal tract organs were extracted by continuous shaking with 15 mL of 50 % methanol in 50 % 0.1 M phosphate buffer (pH 7) containing 20 mM sodium diethyldithiocarbamate. After 30 min, the mixture was centrifuged at 4000 rpm for 20 min. The methanolic supernatant was decanted and the pellet re-extracted a further two times. The three methanolic supernatants were combined and the methanol was removed in vacuo. The remaining aqueous phase was adjusted to pH 3.0 and partitioned three times with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and reduced to dryness in vacuo prior to HPLC-MS analysis. Residual ethyl acetate was removed from the aqueous phase prior to loading onto a pre-conditioned 2g Sep-Pak C₁₈ cartridge. Methanol was used to remove polar compounds, and the extract was dried in vacuo and subsequently analysed by HPLC-MS-MS.

2.8 Measurement of fasting plasma lipids

Fasting plasma lipids including total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol and triacylglycerol (TAG) concentrations were determined by the Routine Lipids Laboratory, Department of Pathological Biochemistry, Glasgow Royal Infirmary using an automated Hitachi 197 multichannel analyser (Roche Diagnostics, Lewes, East Sussex, UK). Total cholesterol and TAG concentrations were determined using enzymatic *in vitro* colorimetric assays. HDL cholesterol concentrations were determined using an enzymatic *in vitro* colorimetric assay after precipitation of apo-B containing lipoproteins with heparin/0.092 M MnCl₂ and LDL cholesterol concentrations were calculated using the Friedewald formula (Friedewald *et al.*, 1972).

2.9 Statistics

Data are presented as mean values \pm standard error of mean (SEM), $n = 3$. Each sample was analysed in triplicate and calibrated against relevant standards where appropriate. Multivariate correlation analysis of experimental data was performed according to Pearson's Correlation (Chapter 3). Calculations quantified the relationship between two sets of experimental variables. The correlation coefficient r , quantified the direction and magnitude of the correlation and ranged from -1 to +1. Independent 2-sample paired t -tests were used to assess differences between groups and an unpaired t -test was used to assess differences within groups (Chapter 4). A one-way ANOVA was used to determine the differences in plasma antioxidant capacity between each time interval (Chapter 5). Values at $P < 0.05$ were considered statistically significant. ns = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared to relevant control. Analysis was carried out using Minitab software version 12 (Minitab Inc., Addison-Wesley Publishing Co., Reading, MA, USA).

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Chapter 3 Identification of the major phenolics in red wine

3.1 Introduction

Phenolic compounds are important components of red wine contributing to the organoleptic and sensory properties, preservation and ageing of wine (Robichaud & Noble, 1990; Soleas, 1997). Phenolic compounds are largely derived from the skins of grapes, particularly the epidermal cells, and seeds, with their concentration being very low in the pulp. Red wine provides an excellent source of various classes of phenolic compounds and may contain between 1000-4000 mg L⁻¹ (Bravo *et al.*, 1998). Although structurally diverse, phenolics fall into one of two major classes - the flavonoids and the non-flavonoids. Flavonoids include the flavonols quercetin, myricetin, kaempferol and Isorhamnetin, existing as aglycones and sugar conjugates; the flavan-3-ols (+)-catechin and (-)-epicatechin; and the anthocyanins, pigments responsible for the colour of red wines. Collectively flavonoid levels are 20-fold higher in red wines than in white wine (Soleas & Goldberg, 1999). The non-flavonoids comprise the hydroxybenzoates, including gallic acid and the hydroxycinnamates, encompassing *p*-coumaric acid, caffeic acid and caftaric acid. The concentration and composition of phenolics in wine depends on a range of factors including grape variety and vintage (Landrault *et al.*, 2001), variations in climate and vinification practices (Goldberg *et al.*, 1998; Price *et al.*, 1995), and the ageing process (Shahidi & Naczki, 1995). As a result the phenolic content and antioxidant capacity of red wine is highly variable.

Recently much effort has been expended on the analysis of red wine, particularly the relationship between the phenolic content and antioxidant activity. The antioxidant activity of red wines have been associated with their total phenolic content (Minussi *et al.*, 2002; Fogliano *et al.*, 1999; Sato *et al.*, 1996), yet the contribution of individual phenolic compounds to the total antioxidant activity has been little studied. Results to date have been inconsistent with studies reporting associations between the antioxidant properties of red wines with the levels of flavan-3-ols (Teissedre *et al.*, 1996; Simonetti *et al.*, 1997), anthocyanins (Ghiselli *et al.*, 1998; Pellegrini *et al.*, 2000) and tannic acid (Larrauri *et al.*, 1999).

The existing methods for the separation and identification of phenolic compounds in wine have been largely based on standard HPLC techniques with absorbance (Burns *et al.*, 2000) and fluorimetric (Rodriguez-Delgado *et al.*, 2002) detection. Among other detection systems used, mass spectrometry has proved to be effective and of considerable value. The technique of fractionation has been employed in an attempt to separate phenolic compounds in foods and beverages using both solid phase and liquid-liquid extraction. Previous studies have demonstrated that red wine phenolics have been separated using small-scale liquid-liquid extraction (C₁₈ Sep-Pak cartridge) methods (Ghiselli *et al.*, 1998; Oszmianski *et al.*, 1998; Sun *et al.*, 1998). However one of the major disadvantages with this method is the lack of selectivity due to the differences in polarity between different phenolics within a group. Therefore, the accurate identification of single phenolic compounds in complex mixtures, such as red wine, is not straight forward. Recently large-scale preparative HPLC has been used for the separation of phenolic compounds in raspberries (Mullen *et al.*, 2002a) and tea (M^cGinn *et al.*, 2001). This facilitated the identification of sanguin-H6 as the major antioxidant in raspberries, while in green tea ECGC was the main antioxidant component.

This chapter reports on a study in which the major phenolic contributors to the antioxidant capacity of red wines were investigated. Thirty-one red wines were analysed using several complimentary techniques to determine their phenolic content and antioxidant activity. HPLC with diode array detection and tandem mass spectrometry was used to identify the phenolic composition of the wines. Antioxidant activity was determined in the FRAP and ESR-derived antioxidant assays. Preparative HPLC and an on-line ABTS⁺ antioxidant assay were carried out to identify specific compounds in wine possessing antioxidant activity.

3.2 Phenolic content and antioxidant activity of wines

Thirty one red wines were analysed for their phenolic content and antioxidant capacity. Samples were selected to represent a variety of wines produced from various grape cultivars and geographical regions. Details of vintage, origin and varietal composition of each wine are detailed in Chapter 2 (Table 2.1). The total phenolic content of each wine was determined in the Folin-Ciocalteu colorimetric assay (Singleton and Rossi, 1965). Total, free and polymeric anthocyanins were measured in the colorimetric method of Ribereau-Gayon and Stonestreet (1965), and the FRAP-derived antioxidant assay of Benzie and Strain (1996) was used to estimate the antioxidant capacity of each wine. The results obtained are presented in Table 3.1.

3.2.1 Determination of total phenolics

There were almost four-fold differences in the concentrations of total phenolics observed in the different wines analysed. Total phenolic content varied from 587 to 2827 mg L⁻¹ gallic acid equivalents (GAE). These figures are comparable with values obtained for red wines by other investigators (Burns *et al.*, 2000; Frankel *et al.*, 1995; Sato *et al.*, 1996). In the current study phenolic-rich wines included a 2000 Young Vatted Spanish Tempranillo (wine 13), a 2000 South African Cabernet Sauvignon (wine 21), a 2001 Young Vatted Bulgarian Merlot (wine 15) and a 2000 Young Vatted Bulgarian Cabernet Sauvignon (wine 31). Lowest concentrations were detected in wine 3, a 2001 Beaujolais, and a 2001 Vin de pays L'Ardeche (wine 16) with values of 1389 and 587 mg L⁻¹ obtained, respectively.

3.2.2 Determination of anthocyanins

Polymeric anthocyanins were present in all wines in larger amounts than free anthocyanins. The highest total anthocyanin concentrations, 212.1 and 202 mg L⁻¹ malvidin-3-glucoside equivalents, were detected in wine 6, a 2001 French Syrah and wine 15, a Young Vatted Bulgarian Merlot, respectively (Table 3.2). The lowest levels, 28 and 43.3 mg L⁻¹ malvidin-3-glucoside equivalents, were observed in wine 16, a 2001 Vin de pays L'Ardeche and wine 9, a 1999 Chianti Classico, respectively.

3.2.3 Measurement of reducing ability

The ability of each wine to reduce a $\text{Fe}^{(\text{III})}$ -2,4,6-Tri-(2-pyr-idyl)-s-triazine (iron chloride-TPTZ) complex to $\text{Fe}^{(\text{II})}$ -TPTZ (iron-sulphate TPTZ) was assessed in the FRAP-derived antioxidant assay and values ranging from 6.3 to 29.4 mM $\text{Fe}^{(\text{II})}$ were obtained. Wine 3, a French Beaujolais and Vin de pays L'Ardeche (wine 16) showed the lowest activities, while a Spanish Young Vatted Tempranillo (wine 13) and a South African Cabernet Sauvignon (wine 21) were ranked first and second, respectively. The relationship between phenolic content with reducing ability in the FRAP-derived antioxidant assay of each wine was analysed using Pearson's correlations. FRAP-derived antioxidant activity was highly and significantly correlated with total phenolic concentration ($r=0.972$, $P < 0.001$) and moderately correlated with total anthocyanin content ($r=0.434$, $P < 0.05$) and polymeric pigment content ($r^2=0.594$; $P < 0.001$).

Table 3.1 Phenolic composition and antioxidant capacity of red wines.

wine	TP ^a	P _R ^b	TA ^c	PP ^d	FA ^e
1. Claret non-vintage	2245 ± 30	22 ± 0	138 ± 6	66 ± 0	72 ± 7
2. Beaune	2458 ± 36	23 ± 1	61 ± 7	27 ± 5	34 ± 1
3. Beaujolais	1389 ± 7	12 ± 0	72 ± 4	46 ± 4	27 ± 1
4. Cotes du Rhone	2034 ± 24	18 ± 0	89 ± 2	64 ± 8	25 ± 0
5. Vin de pays de L'Ardeche	1983 ± 11	20 ± 1	101 ± 2	85 ± 10	16 ± 0
6. Etienne Barret Crozes Hermitage	2004 ± 13	18 ± 0	212 ± 6	66 ± 3	146 ± 3
7. Minervois	2038 ± 32	19 ± 0	123 ± 10	73 ± 1	50 ± 9
8. Corbieres	2352 ± 25	23 ± 1	144 ± 0	108 ± 2	36 ± 0
9. Chianti Classico	2424 ± 23	24 ± 1	43 ± 34	57 ± 5	n.d
10. Valpolicella	1762 ± 38	18 ± 1	97 ± 6	50 ± 8	47 ± 0
11. Aged in Oak Valdepenas Reserva	2202 ± 69	23 ± 0	93 ± 4	71 ± 1	22 ± 3
12. Castillo de Sierra Rioja	1537 ± 42	16 ± 0	81 ± 1	46 ± 6	35 ± 0
13. Young Vatted Tempranillo	2827 ± 26	29 ± 1	118 ± 0	77 ± 2	41 ± 0
14. Bairrada	2032 ± 40	20 ± 0	83 ± 4	38 ± 4	45 ± 0
15. Young Vatted Merlot	2729 ± 69	29 ± 0	202 ± 1	105 ± 1	97 ± 0
16. Vin de Pays L'Ardeche Rose	587 ± 10	6 ± 0	28 ± 1	10 ± 5	18 ± 0
17. Australian Red	1819 ± 15	19 ± 1	86 ± 1	61 ± 5	25 ± 0
18. Australian Oaked Cab. Sauv.	1811 ± 10	18 ± 0	113 ± 2	55 ± 0	59 ± 2
19. Young Vatted Pinotage	2578 ± 30	27 ± 1	127 ± 6	83 ± 6	43 ± 1
20. Cinsault	1611 ± 13	16 ± 1	81 ± 1	41 ± 5	40 ± 0
21. Cabernet Sauvignon	2776 ± 8	28 ± 1	139 ± 3	83 ± 2	57 ± 1
22. Caballo de Plata	1666 ± 32	16 ± 0	72 ± 6	52 ± 6	21 ± 0
23. Cabernet Sauvignon	2048 ± 36	21 ± 0	112 ± 1	61 ± 2	51 ± 0
24. Argentinian Shiraz	1784 ± 39	19 ± 0	109 ± 3	65 ± 6	44 ± 0
25. Inti Malbec	2133 ± 12	22 ± 0	156 ± 4	61 ± 1	95 ± 3
26. Chilean Dry Red	1801 ± 16	18 ± 0	132 ± 0	95 ± 7	37 ± 0
27. Chilean Cabernet Merlot	1965 ± 13	20 ± 0	140 ± 0	82 ± 0	58 ± 0
28. Chilean Cab. Sauv.	2246 ± 26	23 ± 0	152 ± 1	92 ± 9	61 ± 0
29. Granverano Carmenere	2115 ± 11	22 ± 1	172 ± 0	94 ± 2	78 ± 0
30. Pinot Noir Estancia	2282 ± 23	24 ± 1	97 ± 1	44 ± 5	53 ± 0
31. Young Vatted Cab. Sauv.	2597 ± 131	23 ± 3	159 ± 4	105 ± 9	53 ± 0

^a TP-total phenolics expressed as gallic acid equivalents (mg L⁻¹); ^b P_R-ferric reducing power (FRAP) expressed as concentration of Fe^(II) produced (mM); ^{c,d,e} TA-total anthocyanins; PP-polymeric pigments and FA-free anthocyanins. Results are expressed as malvidin-3-glucoside equivalents (mg L⁻¹) ± standard error, where *n* = 3., n.d. not detected.

3.3 HPLC-tandem mass spectrometry analysis of wines

The levels of individual phenolics were determined by HPLC-tandem mass spectrometry (MS-MS) and diode array detection. A selection of the wines exhibiting high and low phenolic contents and antioxidant activities were analysed including a 2000 Young Vatted Bulgarian Cabernet Sauvignon (wine 31), a 2000 Young Vatted Spanish Tempranillo (wine 13), a 2001 Young Vatted Bulgarian Merlot (wine 15), a 2001 Chilean Cabernet Sauvignon (wine 28) and wine 3, a 2001 French Beaujolais. Samples were analysed on a Surveyor HPLC system using a 60 min, 5-40 % gradient of acetonitrile in 1 % aqueous formic acid. The column eluate being directed first to a diode array absorbance monitor and then to a mass spectrometer with an electrospray interface operating in full scan MS/MS mode. Samples were analysed in both positive and negative ionization mode. In total 19 phenolic compounds were identified in each wine based on MS/MS data and λ_{\max} . The results are summarised below and presented in Table 3.2.

Peak 1 ($t_R = 5.3$ min, $\lambda_{\max} = 265$ nm) had a $[M-H]^-$ at m/z 169 with MS^2 yielding a charged fragment ion at m/z 125. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is gallic acid.

Peak 2 ($t_R = 6.9$ min, $\lambda_{\max} = 265$ nm) had a $[M-H]^-$ at m/z 865 with MS^2 yielding two charged fragment ions at m/z 577 and 289. Based on the mass spectral data this compound is a procyanidin trimer and its elution prior to dimer B_1 (Santos-Buelga *et al.*, 1995) indicates that it is likely to be the trimer procyanidin C_2 , a known component of red wine (De Pascual-Teresa *et al.*, 2000b).

Peak 3 ($t_R = 13.4$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 577, with MS^2 producing three major charged fragment ions at m/z 425, 407 and 289. Based on the mass spectral data this compound is a procyanidin dimer. Co-chromatography with a standard indicated the dimer was procyanidin B_1 , a known component of red wine (De Pascual-Teresa *et al.*, 2000b).

Peak 4 ($t_R = 14.3$ min, $\lambda_{\max} = 515$ nm) had a $[M-H]^+$ at m/z 465 which fragmented with MS^2 to produce a delphinidin-like ion at m/z 303 with the $[M-H]^+ - 162$, corresponding to loss of a glucose moiety. This peak is, therefore, the anthocyanin delphinidin-3-glucoside, previously identified in red wine by Burns *et al.* (2002).

Peak 5 ($t_R = 15.4$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 289, yielding two charged ions at m/z 245 and 205. This mass spectrum and co-chromatography with an authentic standard established that peak 4 is the flavan-3-ol (+)-catechin.

Peak 6 ($t_R = 17.8$ min, $\lambda_{\max} = 515$ nm) had a $[M-H]^+$ at m/z 479 which on MS^2 yielded a fragment ion at m/z 317 which corresponds with petunidin ($[M-H]^+ - 162$, cleavage of a glucosyl unit) indicating the presence of petunidin-3-glucoside which has previously been detected in red wines (Burns *et al.*, 2002).

Peak 7 ($t_R = 18.3$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 577 with MS^2 yielding three charged fragment ions at m/z 425, 405 and 289. Based on the mass spectral data this peak is a procyanidin dimer and co-chromatography with a reference compound revealed that it is procyanidin B₂, previously detected in red wine by De Pascual-Teresa *et al.* (2000b).

Peak 8 ($t_R = 19.8$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 289, which on MS^2 yielding ions at m/z 245, 205 and 179. Co-chromatography with an authentic standard confirmed the identification of peak 7 as (-)-epicatechin.

Peak 9 ($t_R = 20$ min, $\lambda_{\max} = 510$ nm) had a $[M-H]^+$ at m/z 463 which on MS^2 exhibited a 162 amu loss, corresponding to cleavage of a glucosyl unit, producing a fragment ion at m/z 301 which corresponds to peonidin. This is in keeping with the presence of peonidin-3-*O*-glucoside, a known component of red wine (Burns *et al.*, 2002).

Peak 10 ($t_R = 21$ min, $\lambda_{\max} = 515$ nm) had a $[M-H]^+$ at m/z 493, MS^2 produced a fragment ion at m/z 331 ($[M-H]^+ - 162$, loss of a glucosyl unit). This peak was confirmed as malvidin-3-*O*-glucoside by reference to an authentic standard.

Peak 11 ($t_R = 21.7$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 865 with MS^2 yielding two charged fragment ions at m/z 577 and 289. Based on the mass spectral data this compound is a procyanidin trimer and its elution after (-)-epicatechin (Santos-Buelga *et al.*, 1995) indicates that it is procyanidin C₁, a known component of red wine (Sánchez-Moreno *et al.*, 2003).

Peak 12 ($t_R = 25$ min, $\lambda_{\max} = 510$ nm) had a $[M-H]^+$ at m/z 517 with MS^2 yielding a charged fragment ion at m/z 355. Although a reference compound is not available, the elution order of this peak after malvidin-3-glucoside and the mass spectral data indicate that it may be vitisin B which has been detected in red wines by Morata *et al.* (2003) and is the adduct resulting from the reaction between malvidin-3-glucoside and acetaldehyde.

Peak 13 ($t_R = 25$ min, $\lambda_{\max} = 370$ nm) had a $[M-H]^-$ at m/z 479 with MS^2 yielding a fragment ion at m/z 317, (162 amu loss, cleavage of a glucosyl unit) which corresponds with myricetin indicating that this compound is a myricetin glucoside.

Peak 14 ($t_R = 29.1$ min, $\lambda_{\max} = 515$ nm) had a $[M-H]^+$ at m/z 533 which on MS^2 yielded a charged fragment ion at m/z 331 ($[M-H]^+ - 204$, loss of an acetylglucosyl unit). On the basis of the MS^2 spectra and the elution order of anthocyanins, this peak is identified as malvidin-3-acetylglucoside previously detected in red wines (Burns *et al.*, 2002).

Peak 15 ($t_R = 29.6$ min, $\lambda_{\max} = 350$ nm) had a $[M-H]^-$ at m/z 471, MS^2 yielded a fragment ion at m/z 301 ($[M-H]^- - 176$, cleavage of a glucuronyl group). On the basis of the MS^2 spectral data and co-chromatography with an authentic standard this peak is identified as quercetin-3-glucuronide.

Peak 16 ($t_R = 34.4$ min, $\lambda_{\max} = 350$ nm) had a $[M-H]^-$ at m/z 317 which corresponds with the flavonol aglycone myricetin. Co-chromatography with an authentic standard confirmed the presence of myricetin.

Peak 17 ($t_R = 35.2$ min, $\lambda_{\max} = 525$ nm) had a $[M-H]^+$ at m/z 639 fragmentation by MS^2 yielded a charged fragment ion at m/z 331 ($[M-H]^+ - 308$, loss of a *p*-coumaroylglucoside group). Based on the absorbance and mass spectral data and the elution profile of this compound it is likely to be the anthocyanin malvidin-3-(*p*-coumaroyl)-glucoside (Burns *et al.*, 2002).

Peak 18 ($t_R = 43.5$ min, $\lambda_{\max} = 370$ nm) had a $[M-H]^-$ at m/z 301 and the identity of this peak was confirmed as the flavonol aglycone quercetin by co-chromatography.

Peak 19 ($t_R = 51$ min, $\lambda_{\max} = 370$ nm) had a $[M-H]^-$ at m/z 285 which indicates the presence of kaempferol and this identification was confirmed by co-chromatography with an authentic standard.

Table 3.2 Identification of flavonoids and phenolic compounds in red wine based on HPLC retention times (R_t), λ_{\max} and MS-MS data obtained in full scan negative and positive ion mode^a.

Peak	R_t (min)	λ_{\max} (nm)	Compound	[M-H] ⁻ (m/z)	MS ² ions (m/z)
1	5.3	265	gallic acid	169 ⁻	125
2	6.9	280	procyanidin trimer C ₂	865 ⁻	577
3	13.4	280	procyanidin dimer B ₁	577 ⁻	425, 407
4	14.3	515	delphinidin-3- <i>O</i> -glucoside	465 ⁺	303 [Del], ([M-H]) ⁺ -Glc]
5	15.3	280	(+)-catechin	289 ⁻	163, 125
6	17.8	515	petunidin-3- <i>O</i> -glucoside	479 ⁺	317 [Pet], ([M-H]) ⁺ -Glc]
7	18.9	280	procyanidin dimer B ₂	577 ⁻	425, 407
8	19.8	280	(-)-epicatechin	289 ⁻	163; 125
9	20.0	510	peonidin-3- <i>O</i> -glucoside	463 ⁺	301 [Peo], ([M-H]) ⁺ -Glc]
10	21.0	515	malvidin-3- <i>O</i> -glucoside	493 ⁺	331 [Mal], ([M-H]) ⁺ -Glc]
11	21.8	280	procyanidin trimer C ₁	865 ⁻	577
12	25.0	510	vitisin B	517 ⁺	355
13	25	370	myricetin glucoside	479 ⁻	317 [Myr], ([M-H]) ⁺ -Glc]
14	29.1	515	malvidin-(6-acetyl)-3-glucoside	535 ⁺	331 [Mal], ([M-H]) ⁺ -AGlc]
15	29.6	350	quercetin-3-glucuronide	477 ⁻	301 [Q], ([M-H]) ⁺ -GlcUA]
16	34.4	350	myricetin	317 ⁻	—
17	35.2	525	malvidin-(6- <i>O</i> - <i>p</i> -coumaroyl)-3-glucoside	639 ⁺	331 [Pet], ([M-H]) ⁺ -PGlc]
18	43.5	370	quercetin	301 ⁻	—
19	51.0	370	kaempferol	285 ⁻	—

3.4 Levels of phenolics in selected wines

The levels of 19 phenolic and polyphenolic compounds were measured by HPLC-MS-MS and the results obtained are summarised below and presented in Table 3.3. There was a wide variation in the levels of phenolics in each wine; however gallic acid, the flavan-3-ols and the anthocyanins were the most abundant compounds.

The levels of gallic acid varied almost 4-fold from 69 mg L⁻¹ in a French Beaujolais (wine 3) to 282 mg L⁻¹ in wine 31, a Young Vatted Bulgarian Cabernet Sauvignon. (+)-Catechin, (-)-epicatechin and procyanidin dimers B₁ and B₂ were identified by LC-MS, with total flavan-3-ols ranging from 145 mg L⁻¹ to 260 mg L⁻¹ in wine 15, a Young Vatted Bulgarian Merlot and a Spanish Tempranillo, respectively. (+)-Catechin was invariably present in larger amounts than (-)-epicatechin in all wines analysed. The highest concentration was found in wines 31 and 13 contributing 22 % and 17 % of the total flavan-3-ols, respectively.

Seven anthocyanins (two of them acylated) were identified in red wines: malvidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-acetyl-glucoside, malvidin-3-(*p*-coumaroyl) glucoside and vitisin B. Total anthocyanins ranged from 80 mg L⁻¹ to 477 mg L⁻¹ in wine 3 and 28, respectively. Malvidin-3-glucoside constituted the main anthocyanin in wines and levels varied almost 8-fold from 43 mg L⁻¹ in wine 3, a French Beaujolais to 305 mg L⁻¹ in wine 28, a Chilean Cabernet Sauvignon.

Flavonols were minor components in the wines compared with other classes of phenolics identified, with quercetin, myricetin, kaempferol and conjugates of quercetin and myricetin detected. Total flavonols ranged from 4 to 17.3 mg L⁻¹ in wine 3, a French Beaujolais and a Spanish Tempranillo (wine 13), respectively.

Table 3.3 Levels of flavonoids and phenolic compounds in selected red wines ^a

Compound	wine 3 (Beaujolais)	wine 13 (Tempranillo)	wine 15 (Merlot)	wine 28 (Cab. Sauv.)	wine 31 (Cab. Sauv.)
gallic acid	69 ± 2.3	104 ± 1.1	116 ± 0.8	146 ± 3.1	282 ± 10
procyanidin trimer C ₁	7.0 ± 0.8	6.1 ± 1.2	14.4 ± 4.4	5.5 ± 1.7	14.3 ± 4.6
(+)-catechin	25.2 ± 0.3	43.0 ± 0.2	40.0 ± 1.3	42.4 ± 0.2	43.0 ± 0.5
(-)-epicatechin	14.2 ± 1.0	37.0 ± 0.2	28.0 ± 1.0	28.0 ± 1.0	42.3 ± 1.6
procyanidin dimer B ₁	38 ± 0.4	51.0 ± 6.0	35.1 ± 1.0	44 ± 2.4	39.3 ± 1.1
procyanidin dimer B ₂	59 ± 3.5	104 ± 6.5	16.1 ± 1.0	43.1 ± 3.9	44.7 ± 0.3
procyanidin trimer	8.1 ± 2.0	19.1 ± 5.4	12 ± 2.4	7 ± 2.5	8.4 ± 0.6
delphinidin-3- <i>O</i> -glucoside	5 ± 0.1	11.4 ± 0.3	11 ± 0.5	29 ± 0.4	7.1 ± 0.3
peonidin-3- <i>O</i> -glucoside	8.4 ± 0.4	12 ± 0.3	15.0 ± 0.2	31 ± 0.4	7.6 ± 0.2
petunidin-3- <i>O</i> -glucoside	9.3 ± 0.3	7 ± 0.1	9.4 ± 0.1	19 ± 0.2	3.0 ± 0.0
malvidin-3- <i>O</i> -glucoside	43 ± 4.1	62.0 ± 3.7	81.1 ± 3.5	305 ± 3.0	155 ± 4.0
vitisin B	n.d	4.2 ± 0.1	7.3 ± 0.3	n.d	6.4 ± 0.0
malvidin-3-acetylglucoside	6 ± 1.0	7 ± 0.1	16.3 ± 0.3	79.0 ± 3.5	6.3 ± 0.1
malvidin-3-(<i>p</i> -coumaroyl)glucoside	10 ± 0.3	9 ± 0.2	7.1 ± 0.4	15.5 ± 0.2	2.6 ± 0.0
quercetin	1.3 ± 0.0	4.0 ± 0.2	5.1 ± 0.0	2.6 ± 0.0	5.8 ± 0.1
myricetin	1.2 ± 0.1	2.0 ± 0.1	9.3 ± 0.1	4.3 ± 0.0	3.6 ± 0.1
kaempferol	n.d	0.7 ± 0.0	0.2 ± 0.0	n.d	0.3 ± 0.0
quercetin glucuronide	1.2 ± 0.0	5.1 ± 0.1	1.5 ± 0.0	3.3 ± 0.1	2.0 ± 0.1
myricetin-glucoside	n.d	5.5 ± 0.0	n.d	2.4 ± 0.1	1.0 ± 0.0

^a Levels of individual phenolics expressed as mg L⁻¹ (+)-catechin, malvidin-3-glucoside and quercetin equivalents for total flavan-3-ols; anthocyanins and flavonols, respectively, ± standard error, where *n* = 3. n.d. not detected.

3.5 Identification of the major antioxidants in red wine

Two techniques were employed to identify the major phenolic compounds in wine contributing to the *in vitro* antioxidant activity; an on-line ABTS⁺ antioxidant assay and preparative HPLC as detailed in Chapter 2 (section 2.5.3 and 2.5.5, respectively). Although the technique of preparative HPLC has been successful for the separation of compounds in tea and raspberries, it did not allow for the complete separation of compounds in red wine. Nevertheless, the results indicated a significant correlation between the total catechin content of wine fractions with ESR-derived antioxidant activity. On-line analysis of individual compounds in red wine was measured in the ABTS⁺ antioxidant assay. This method allowed the complete separation of compounds in wine, while at the same time, measured their antioxidant activity.

3.5.1 Preparative HPLC

Ten mL of a Chilean Cabernet Sauvignon (wine 28) was injected on a preparative HPLC system comprising two HPLC pumps, a UV absorbance detector, monitoring at 520 and 371 nm, and a preparative injector with a 10 mL injection loop. Separation was carried out using a 40 min gradient of 5 to 30 % ACN in 0.5 % TFA (with a 5 min hold at 30 %) on a 150 x 20 mm i.d. 5 µm ODS-H column eluted at a flow rate of 10 mL/min, with the column oven maintained at 40°C. Sixty fractions were collected 3 min after the injection of the wine collecting 7.5 mL/min of eluent. Each fraction was measured for total phenolic content, anthocyanin content and antioxidant activity in the ESR-derived antioxidant assay. Selected fractions exhibiting high phenolic contents and antioxidant abilities were further analysed for their total catechin content, and HPLC-MS was used to identify their phenolic composition.

3.5.1.2 Total phenolics and ESR-derived antioxidant activity

The total phenolic content of each fraction was determined using the Folin-Ciocalteu method and the ability of the sixty wine fractions to reduce Fremy's salt free radical in the ESR-based antioxidant assay was assessed and results are illustrated in Figure 3.1. Fraction 13 contained the highest total phenolic content with 78.3 mg L⁻¹ GAE obtained, while fractions 12 and 20 were ranked second and third with concentrations of 67 and 62.3 mg L⁻¹ GAE obtained, respectively. The greatest antioxidant activity was observed in fraction 20 with 11.4 x 10²¹ radicals reduced L⁻¹, while fractions 18 and 19 were ranked second and third with antioxidant activities of 11.1 x 10²¹ and 11.0 x 10²¹ obtained, respectively. The total phenolic content of the wine fractions were highly and significantly associated with ESR-derived antioxidant activity ($r=0.816$, $P < 0.001$) using Pearson's correlations.

3.5.1.3 Determination of total anthocyanins

The total anthocyanin content of each wine fraction was determined using a pH shift method as described in Chapter 2 (section 2.3.2) and results are illustrated in Figure 3.2. Fraction 31 contained the highest concentration of total anthocyanins with 52 mg L⁻¹ malvidin-3-glucoside equivalents obtained, followed by fraction 42 with a concentration of 20.1 mg L⁻¹ malvidin-3-glucoside equivalents obtained. The relationship between total anthocyanin content with ESR-derived antioxidant activity of each fraction was assessed using Pearson's correlations. There was no correlation between the total anthocyanin content of wine fractions with antioxidant activity ($r=0.188$, $p=0.151$).

3.5.1.4 *Total catechin content of selected fractions*

Selected fractions were analysed for their total catechin content using a colorimetric method by Kivitis *et al.*, (1997) as detailed in Chapter 2 (section 2.3.3). Results are illustrated in Figure 3.3. Seventeen fractions were analysed, incorporating fractions exhibiting high and low phenolic contents and antioxidant abilities. Fraction 5 contained the highest concentration with 75.1 mg L⁻¹ total catechin obtained, while the lowest concentration was observed in fraction 31 with a value of 1.5 mg L⁻¹ total catechin. The total catechin content of selected fractions was highly and significantly associated with ESR-derived antioxidant activity ($r^2 = 0.591$, $P < 0.01$) when analysed using Pearson's correlations.

3.5.1.5 *HPLC-MS analysis of selected wine fractions*

Analysis of the individual fractions were analysed by LC-MS/MS. Separation was carried out using a 250 x 4.6 mm i.d. 4 µm RP-Max column (eluted at a flow rate of 1 mL/min). A 5-40 % gradient over 60 min of 1 % formic acid and acetonitrile was used for the complete separation of all compounds. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 mL was directed to a LCQ Deca XP ion trap mass spectrometer fitted with an electrospray interface. Analysis was carried out in full scan mode from 100-2000 amu using the positive ion mode when analysing anthocyanins and with negative ionisation for all other compounds. LC-MS/MS established the presence of gallic acid ($t_R=5.84$ min, λ_{max} 280 nm) and procyanidin trimer ($t_R=8.52$ min, λ_{max} 277 nm) in fractions 4 and 5. Two further compounds were detected in these fractions but could not be identified. Fractions 13-20 contained procyanidin dimer B₁ ($t_R=10.8$ min, λ_{max} 285 nm); (+)-catechin ($t_R=15.5$ min, λ_{max} 280 nm); (-)-epicatechin ($t_R=20.5$ min, λ_{max} 280 nm) and procyanidin B₂ ($t_R=19.6$ min, λ_{max} 280 nm), consistent with the data obtained by colorimetric analysis of total catechins. Fractions 26-31 contained anthocyanins, consistent with the data obtained by colorimetric analysis, with petunidin-3-glucoside ($t_R=16.92$ min, λ_{max} 519 nm) and malvidin-3-glucoside ($t_R=25.98$ min, λ_{max} 525 nm) identified in Fraction 31.

3.5.2 ABTS⁺ on-line decolourisation assay

The antioxidant activity of individual components in the 5 selected wines was determined using the ABTS⁺ on-line decolourisation assay based on the methods of Dapkevicius *et al.* (2001) and Koleva *et al.* (2001) described in Chapter 2 (section 2.5.3). In brief, a 2 mM ABTS⁺ stock solution containing 3.5 mM potassium persulfate was prepared and incubated at room temperature in darkness overnight. ABTS⁺ reagent was prepared by diluting the stock solution 8-fold in phosphate buffer at pH 8. Twenty μL of a 1:5 dilution of wine was injected into a HPLC system comprising a LC pump, a PDA detector and a UV-VIS detector. Separation was carried out using a 250 x 4.6 mm i.d. 4 μm RP-Max column maintained at 40°C and eluted at a flow rate of 1 mL/min with a 60 min gradient at 4-25 % acetonitrile and 0.1 % formic acid. Following separation of the compounds, the eluent was mixed with the ABTS⁺ reagent at a flow rate of 0.5 mL/min supplied by a Shimadzu LC-10 AP VP liquid chromatography pump. A Shimadzu GT-1543 vacuum degasser was used to remove any oxygen in the reagent prior to mixing. After mixing through a 3 m x 0.25 mm i.d. loop, the absorbance was measured by a UV detector at 720 nm. The results obtained are presented in Table 3.4 and shown in Figures 3.4-3.8.

The major antioxidants detected in each wine had an absorbance maximum at 280 nm, with 9 peaks being identified. Antioxidant activity was clearly associated with the presence of gallic acid and the flavan-3-ols. Gallic acid was identified as a major antioxidant component of red wine with values ranging from 103 mg L⁻¹ to 269.2 mg L⁻¹ Trolox equivalents in a French Beaujoalis (wine 3) and a Young Vatted Bulgarian Cabernet Sauvignon (wine 31), respectively. The flavan-3-ols were also identified as major antioxidants in red wines with (+)-catechin and (-)-epicatechin being the most abundant in all wines analysed. Values ranging from 67 to 130.4 mg L⁻¹ Trolox equivalents were obtained for wine 3 and wine 31, respectively. Procyanidin dimer B₁ ranged from 32.4 mg L⁻¹ in wine 15 to 128.3 mg L⁻¹ Trolox equivalents in wine 3, contributing 6 % and 29 % of the total antioxidant activity, respectively. The Chilean Cabernet Sauvignon (wine 28) exhibited the highest activity for dimer B₂, while wine 15 had the lowest activity, with values ranging from 8.0 to 52 mg L⁻¹ Trolox equivalents.

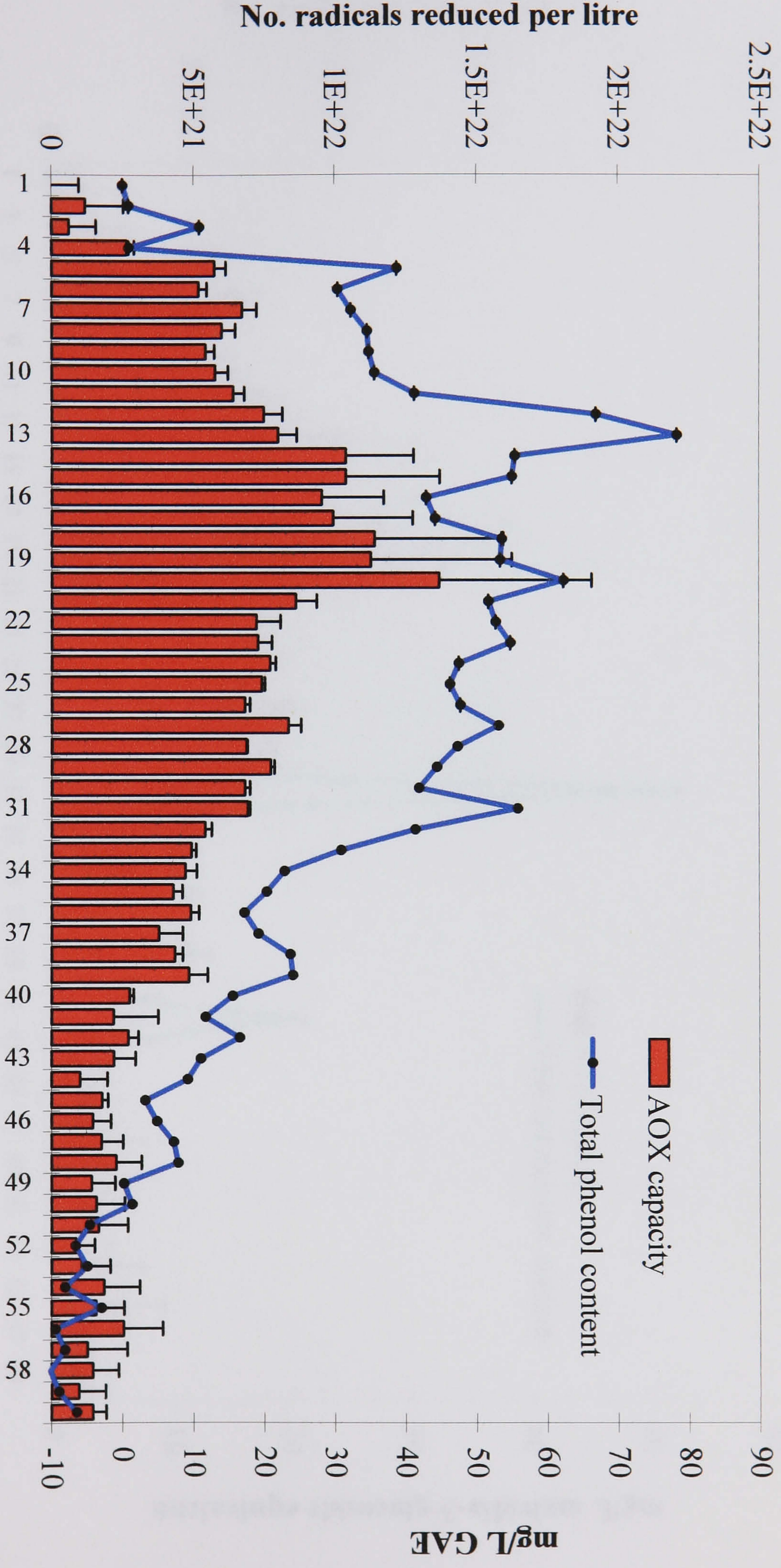


Figure 3.1 Total phenolic content and ESR-derived antioxidant activity of wine 28 fractions 1-60. Results are expressed as mg L⁻¹ GAE and no. Fremys radicals reduced (x 10²¹) per litre of wine ± standard error, where n=3.

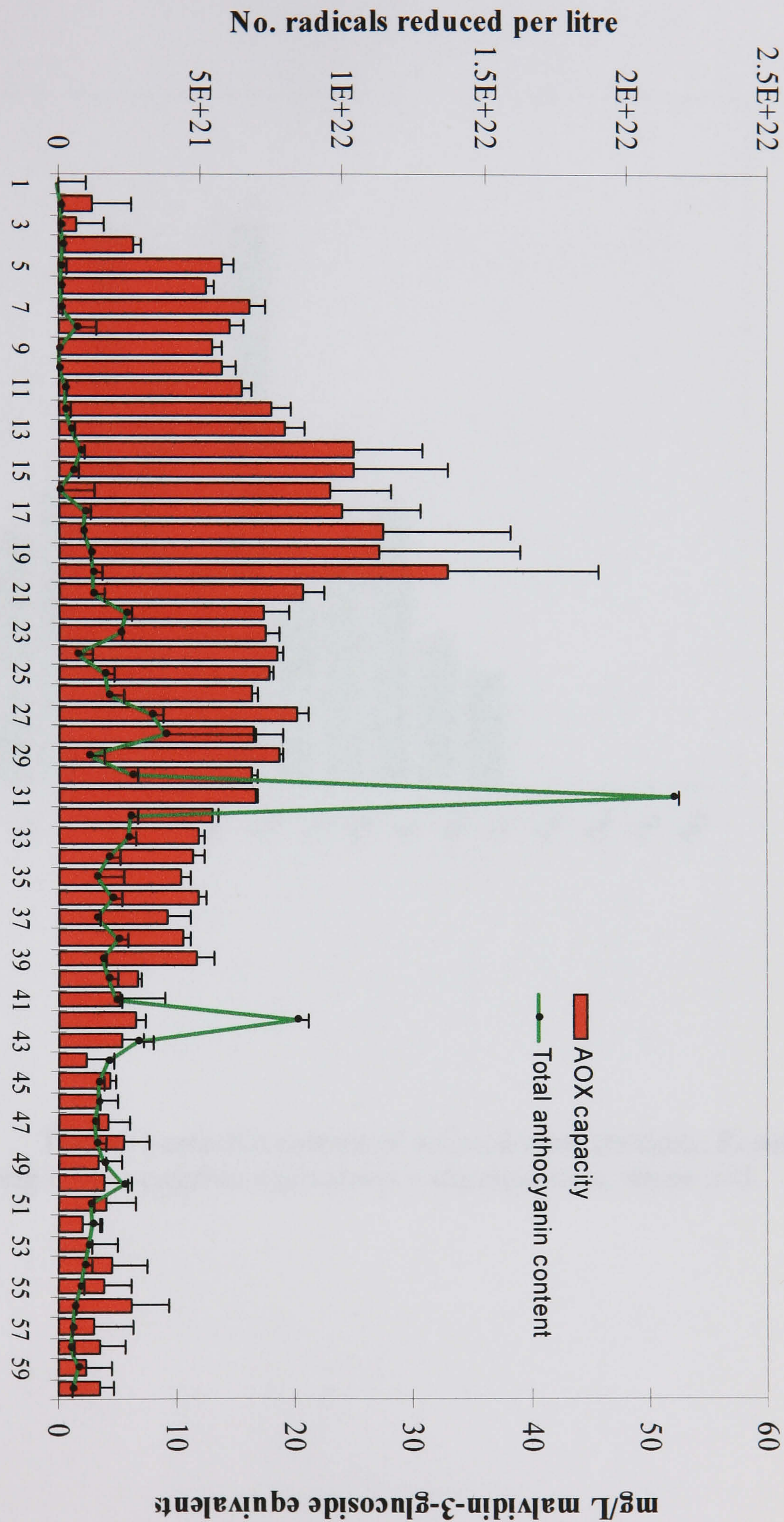


Figure 3.2 Total anthocyanin content and ESR-derived antioxidant activity and of wine 28 fractions 1-60. Results are expressed as mg L⁻¹ malvidin-3-glucoside equivalents and no. Fremys radicals reduced (x 10²¹) per litre of wine ± standard error, where n=3.

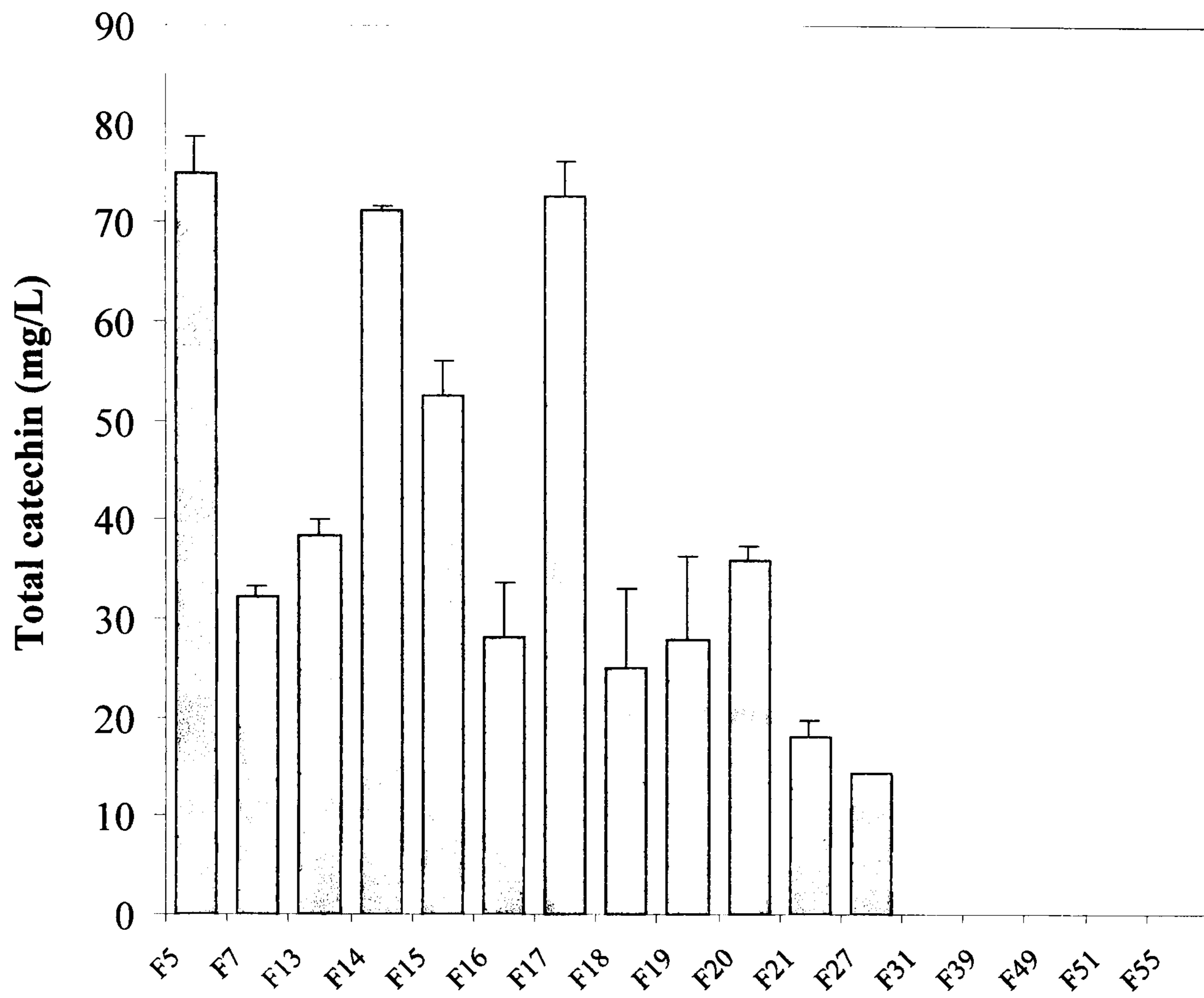


Figure 3.3 Total (+)-catechin content of selected wine fractions. Results are expressed as mg L^{-1} (+)-catechin equivalents \pm standard error, where $n=3$.

Table 3.4 On-line ABTS antioxidant analysis of selected red wines ^a.

Compound	tr (min)	wine				
		3	13	15	28	31
gallic acid	6.9	103 ± 0.2 (23)	151 ± 14 (36)	213 ± 24 (41)	237.4 ± 4 (31)	269.2 ± 1.3 (44)
procyanidin trimer C ₂	11.0	6.0 ± 0.8 (1)	24 ± 0.4 (6)	7.8 ± 0 (2)	12 ± 0 (2)	25.0 ± 0.1 (4)
procyanidin dimer B ₁	20.1	128.3 ± 1.7 (29)	37 ± 2.2 (9)	32.4 ± 1.1 (6)	75.2 ± 4.2 (10)	60.4 ± 3.2 (10)
(+)-catechin	22.5	67 ± 1.0 (15)	69.3 ± 7.0 (17)	80.2 ± 1.0 (16)	107.2 ± 1.1 (14)	130.4 ± 1.0 (21)
procyanidin trimer	25.0	66.4 ± 0.2 (15)	51 ± 19 (12)	74.5 ± 1.0 (14)	112.4 ± 0 (15)	4.0 ± 0.1 (1)
procyanidin dimer B ₂	28.6	34 ± 2.0 (8)	30 ± 1.2 (7)	8 ± 0.1 (2)	52 ± 1.6 (7)	26 ± 0.2 (4)
(+)-epicatechin	31.2	17.5 ± 0.3 (4)	30 ± 1.4 (7)	46.3 ± 0.5 (9)	66.3 ± 4.0 (9)	74.4 ± 1.2 (12)
procyanidin trimer	34.4	1.0 ± 0 (0.2)	5.4 ± 0 (0.3)	4.9 ± 0 (1)	48.1 ± 1.7 (6)	5.8 ± 0 (1)
procyanidin tetramer	40.0	24.0 ± 0.2 (5)	12.4 ± 0 (3)	48.2 ± 1.4 (9)	49.1 ± 1.0 (7)	14.4 ± 0.2 (2)

^a tr values refers to peaks in Figures 3.4-3.8. Antioxidant activity expressed as mg L⁻¹ trolox equivalents ± standard error, where n=3, and in parenthesis as a % of the total antioxidant activity.

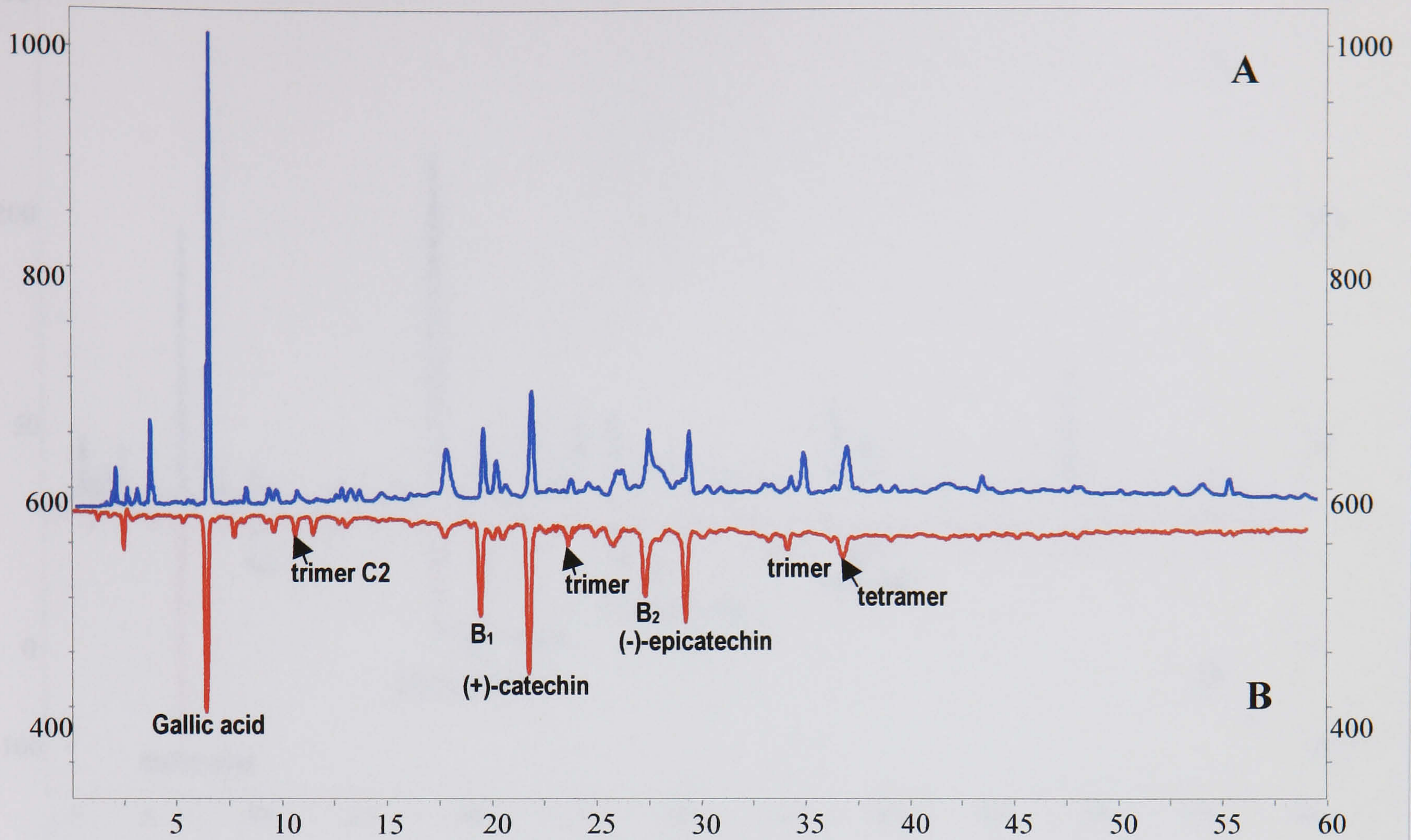


Figure 3.4 On-line ABTS+ analysis of a Bulgarian Cabernet Sauvignon (wine 31), showing UV absorbance at 280 nm (A) and 720 nm (B).

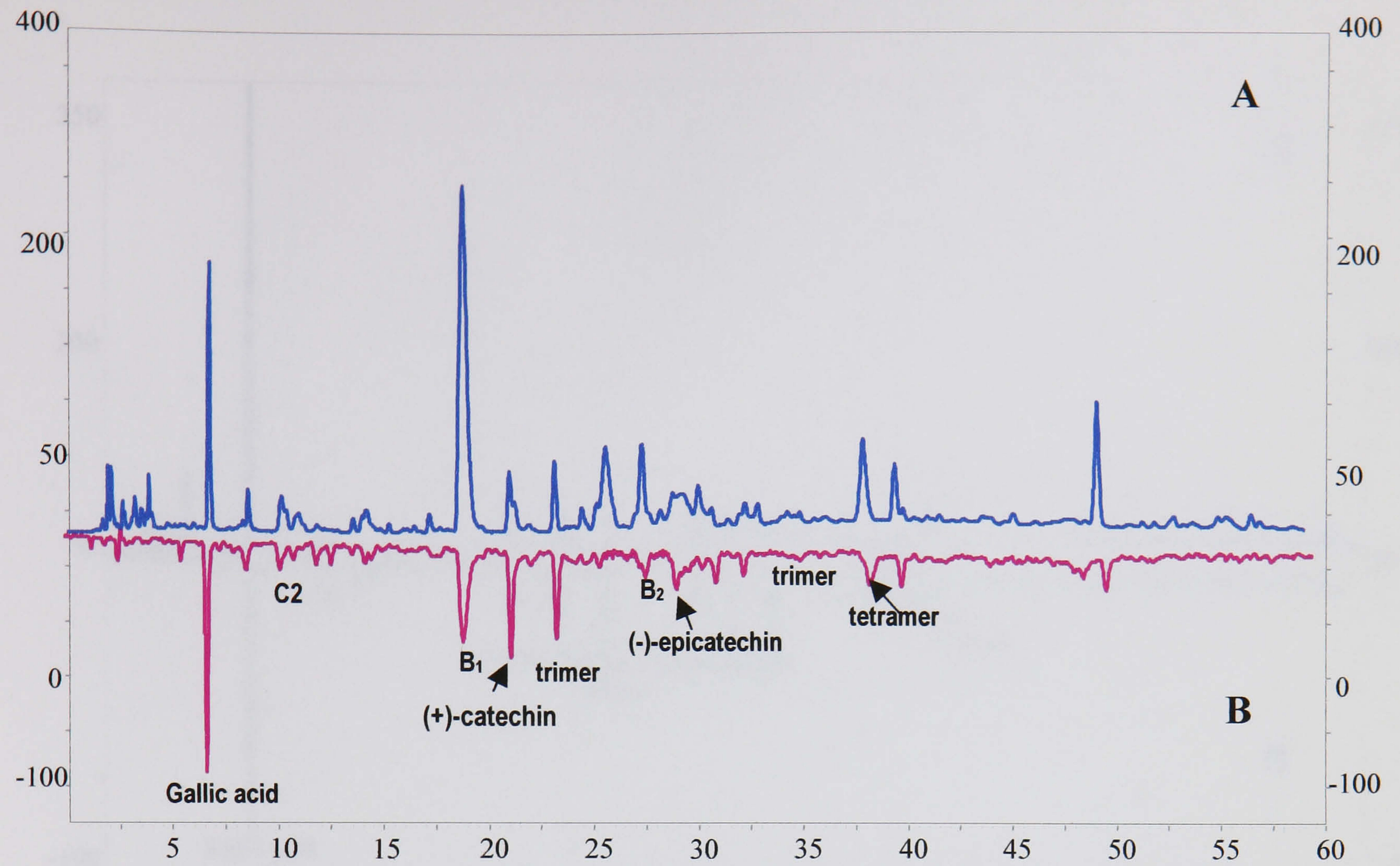


Figure 3.5 On-line ABTS+ analysis of a French Beaujolais (wine 3), showing UV absorbance at 280 nm (A) and 720 nm (B).

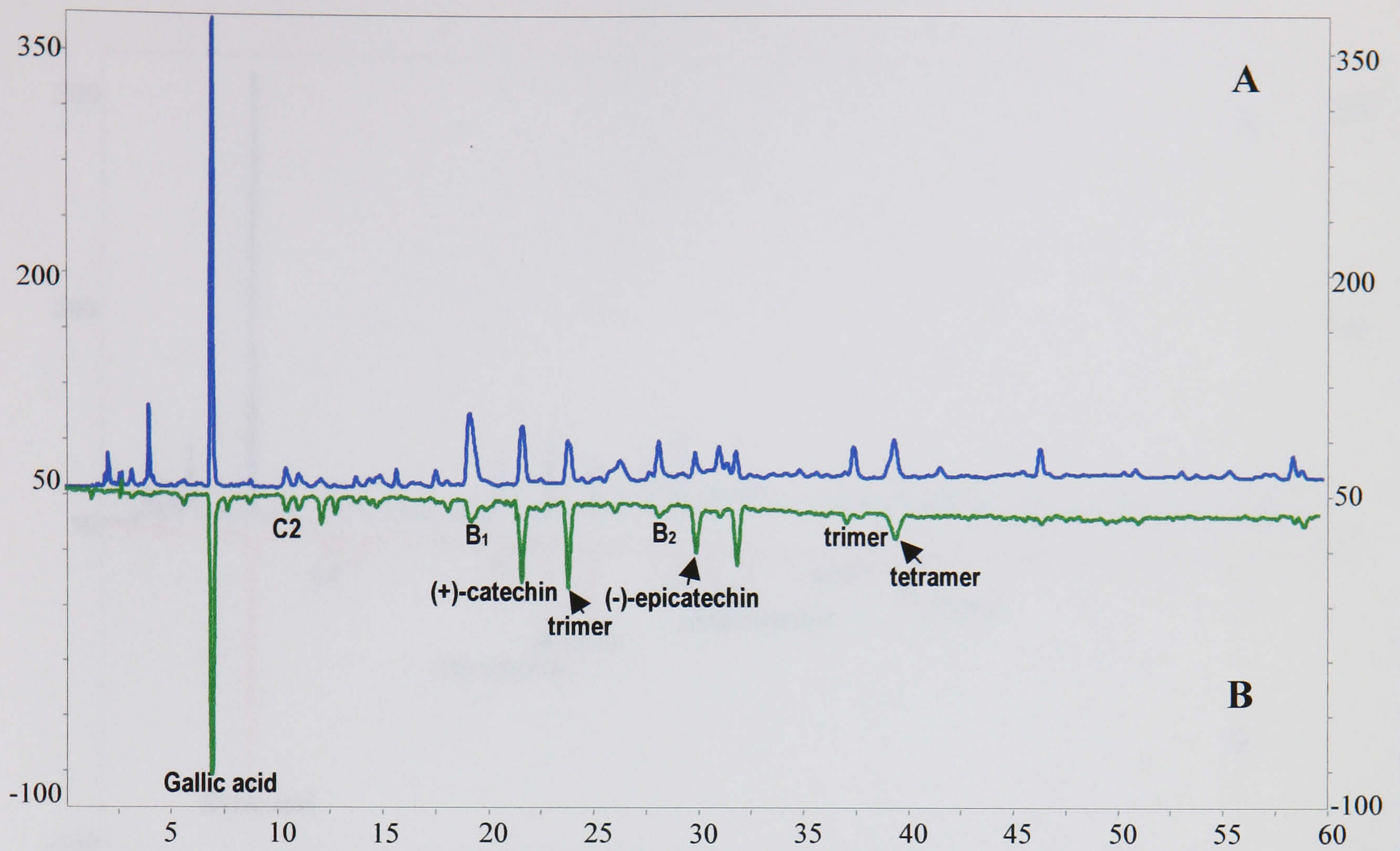


Figure 3.6 On-line ABTS+ analysis of a Bulgarian Merlot (wine 15), showing UV absorbance at 280 nm (A) and 720 nm (B).

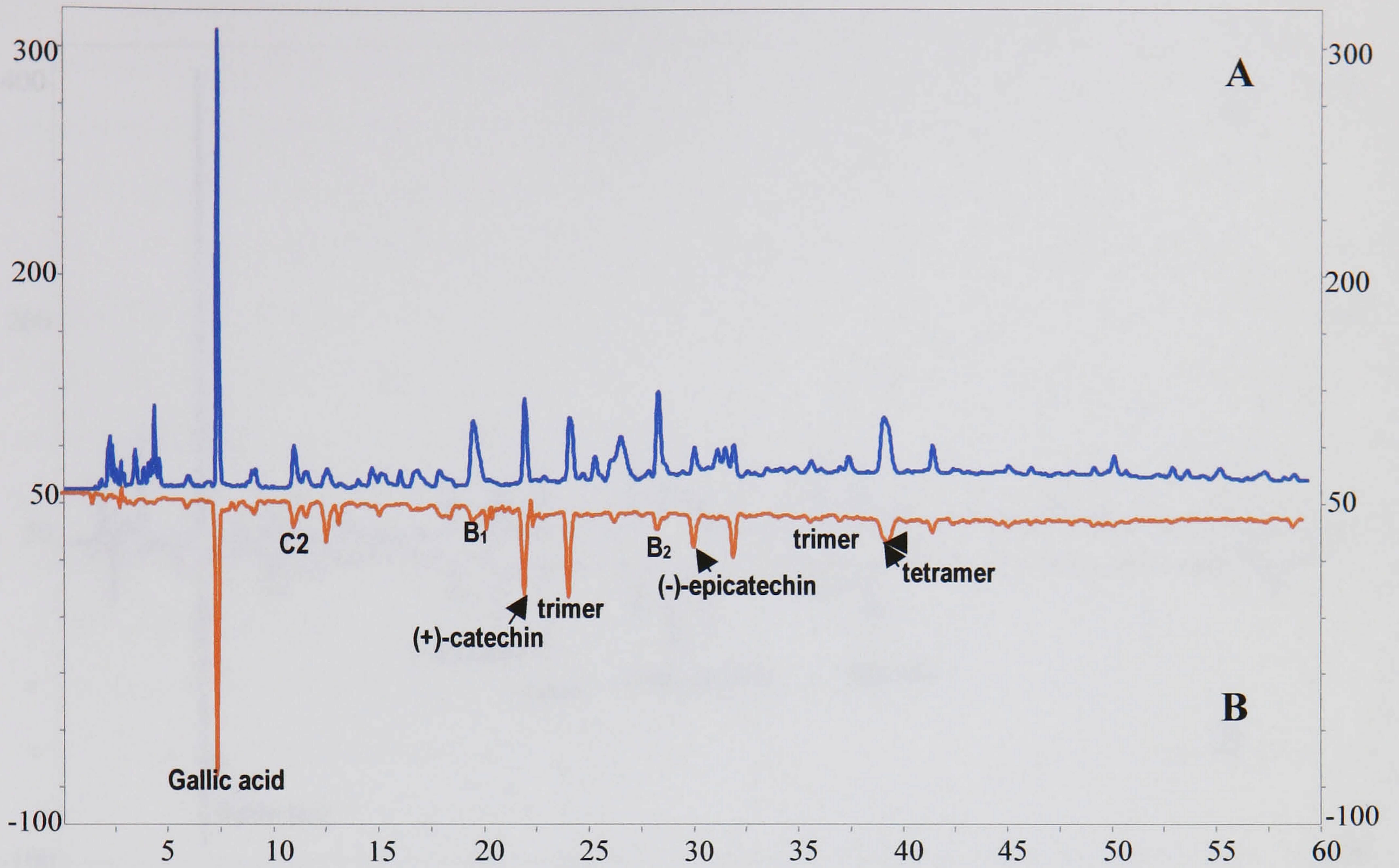


Figure 3.7 On-line ABTS+ analysis of a Spanish tempranillo (wine 13), showing UV absorbance at 280 nm (A) and 720 nm (B).

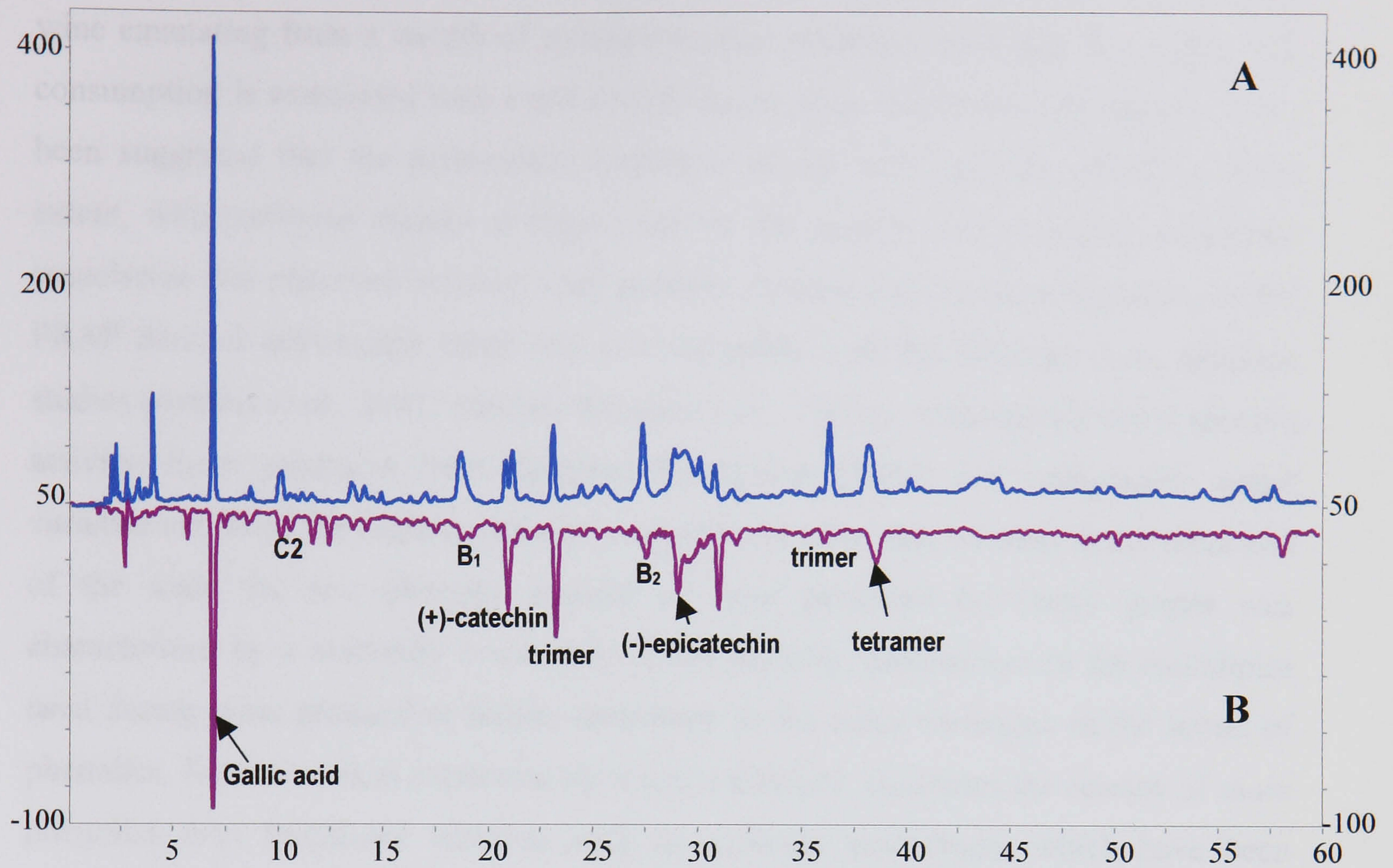


Figure 3.8 On-line ABTS+ analysis of a Chilean Cabernet Sauvignon (wine 28), showing UV absorbance at 280 nm (A) and 720 nm (B).

3.6 Discussion

In recent years there has been a great interest in the antioxidant properties of red wine emanating from a wealth of epidemiological evidence indicating that a moderate consumption is associated with a reduced mortality from cardiovascular disease. It has been suggested that the antioxidant properties of red wine may be linked, to some extent, with particular classes of flavonoids. In the present study a highly significant association was observed between total phenolic content and the reducing ability in the FRAP derived antioxidant assay and is comparable with the findings from previous studies (Arnous *et al.*, 2001; Sánchez-Moreno *et al.*, 1999a). Although all wines showed activity, those produced from Cabernet Sauvignon, Merlot and Tempranillo grape varieties exhibited the highest phenolic contents and activities, whereas at the other end of the scale the low phenolic content of wine produced by Gamy grapes was characterized by a markedly lower antioxidant capacity. Differences in the techniques used during wine production further contribute to the large variations in the levels of phenolics. Extensive skin extraction by rotary extraction facilitates the release of more phenolics than traditional methods such as carbonic maceration, which have been described for the Young Vatted Bulgarian Cabernet Sauvignon and Beaujolais, respectively (Burns *et al.*, 2000).

Gallic acid, the flavan-3-ols and the anthocyanins were the most abundant phenolics identified in red wines. Although the flavonols have been extensively investigated recent studies have shown that they are not the major antioxidants present in red wine (Gardner *et al.*, 1999). In the present study free and conjugated flavonols were detected in the wines but their concentration was comparatively much lower than any other class of phenolic identified. Comparable findings have been documented with levels of quercetin ranging from 0.5-5.3 mg L⁻¹ (Soleas *et al.*, 1997), while values of 0.8 and 1.1 mg L⁻¹ were obtained for myricetin and kaempferol in red wine, respectively (Rodriguez-Delgado *et al.*, 2002). The flavonols had little impact on the antioxidant capacity.

Total anthocyanin content of each wine was moderately associated with FRAP-derived antioxidant activity ($P < 0.05$), however no correlation was observed between the anthocyanin content of wine fractions with ESR-derived antioxidant activity. Anthocyanins have been shown to exhibit antioxidant properties in a number of *in vitro* systems (Rice-Evans *et al.*, 1995; Wang *et al.*, 1997), however other studies have indicated that they are not the major antioxidants in red wine (Frankel *et al.*, 1995; Burns *et al.*, 2000). The determination of anthocyanins by spectrophotometry has been known to provide an inaccurate estimation of the total monomeric anthocyanins (Somers and Evans, 1977). This was further demonstrated by Bakker *et al.* (1986), who reported the large differences observed in total anthocyanin content with spectrophotometry and HPLC analysis. The weak association between antioxidant activity and total anthocyanin content may also be due to the fact that total anthocyanin values also represent polymeric and other types of pigments in wine which may not possess any antioxidant activity. Arnous *et al.* (2002) reported that the correlation between coloured anthocyanins, or polymeric pigments, with antioxidant activity was higher compared with total anthocyanin content, suggesting that the ionization state of anthocyanins may be important with respect to their antiradical activity. In the present study a similar pattern was observed in that there was a higher correlation between polymeric anthocyanin content and FRAP-derived antioxidant activity ($r^2=0.594$; $P < 0.001$) than with total anthocyanin content ($r^2=0.434$; $P < 0.01$). This is supported by the fact that pseudo-base and quinoidal-base of malvidin-3-glucoside, generated at pH 4.0 and 7.0, respectively show different antioxidant behaviours (Lapidot *et al.*, 1999). In the present study the anthocyanins in red wine exhibited modest antioxidant activity, however previous studies have shown that they may act via different mechanisms. There is evidence that anthocyanins derived from red wine induce vasorelaxation mediated via the release of nitric oxide (Andriambelason *et al.*, 1998) and protect the integrity of capillaries against free radical damage in animals (Cao *et al.*, 1999). Additional work demonstrated that delphinidin, but not malvidin or cyanidin, was able to elicit endothelium dependent vasorelaxation in vascular tissue (Fitzpatrick *et al.*, 1993).

McGinn *et al.* (2000) showed an inverse relationship between vasodilatory and antioxidant activity of grape extracts, indicating that the higher the antioxidant activity of the extracts, the less active they were as vasodilators. The authors further reported that the compounds responsible for inducing antioxidant activity were distinct from those exhibiting vasodilator activities.

Further studies have reported a strong correlation between the total flavan-3-ol content of red wine with the antioxidant activity (Simonetti *et al.*, 1997; Burns *et al.*, 2000). In this study the total catechin content of selected wine fractions separated by preparative HPLC was highly and significantly associated with ESR-derived antioxidant activity ($P < 0.01$). Monomeric and oligomeric flavanols; (+)-catechin, (-)-epicatechin, procyanidin dimers B1 and B2, were major antioxidant components of red wine, while trimers and tetramers exhibited a moderate antioxidant activity. Low molecular weight flavan-3-ols, especially the monomers and dimers have been found to exert greater antioxidant activities than high molecular weight compounds. (+)-Catechin, (-)-epicatechin, procyanidin dimers B₁, B₂, B₃, B₄ and procyanidin trimers C₁ and C₂ inhibit LDL oxidation *in vitro* (Teissedre *et al.*, 1996) more efficiently than high molecular weight polymers such as hexamers (Lotito *et al.*, 2000). Collectively the flavan-3-ols contributed > 50 % of the total antioxidant capacity of each wine, with the greatest activity observed in the Young Vatted Bulgarian Cabernet Sauvignon. Gallic acid was also a major antioxidant component of red wine contributing between 23-44 % of the total antioxidant capacity in a French Beaujolais and a Bulgarian Cabernet Sauvignon, respectively. Shahidi & Naczki, (1995) similarly reported the presence of gallic acid as a major phenolic component of red wine, followed by (+)-catechin and (-)-epicatechin. A high concentration of gallic acid would be expected in red wine since this phenolic acid is principally formed by the hydrolysis of flavonoid gallate esters, which are largely absent in white wines, due to the lack of skin extraction (Frankel *et al.*, 1995). Previous studies have shown a high association between total antioxidant activity and gallic acid content of red wine (Minussi *et al.*, 2003; Burns *et al.*, 2000; Soleas *et al.*, 1997), while Sanchez-Moreno *et al.* (1999b) observed that gallic acid exhibited the greatest free-radical scavenging activity in wine and grape juice. Similarly, Bohm, (2000) demonstrated that gallic acid exerted the strongest antioxidant activity in TEAC, TRAP and LDL oxidation assays.

There is some evidence to suggest that flavan-3-ols and gallic acid are bio-available in the human body. Monomeric and dimeric flavan-3-ols are absorbed into the bloodstream following the ingestion of red wine (Bell *et al.*, 2000) and cocoa (Holt *et al.*, 2002), and gallic acid and its metabolite 4-*O*-methylgallic acid have been detected in human plasma and urine following oral administration of 50 mg of gallic acid and black tea (Shahrzad & Bitsch, 1998; Shahrzad *et al.*, 2001).

3.7 Conclusion

The antioxidant properties of red wines appear to be governed by the total phenolic content, specifically gallic acid and monomeric and dimeric flavan-3-ols; (+)-catechin, (-)-epicatechin and procyanidin dimers B₁ and B₂, while the anthocyanins and flavonols had little impact on the antioxidant capacity of red wine. The findings from this study highlight the importance of these compounds as potent antioxidants *in vitro*, however further information regarding their bioavailability and protective effects *in vivo* is warranted.

CHAPTER 4 THE INFLUENCE OF MODERATE RED WINE CONSUMPTION ON ANTIOXIDANT STATUS AND INDICES OF OXIDATIVE STRESS ASSOCIATED WITH CORONARY HEART DISEASE IN HEALTHY VOLUNTEERS108

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Chapter 4 The influence of moderate red wine consumption on antioxidant status and indices of oxidative stress associated with coronary heart disease in healthy volunteers

4.1 Introduction

There is much public, media and scientific interest in the possibility that red wine may be protective against CHD. Mounting evidence suggests that moderate red wine consumption is inversely associated with CHD, an association popularised as the ‘French Paradox’ (Renaud & de Logeril, 1993). The paradox describes the seemingly low mortality rates in certain populations of France despite high levels of associated risk factors; serum cholesterol, systolic blood pressure, dietary fat intakes and low consumption of fruits and vegetables. It is speculated that this paradox is associated with the widespread consumption of red wine (Criqui & Ringel, 1994). Although there is evidence to suggest that light to moderate intake of alcohol is beneficial to health (Albert *et al.* 1999), it has been shown that red wine provides additional health benefits beyond those of alcohol alone (Rimm *et al.*, 1996, Burns *et al.*, 2001a).

The putative cardio-protective effects of red wine have been largely attributed to the abundance of phenolic compounds. Plant-derived phenolics are widely distributed in nature and are present in varying amounts in commonly consumed fruits, vegetables and beverages. Red wine provides an especially rich source of these compounds which originate from grape seeds, skins and vine stems (Singleton, 1982). The levels of phenolic acids, major catechins and anthocyanins in red wines vary in concentration depending on different grape varieties and vintages (Landrault *et al.*, 2001), together with variations in climate and vinification practices (Goldberg *et al.*, 1998, Burns *et al.*, 2001b). Generally red wines contain between 1000 and 4000 mg/L of phenolics, the majority of which are derived from anthocyanins and proanthocyanidins (Burns *et al.*, 2000). Although the exact mechanisms of protection conferred by red wine are unknown, there is evidence that phenolic compounds act as antioxidants by protecting the body from damaging oxidation reactions caused by ‘free radicals’ (Kanner *et al.*,

1994). There is further evidence that they may inhibit oxidative modification of LDL based on their ability to increase the resistance of isolated LDL to copper oxidation *in vitro* (Frankel *et al.*, 1995). Several studies have demonstrated that many dietary phenolics protect against the oxidative modification of LDL *in vitro* (Vinson & Hontz, 1995; Teissedre *et al.*, 1996). Despite increasing evidence for the *in vitro* effectiveness of phenolics, there remains a dearth of information regarding their efficacy *in vivo* and this may be due, in part, to a lack of knowledge of their bioavailability in humans. The results from wine trials to date have shown conflicting results with some showing a protective effect following red wine intake (Serafini *et al.*, 1998; Natella *et al.*, 2001), while others have not demonstrated such an effect (van Golde *et al.*, 1999).

This chapter reports on the results of an intervention study in which the influence of red wine consumption on the antioxidant status and on indices of oxidative stress associated with CHD was investigated in healthy human volunteers. The total concentration of phenolics and individual phenolics in the wine and plasma were analysed in the Folin-Ciocalteu assay and by HPLC-tandem mass spectrometry. The antioxidant capacity of plasma was measured with ESR while homocysteine, fasting plasma lipids and the production of conjugated dienes and TBARS in copper oxidised LDL were further determined.

4.2 Study design

A randomised, controlled study was performed with 20 free-living healthy male and female volunteers. Subjects were non-smokers aged between 23-50 and taking no medications or vitamin supplements. There were no statistically significant differences observed in mean age (years); height (m); weight (kg); or body mass index (kg/m^2), in the red wine and control group, respectively (Table 4.1). The study protocol was approved by the Research Ethics Committee, Glasgow Royal Infirmary, United Kingdom. Written informed consent was obtained from each volunteer. Subjects were randomised into two groups; red wine and control. Subjects in the red wine group were asked to consume 375 ml red wine (Safeway, 1999 Bulgarian Young Vatted Cabernet Sauvignon, 12 % alcohol) each day for 2 weeks. Fasting blood samples obtained at weekly intervals were collected into EDTA and lithium heparin tubes. Plasma was immediately separated by low speed centrifugation ($2500 \times g$ for 10 min at 4°C) and stored at -80°C prior to analysis. For vitamin C analysis 0.6 mL plasma was mixed with an equal volume of 10 % (w/v) meta-phosphoric acid and snap frozen in liquid nitrogen and stored at -80°C prior to analysis.

4.2.1 Dietary Assessment

Volunteers were required to avoid all alcohol and foods/beverages rich in polyphenols, and to limit fruit and vegetable intake over the study period. A record of food and beverage intake was monitored over the study period and this information was used to assess compliance to the dietary instructions given. Subjects were asked to keep a record of their food and beverage intake for 3 days (one of which was at the weekend) and oral and written instructions were given on keeping the diet records and they were supplied with diaries and portion size photographs to aid with quantification (Appendix I). Data collected was analysed using a dietary analysis software package, Diet 5™ for windows (Robert Gordon University, Aberdeen, UK).

Table 4.1 Anthropometric characteristics of red wine (*n* 12) and control (*n* 8) subjects.

	Red wine group (n 12)		Control group (n 8)	
	Mean ± SEM	Range	Mean ± SEM	Range
Age (years)	35.0 ± 2.5	24-50	29 ± 2.5	23-45
Height (m)	1.67 ± 0.1	1.54-1.85	1.65 ± 0.1	1.55-1.75
Weight (kg)	68.3 ± 1.9	55-77	67.0 ± 3.2	55-77.5
Body mass index (kg/m ²)	22.5 ± 0.3	20-24.1	23.2 ± 1.2	18.8-28.3

Results are expressed as mean values ± standard error (SE) and range.

4.3 Phenolic composition of wine

The red wine used in this study was a Bulgarian Young Vatted Cabernet Sauvignon and analysis by HPLC-MS-MS and diode array detection revealed the presence of a number of flavonoids and phenolic compounds of which 19 were identified. The results obtained are illustrated in Figure 4.1 and are summarised below and in Table 4.2.

Peak 1 ($t_R = 5.3$ min, $\lambda_{\max} = 265$ nm) had a $[M-H]^-$ at m/z 169 with MS^2 yielding a charged fragment ion at m/z 125. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is gallic acid.

Peak 2 ($t_R = 6.9$ min, $\lambda_{\max} = 265$ nm) had a $[M-H]^-$ at m/z 865 with MS^2 yielding two charged fragment ions at m/z 577 and 289. Based on the mass spectral data this compound is a procyanidin trimer and its elution prior to dimer B₁ (Santos-Buelga *et al.*, 1995) indicates that it is likely to be the trimer procyanidin C₂, a known component of red wine (De Pascual-Teresa *et al.*, 2000b).

Peak 3 ($t_R = 13.4$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 577, with MS^2 producing three major charged fragment ions at m/z 425, 407 and 289. Based on the mass spectral data this compound is a procyanidin dimer. Co-chromatography with a standard indicated the dimer was procyanidin B₁, a known component of red wine (De Pascual-Teresa *et al.*, 2000b).

Peak 4 ($t_R = 14.3$ min, $\lambda_{\max} = 515$ nm) had a $[M-H]^+$ at m/z 465 which fragmented with MS^2 to produce a delphinidin-like ion at m/z 303 with the $[M-H]^+ - 162$, corresponding to loss of a glucose moiety. This peak is, therefore, the anthocyanin delphinidin-3-glucoside, previously identified in red wine by Burns *et al.* (2002).

Peak 5 ($t_R = 15.4$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 289, yielding two charged ions at m/z 245 and 205. This mass spectrum and co-chromatography with an authentic standard established that peak 4 is the flavan-3-ol (+)-catechin.

Peak 6 ($t_R = 17.8$ min, $\lambda_{\max} = 515$ nm) had a $[M-H]^+$ at m/z 479 which on MS^2 yielded a fragment ion at m/z 317 which corresponds with petunidin ($[M-H]^+ - 162$, cleavage of a glucosyl unit) indicating the presence of petunidin-3-glucoside which has previously been detected in red wines (Burns *et al.*, 2002).

Peak 7 ($t_R = 18.3$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 577 with MS^2 yielding three charged fragment ions at m/z 425, 405 and 289. Based on the mass spectral data this peak is a procyanidin dimer and co-chromatography with a reference compound revealed that it is procyanidin B₂, previously detected in red wine by De Pascual-Teresa *et al.* (2000b).

Peak 8 ($t_R = 19.8$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 289, which on MS^2 yielding ions at m/z 245, 205 and 179. Co-chromatography with an authentic standard confirmed the identification of peak 7 as (-)-epicatechin.

Peak 9 ($t_R = 20$ min, $\lambda_{\max} = 510$ nm) had a $[M-H]^+$ at m/z 463 which on MS^2 exhibited a 162 amu loss, corresponding to cleavage of a glucosyl unit, producing a fragment ion at m/z 301 which corresponds to peonidin. This is in keeping with the presence of peonidin-3-*O*-glucoside, a known component of red wine (Burns *et al.*, 2002).

Peak 10 ($t_R = 21$ min, $\lambda_{\max} = 515$ nm) had a $[M-H]^+$ at m/z 493, MS^2 produced a fragment ion at m/z 331 ($[M-H]^+ - 162$, loss of a glucosyl unit). This peak was confirmed as malvidin-3-*O*-glucoside by reference to an authentic standard.

Peak 11 ($t_R = 21.7$ min, $\lambda_{\max} = 280$ nm) had a $[M-H]^-$ at m/z 865 with MS^2 yielding two charged fragment ions at m/z 577 and 289. Based on the mass spectral data this compound is a procyanidin trimer and its elution after (-)-epicatechin (Santos-Buelga *et al.*, 1995) indicates that it is procyanidin C₁, a known component of red wine (Sánchez-Moreno *et al.*, 2003).

Peak 12 ($t_R = 25$ min, $\lambda_{\max} = 510$ nm) had a $[M-H]^+$ at m/z 517 with MS^2 yielding a charged fragment ion at m/z 355. Although a reference compound is not available, the elution order of this peak after malvidin-3-glucoside and the mass spectral data indicate that it may be vitisin B which has been detected in red wines by Morata *et al.* (2003) and is the adduct resulting from the reaction between malvidin-3-glucoside and acetaldehyde.

Peak 13 ($t_R = 25$ min, $\lambda_{\max} = 370$ nm) had a $[M-H]^-$ at m/z 479 with MS^2 yielding a fragment ion at m/z 317, (162 amu loss, cleavage of a glucosyl unit) which corresponds with myricetin indicating that this compound is a myricetin glucoside.

Peak 14 ($t_R = 29.1$ min, $\lambda_{\max} = 515$ nm) had a $[M-H]^+$ at m/z 533 which on MS^2 yielded a charged fragment ion at m/z 331 ($[M-H]^+ - 204$, loss of an acetylglucosyl unit). On the basis of the MS^2 spectra and the elution order of anthocyanins, this peak is

identified as malvidin-3-acetylglucoside previously detected in red wines (Burns *et al.*, 2002).

Peak 15 ($t_R = 29.6$ min, $\lambda_{\max} = 350$ nm) had a $[M-H]^-$ at m/z 471, MS^2 yielded a fragment ion at m/z 301 ($[M-H]^- - 176$, cleavage of a glucuronyl group). On the basis of the MS^2 spectral data and co-chromatography with an authentic standard this peak is identified as quercetin-3-glucuronide.

Peak 16 ($t_R = 34.4$ min, $\lambda_{\max} = 350$ nm) had a $[M-H]^-$ at m/z 317 which corresponds with the flavonol aglycone myricetin. Co-chromatography with an authentic standard confirmed the presence of myricetin.

Peak 17 ($t_R = 35.2$ min, $\lambda_{\max} = 525$ nm) had a $[M-H]^+$ at m/z 639 fragmentation by MS^2 yielded a charged fragment ion at m/z 331 ($[M-H]^+ - 308$, loss of a *p*-coumaroylglucoside group). Based on the absorbance and mass spectral data and the elution profile of this compound it is likely to be the anthocyanin malvidin-3-(*p*-coumaroyl)-glucoside (Burns *et al.*, 2002).

Peak 18 ($t_R = 43.5$ min, $\lambda_{\max} = 370$ nm) had a $[M-H]^-$ at m/z 301 and the identity of this peak was confirmed as the flavonol aglycone quercetin by co-chromatography.

Peak 19 ($t_R = 51$ min, $\lambda_{\max} = 370$ nm) had a $[M-H]^-$ at m/z 285 which indicates the presence of kaempferol and this identification was confirmed by co-chromatography with an authentic standard.

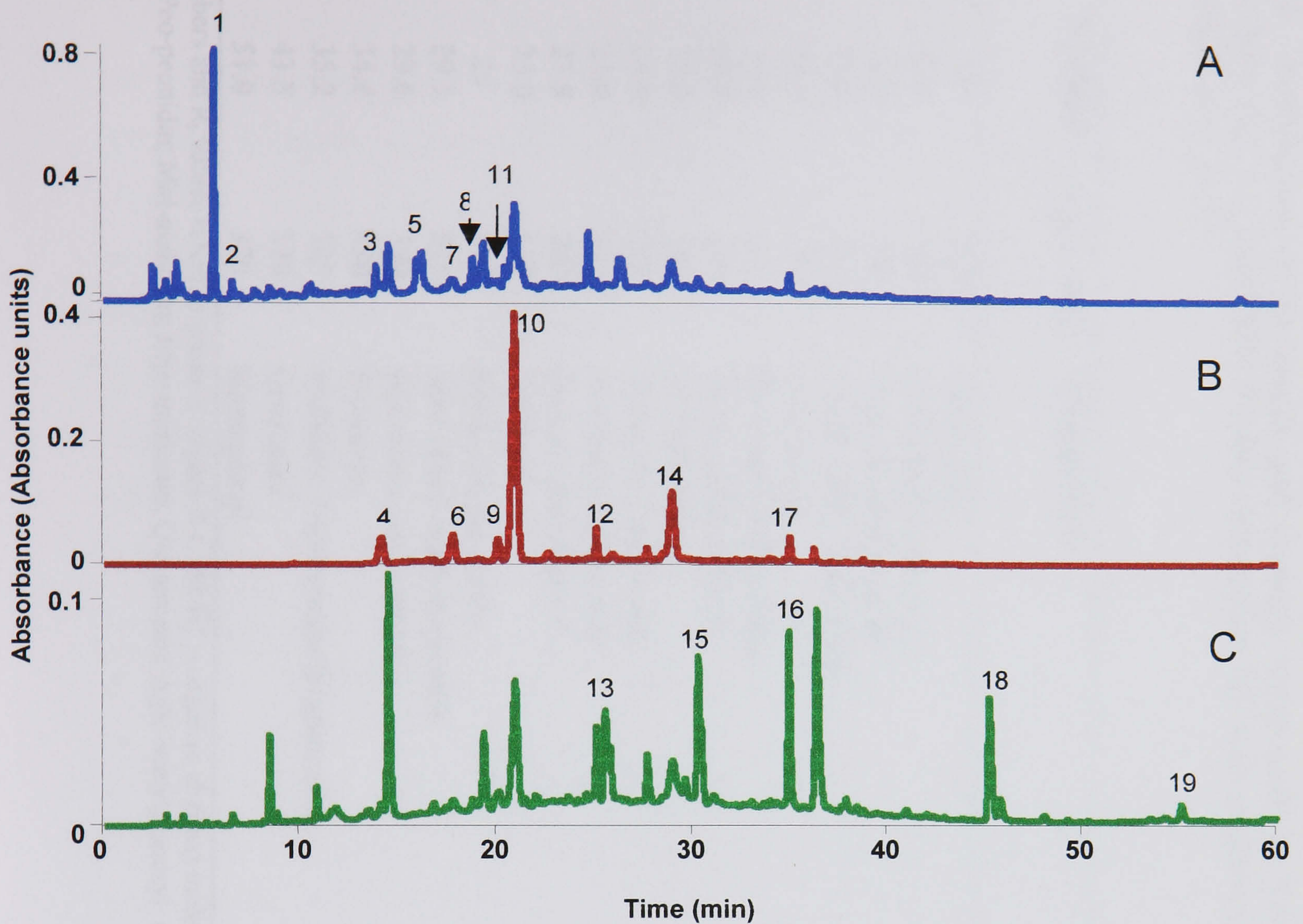


Figure 4.1 Gradient HPLC chromatogram of phenolics identified in the Bulgarian Young Vatted Cabernet Sauvignon with diode array detector operating at [A] 280 nm; [B] 520 nm and [C] 365 nm for the detection of flavan-3-ols; anthocyanins and flavonols, respectively. (For MS-MS data and identification and quantification of peaks 1-19, see Table 4.2).

Table 4.2 Identification of flavonoids and phenolic compounds in Young Vatted Bulgarian Cabernet Sauvignon red wine based on HPLC retention times (R_t), λ_{\max} and MS-MS data obtained in full scan negative and positive ion mode^a. Amount ingested daily expressed as mg per 375 ml \pm standard error ($n=3$).

Peak	R_t (min)	λ_{\max} (nm)	Compound	[M-H] ⁻ (m/z)	MS ² ions (m/z)	Amount ingested daily
1	5.3	265	Gallic acid	169	125	59.6 \pm 2.0
2	6.9	280	Procyanidin trimer C ₂	865	577	3.2 \pm 0.2
3	13.4	280	Procyanidin dimer B ₁	577	425, 407	14.7 \pm 0.4
4	14.3	515	Delphinidin-3-glucoside	465*	303 [Del], ([M+H] ⁺ -Glc]	2.7 \pm 0.1
5	15.3	280	(+)-Catechin	289	163, 125	16.1 \pm 0.2
6	17.8	515	Petunidin-3-glucoside	479*	317 [Pet], ([M+H] ⁺ -Glc]	2.8 \pm 0.1
7	18.9	280	Procyanidin dimer B ₂	577	425, 407	16.8 \pm 0.1
8	19.8	280	(-)-Epicatechin	289	163; 125	15.9 \pm 1.0
9	20.0	510	Peonidin-3-glucoside	463*	301 [Peo], ([M+H] ⁺ -Glc]	1.1 \pm 0.1
10	21.0	515	Malvidin-3-glucoside	493*	331 [Mal], ([M+H] ⁺ -Glc]	16.0 \pm 1.6
11	21.8	280	Procyanidin trimer C ₁	865	577	5.4 \pm 1.7
12	25.0	510	Vitisin B	517*	355	2.4 \pm 0.1
13	25	370	Myricetin glucoside	479	317, [Myr], ([M-H] ⁻ -Glc]	0.4 \pm 0.0
14	29.1	515	Malvidin-3-acetylglucoside	535*	331 [Mal], ([M+H] ⁺ -AGlc]	2.4 \pm 0.0
15	29.6	350	Quercetin-3-glucuronide	477	301 [Q], ([M-H] ⁻ -GlcUA]	0.7 \pm 0.0
16	34.4	350	Myricetin	317*		1.4 \pm 0.0
17	35.2	525	Malvidin-3-(<i>p</i> -coumaroyl)glucoside	639*	331 [Mal], ([M+H] ⁺ -PGlc]	1.0 \pm 0.1
18	43.5	370	Quercetin	301		2.2 \pm 0.0
19	51.0	370	Kaempferol	285		0.1 \pm 0.0

^a Peak numbers and R_t values refers to peaks in Figure 4.1. [M-H]⁻ - negative charged molecular ion; *indicates positively charged molecular ion ([M+H]⁺); Del-delphinidin; Pet-petunidin; Peo-peonidin; Mal-malvidin; Myr-myricetin; Q-quercetin; Aglc-acetylglucosyl; Glc-glucosyl; PGlc-*p*-coumaroylglucosyl; GlcUA- glucuronyl.

4.4 Plasma content and activity

Total phenolics in plasma were estimated with Folin Ciocalteu reagent using a modification of the method of Swain and Hillis, (1959) which avoids interference from proteins in biological samples (Serafini *et al.*, 1998) as described previously in Chapter 2 (section 2.3.1). Two separate HPLC methods were used to determine the plasma levels of six carotenoids (lutein/zeaxanthin, β -cryptoxanthin, all trans-lycopene, α -carotene and all trans β -carotene), retinol, α - and γ -tocopherol and ascorbic acid (see sections 2.5.1 and 2.5.2). Antioxidant capacity of plasma was measured in the ESR-derived antioxidant assay and HPLC-MS-MS in the selective reaction monitoring mode (SRM) was used to identify plasma metabolites. Results obtained are shown in Tables 4.3 and 4.4.

4.4.1 Total phenolic concentration

Twelve healthy volunteers consumed 375 mL of the red wine on a daily basis for two weeks and the amounts of the individual flavonoids and phenolics ingested per day are presented in Table 4.2. The main individual component was gallic acid at 59.6 mg per day while 28.4 mg of anthocyanins, 8.4 mg of flavonols and 72 mg of monomeric flavan-3-ols and proanthocyanidins were consumed each day. Consumption of red wine was associated with a small but significant 4.4 μ M GAE increase (unpaired t-test) in plasma total phenolics which was associated with a 7 % increase in plasma antioxidant capacity which failed to achieve statistical significance (Table 4.3). Total phenolic levels were increased highly significantly ($P=0.000$) from 12.4 μ M GAE at baseline to 16.6 μ M GAE following 2-weeks of daily red wine consumption in the red wine group. No change in plasma total phenolic concentration was observed in the control group.

4.4.2 Plasma levels of carotenoids, tocopherols, retinol & ascorbic acid

The levels of six carotenoids (lutein/zeaxanthin, β -cryptoxanthin, all trans-lycopene, α -carotene and all trans β -carotene), retinol and α - and γ -tocopherol in plasma were measured by reversed phase HPLC with absorbance and fluorimetric detection according to the method of Hess *et al.* (1991). The analysis was conducted under the auspices of the US National Institute of Standards Quality Assurance Scheme for fat-soluble vitamins and full details of the method are described in Chapter 2 (section 2.5.1) and in Duthie (1999). Ascorbic acid content was determined from 0.6 mL plasma and measured by reversed phase HPLC using an ion-pairing reagent with UV detection (Ross, 1994). Analysis was conducted under the US National Institute of Standards Quality Assurance Scheme for Vitamin C analysis in biological fluids and the method is described in detail in Chapter 2 (section 2.5.2) and in Duthie (1999). The plasma levels of carotenoids, tocopherols, retinol and ascorbic acid obtained are in agreement with previous studies (Mezzano *et al.*, 2001; Higgins *et al.*, 2001), however there were no significant changes in the plasma concentrations of α - and γ -tocopherols, retinol and ascorbic acid following red wine consumption. Similarly no differences were observed between the groups in plasma concentrations of the individual carotenoids and data have therefore been presented as total carotenoids in Table 4.3.

4.4.3 ESR-derived antioxidant activity

Antioxidant capacity of plasma was measured in the ESR-derived antioxidant assay as described by Gardner *et al.* (1998). This method is based on the ability of antioxidants in plasma to donate a hydrogen atom or electron to the synthetic free radical potassium nitro-disulphonate (Fremy's salt). A 300 μ L aliquot of plasma was added to 2.7 mL PBS and mixed with an equal volume of 50 μ M of Fremy's radical in ethanol/water (12:88, v/v). After 20 min following completion of the reaction the ESR spectra of the low field resonance of the Fremy's radical was obtained. Signal intensity was obtained by double integration and the concentration was calculated by comparison with a control reaction using ethanol/water (12:88, v/v) without sample.

Values obtained ranged from 37.4% and 41 % radicals reduced/L at baseline and after 2-weeks of wine consumption, respectively. However, the results obtained did not reach statistical significance ($p=0.303$).

4.4.4 HPLC-MS detection of metabolites in plasma

One hundred μL of plasma was extracted according to a modified method of Day *et al.* (1999) as described previously in Chapter 2 (section 2.7.1). HPLC-MS-MS in the SRM mode detected trace amounts of flavan-3-ol glucuronides of (+)-catechin and (-)-epicatechin in plasma of subjects in the red wine group post intervention (Table 4.4).

Peaks M-1 ($t_R = 16.6$ min, $\lambda_{\text{max}} = 280$ nm) and *M-2* ($t_R = 23$ min, $\lambda_{\text{max}} = 280$ nm) both had a $[\text{M-H}]^-$ at m/z 465 which yielded MS^2 fragments (M-176, loss of a glucuronyl unit) corresponding to (+)-catechin/(-)-epicatechin at m/z 289 and 245. The $[\text{M-H}]^-$ -176 loss is in keeping with the cleavage of a glucuronyl unit. The MS fragmentation pattern, together with the HPLC elution order, indicates that the earlier eluting *M-1* peak is a (+)-catechin glucuronide while *M-2* is an (-)-epicatechin glucuronide.

Peak M-3 ($t_R = 21.6$ min, $\lambda_{\text{max}} = 280$ nm) and *M-4* ($t_R = 27.1$ min, $\lambda_{\text{max}} = 280$ nm) also had similar mass spectra with a $[\text{M-H}]^-$ at m/z 479 which on loss of 176 amu (loss of a glucuronyl unit) yielded a MS^2 ion at m/z 303. These fragments are 14 amu higher than the equivalent ions in the spectra of *M-1* and *M-2*, indicating the presence of methylated flavan-3-ol glucuronides. *M-3* is therefore probably a methyl-catechin glucuronide and the later eluting *M-4* a methyl-epicatechin glucuronide (Table 4.4). Without reference compounds it was not possible to determine the positions on the flavan-3-ol skeleton of the methyl and glucuronic acid substituents. The most likely candidates for *M-2* and *M-4* are (-)-epicatechin-3'-*O*-glucuronide and 4'-*O*-methyl(-)-epicatechin-3'-*O*-glucuronide which have been identified as the main metabolites in human plasma after ingestion of (-)-epicatechin. Other methylated and glucuronide conjugates of (-)-epicatechin were, however, also present, albeit in smaller amounts (Natsume *et al.*, 2003). None of the four flavan-3-ol metabolites were detected in the plasma of subjects from the control group.

Table 4.3 Antioxidant capacity ((% Fremy's radical reduced/L), total phenolic content (μMGAE), tocopherols, retinol, carotenoids (μM) and ascorbic acid (μM) of plasma collected from human volunteers consuming 375 mL red wine daily for a 2 week period ($n = 12$) and from a control group ($n = 8$).

Parameter	Red Wine Group		Control Group	
	Baseline	After 2-weeks	Baseline	After 2-weeks
Antioxidant capacity (%)	37.4 \pm 2.5	40.5 \pm 3.3	35.3 \pm 1.8	34.0 \pm 2.3
Total phenolics ($\mu\text{M GAE}$)	12.4 \pm 5.9	16.6 \pm 5.9 ^{**†}	11.8 \pm 0.0	13.0 \pm 5.9
α -tocopherol (μM)	25.3 \pm 1.9	23.2 \pm 0.9	23.2 \pm 1.9	23.2 \pm 1.9
γ -Tocopherol (μM)	1.0 \pm 0.2	1.2 \pm 0.2	1.4 \pm 0.2	1.9 \pm 0.4
Retinol (μM)	2.4 \pm 0.0	2.1 \pm 0.3	1.8 \pm 0.3	1.8 \pm 0.3
Total carotenoids ((μM)	1.8 \pm 0.2	1.4 \pm 0.2	1.7 \pm 0.0	1.4 \pm 0.0
Ascorbic acid (μM)	63.5 \pm 5.8	54.3 \pm 7.4	51.8 \pm 4.6	55.6 \pm 8.6

Statistical significance between intervention and control groups based on unpaired *t*-test: † $P \leq 0.05$. Statistical significance within each group based on paired *t*-test: ** $P \leq 0.001$, * $P \leq 0.05$. Results are expressed as mean values \pm standard error, where $n=3$.

Table 4.4 HPLC and mass spectral properties of metabolites detected in plasma of human volunteers after consuming 375 mL red wine daily for a 2 week period ^a.

Peak	R _t (min)	Metabolite	[M-H] ⁻ (m/z)	MS ² ions (m/z)
M-1	16.6	(+)-Catechin glucuronide	465	289; 245 (Cat; [M-H] ⁻ -GlcUA)
M-2	23.0	(-)-Epicatechin glucuronide	465	289; 245 (ECat; [M-H] ⁻ -GlcUA)
M-3	21.6	Methyl catechin glucuronide	479	303(M-Cat; [M-H] ⁻ -GlcUA)
M-4	27.1	Methyl epicatechin glucuronide	479	303(M-ECat; [M-H] ⁻ -GlcUA)

^a R_t-retention time; [M-H]⁻-negatively charged molecular ion; C-(+)-catechin; EC-(-)-epicatechin; MC-methyl-catechin; MEC-methyl-epicatechin; GlcUA-glucuronyl unit.

4.5 Measurement of indices of oxidative stress

Oxidative modification of LDL is a lipid peroxidation process and this study focused on measuring lipid hydroperoxides such as TBARS and conjugated dienes in copper oxidised LDL. Fasting plasma lipids including triacylglycerols (TAG), total cholesterol, LDL and HDL cholesterol were measured in fasting plasma and circulatory levels of homocysteine were also determined. Results obtained are shown in Table 4.5 and Table 4.6.

4.5.1 Production of lipid hydroperoxides

LDL samples were adjusted to a concentration of 50 μg LDL protein/mL and 1 μM EDTA in quartz cuvettes and oxidation was initiated by addition of 15 mM CuSO_4 according to the method of Kleinveld *et al.* (1992). Absorbance of the samples was measured at 234 nm, every 2 min at 30 °C. Maximal concentration of conjugated dienes (nmol/mg LDL protein), rate of production of conjugated dienes (nmol/mg LDL protein/min) and lag time (min) were estimated using exact co-ordinates provided by the kinetics software application.

LDL that was oxidized for measurement of conjugated dienes was subsequently used for the measurement of TBARS, based on the method of Buege and Aust (1978). Following 3 h oxidation, the reaction was stopped by addition of 200 μM EDTA and 40 μM butylated hydroxytoluene and the samples were stored at 4°C for a maximum of 7 d. TBARS were determined by adding 1.6 mL TCA-TBA-HCL reagent (15 % trichloroacetic acid: 0.375 % thiobarbituric acid: 0.25 mol HCL) to a 0.8 mL aliquot of the LDL solution (oxidized as above), mixing the sample, and heating it at 100°C for 15 min. After placing the tubes in a bath of cold water for 5 min, samples were centrifuged for 10 min at 2000rpm. Absorbance was measured at 535 nm and quantification of TBARS was performed by comparison with a standard curve of malonaldehyde equivalents generated by acid catalysed hydrolysis of 1,1,3,3,-tetraethoxypropane.

The maximum concentration of conjugated dienes produced in copper-oxidised LDL was reduced significantly (paired t-test) ($P=0.026$) from 854 nmol/mg LDL protein at baseline to 715 nmol/mg LDL protein following wine consumption in the red wine group (Table 4.5). Similarly, the TBARS concentration (nmoles/mg LDL protein) in copper-oxidised LDL was reduced significantly (paired t-test) ($P=0.050$) from 69 nmol/mg LDL protein to 50 nmol/mg LDL protein in the red wine group (Table 4.5). There were no significant changes observed in conjugated dienes or TBARS in the control group.

4.5.2 Fasting plasma lipids

Fasting plasma lipids including total cholesterol, HDL, LDL and triacylglycerol (TAG) concentrations were determined using standard procedures as described previously in Chapter 2 (section 2.8). LDL cholesterol concentrations were calculated using the Friedewald formula (Friedewald *et al.*, 1972). There was a significant increase (paired t-test) in the level of HDL cholesterol ($P=0.020$) after 2-weeks of wine consumption in the red wine group, while no changes were observed in the control group. Values ranging from 0.92 to 2.21 mmol L⁻¹ were obtained for subjects in the red wine group with an average value of 1.5 mmol L⁻¹. No significant change was observed in other fasting lipids in either group (Table 4.6).

4.5.3 Homocysteine concentrations

Homocysteine concentrations were determined in plasma as described in detail in Chapter 2 (section 2.5.4). All subjects were within the normal range for plasma homocysteine levels (Range: ≤ 15.9 $\mu\text{mol L}^{-1}$) (Manilow, 1994). Values ranging from 4.9 to 11.6 $\mu\text{mol L}^{-1}$ were obtained for subjects within the red wine group, with an average value of 7.8 $\mu\text{mol L}^{-1}$. In the control group values ranging from 7.3 to 13.1 $\mu\text{mol L}^{-1}$ were obtained with an average value of 9.8 $\mu\text{mol L}^{-1}$. There appeared to be a reduction in the level of homocysteine in the red wine group compared with the control group, however did not reach statistical significance ($p=0.082$) (Table 4.6).

Table 4.5 Maximum diene concentration (nmol/mg LDL protein) and maximal oxidation rate (nmol/mg LDL protein per min), lag phase (min) before rapid onset of conjugated diene production and thiobarbituric acid-reactive substances (TBARS) in copper oxidised LDL (nmol/mg LDL protein) in plasma of human volunteers consuming 375 mL red wine daily for 2 weeks (n 12) and from a control group (n 8).

Parameter	Red Wine Group		Control Group	
	Baseline	After 2-weeks	Baseline	After 2-weeks
Maximum diene concentration	913 ± 85	715 ± 53*	860 ± 76	868 ± 75
Maximal oxidation rate	9.4 ± 1.2	7.4 ± 0.6	9.3 ± 2.3	8.4 ± 0.1
Lag phase	43.8 ± 3.7	49.8 ± 2.9	44.3 ± 6.8	46.4 ± 7.5
TBARS	69.0 ± 8.3	50.6 ± 5.6*	62.5 ± 12.6	62.4 ± 9.1

Statistical significance within each group based on paired t -test: * $P \leq 0.05$. Results are expressed as mean values ± standard error, where $n=3$.

Table 4.6 Fasting plasma total, LDL and HDL cholesterol concentrations (mmol/L), fasting triacylglycerol (TAG) concentrations (mmol/L) and homocysteine levels ($\mu\text{mol/L}$) in plasma of human volunteers consuming 375 mL red wine daily for 2 weeks (n 12) and from a control group (n 8).

Parameter	Red Wine Group		Control Group	
	Baseline	After 2-weeks	Baseline	After 2-weeks
Total cholesterol	4.5 \pm 0.3	4.9 \pm 0.3	4.2 \pm 0.4	4.1 \pm 0.3
LDL-cholesterol	2.7 \pm 0.2	2.9 \pm 0.2	2.4 \pm 0.3	2.4 \pm 0.3
HDL-cholesterol	1.4 \pm 0.1	1.5 \pm 0.1*	1.4 \pm 0.1	1.4 \pm 0.1
TAG	0.8 \pm 0.1	0.9 \pm 0.1	0.8 \pm 0.2	0.7 \pm 0.1
Homocysteine	8.1 \pm 0.8	7.8 \pm 0.7	9.8 \pm 0.7	10.0 \pm 1.0

Statistical significance within each group based on paired t -test: * $P \leq 0.05$. Results are expressed as mean values \pm standard error, where $n=3$.

4.6 Discussion

There is a growing awareness of the potential health benefit of diets rich in fruits and vegetables and nutritional guidelines indicate that an increase in the consumption of foods rich in antioxidant nutrients may decrease the risk of CHD and certain cancers (The Scottish Office, 1993). Epidemiological studies have reported a reduction in the incidence of CHD with moderate daily red wine consumption, an anomaly referred to as the 'French Paradox'. This effect has been ascribed to the low molecular weight phenolics in many plant based foods which can act as antioxidants because their extensive conjugated π -electron systems allow ready donation of electrons or hydrogen atoms from the hydroxyl moieties to free radicals (Scott, 1997).

In the present study, moderate daily consumption of red wine for 2-weeks significantly increased plasma total phenolic concentrations (Table 4.3) by only 4.4 μ M GAE which was also associated with a non-significant increase in antioxidant capacity. This is in contrast to several studies which have measured total phenols and plasma antioxidant activity at various time intervals up to 24 h following the consumption of red wine (Maxwell *et al.*, 1994; Whitehead *et al.*, 1995; Day *et al.*, 1997; Duthie *et al.*, 1998; Serafini *et al.*, 1998). However, in such studies the increases in the plasma phenolics and antioxidant capacity decrease within 4 h after wine ingestion. In the present investigation, blood samples for the measurement of antioxidant capacity were taken in the morning about 10-12 h after the consumption of red wine the previous evening which may indicate that the majority of the phenolics had cleared from the blood. This suggests that regular intake over 2 weeks does not lead to sustained increase in total phenols in the circulation. In the present study, there was a significant increase in the concentration of HDL cholesterol ($P \leq 0.05$) in the red wine group post-intervention (Table 4.6). HDL cholesterol concentrations are inversely associated with risk of CHD (Marques-Vidal *et al.*, 1995). This is due to the role of these lipoproteins in carrying cholesterol from peripheral cells to the liver, where it is degraded and repackaged, a process known as 'reverse cholesterol transport' (Eisenberg, 1984). It is well documented that alcohol increases the concentration of HDL cholesterol (Gaziano *et al.*, 1993) and there is some evidence to suggest that red wine phenolics may provide a protective effect. Lavy *et al.* (1994) reported an increase in HDL cholesterol

concentrations following 2-weeks of daily moderate red wine consumption in healthy volunteers. These effects were not observed following consumption of white wine indicating that red wine phenolics may influence HDL cholesterol. In the present study while the changes in the concentration of HDL cholesterol were statistically significant, the change is small and unlikely to be of clinical significance. There were no significant differences (unpaired t-tests) in the lag-phase or in any of the other indices of LDL oxidation between the red wine and control groups, post-intervention (Table 4.5). However, within the red wine group (paired t-test); there was a significant reduction in the maximum concentration of conjugated dienes ($P \leq 0.05$) and TBARS ($P \leq 0.05$) post-supplementation. While the lag phase was not significantly increased in the red wine group, there was an increase in 8 of the 12 subjects. Our finding of a significant reduction in the concentration of conjugated dienes and TBARS without a statistically significant increase in the lag phase could lead one to suspect that there had been changes in LDL particle fatty acid composition. However, while we did not directly measure the LDL fatty acid composition, analysis using a computerised version of the food composition tables (Holland *et al.*, 1991) of the subjects' daily diet records for the period of the trial revealed no significant changes in the quantity or in the quality of dietary fat intake. It is very unlikely, therefore, that the fat composition of the LDL particle was altered in the case of 8 of the 12 subjects and the 4 who responded differently were not distinct in this respect. Thus, while there was not a statistically significant reduction in susceptibility of LDL to oxidation compared with the control group, changes within the red wine supplemented group appear to show some protection against LDL oxidation. This is mostly likely to be due to the small but statistically significant increase in the plasma concentration of phenolics, which can bind to LDL and offer protection against LDL oxidation. It is likely that the difference in response was simply due to person to person variation in response to the red wine intervention, as individuals may respond differently to supplementation as has been suggested by Howard *et al.* (2002). Furthermore, the 4 subjects who did not show an increase in the lag-phase did not respond differently in their plasma phenolic concentrations, TBARS or the maximum concentration and the rate of conjugated dienes, leading us to the conclusion that compliance to the intervention was met. Two previous studies (Furhman *et al.*, 1995; Nigdikar *et al.*, 1998) have shown a reduction in LDL oxidation using similar red wine doses (375 mL/d and 400 mL/d, respectively) and intervention periods

(2 weeks) as the current study. However, there may be a number of possible reasons to explain this apparent discrepancy. The phenolic composition of the wine used in wine trials is important with regard to their effect on LDL oxidation (Howard *et al.*, 2002). In the present study a Young Vatted Bulgarian Cabernet Sauvignon was used and the intake of (mg/d) phenolic compounds from the 375 mL serving of red wine was 165 mg/d, with the majority of the phenolics derived from anthocyanins, gallic acid and total flavan-3-ols (Table 4.2). The overall levels of phenolics in the wines used in the studies of Nigdikar *et al.* (1998) and Fuhrman *et al.* (1995) were somewhat higher than in the present study, for example the amount of red wine phenolics ingested in the study of Nigdikar *et al.* (1998) was 248 mg/d in a 375 mL serving. This difference in dose may explain why we observed no statistically significant effect on LDL oxidation. A further consideration is that in the studies of Nigdikar *et al.* (1998) and Fuhrman *et al.* (1995) the LDL oxidation assays were carried out on fresh plasma samples. In this investigation EDTA-treated plasma was stored at -80°C for several weeks prior to being assayed. Our experience (Higgins *et al.*, 2001), along with that of Esterbauer *et al.* (1993) and Kleinveld *et al.* (1992) is that this does not adversely affect the reliability of the oxidation assay. Another difference between the present investigation and the studies of Nigdikar *et al.* (1998) and Fuhrman *et al.* (1995) is that in both these studies the volunteers were male, while both male and female volunteers participated in our study. This could have increased the inter-individual variability in the response to the red wine intervention making it more difficult to see a statistically significant difference in the lag phase between the intervention and control groups. However, we did not find any differences in any of the parameters measured between male and female volunteers. A power calculation was carried out post-hoc to determine whether the number of subjects used in this study was adequate to see a statistically significant difference in the lag phase. The sample size was calculated based on a two-sample t-test (wedstaff@stat.ucla.edu). The number of volunteers required to see a difference was calculated to be $n=16$. Thus the number of subjects used in the present study was slightly less than the number required to see a difference at 5 % significance level and 80 % power. Although there was a trend towards a reduction in the plasma concentration of homocysteine in the red wine group the results were not statistically significant (Table 4.6). Elevated circulating levels of homocysteine are associated with an increased risk of cardiovascular disease (Refsum *et al.*, 1998) and are affected by

diet, mainly due to inadequate intakes of folate and vitamin B12. There is evidence that a light to moderate consumption of alcohol is associated with lower fasting plasma concentrations of homocysteine, and this has previously been reported for beer (van der Gaag *et al.*, 2002) and red wine (Dixon *et al.*, 2002). Although beer contains folate and vitamin B6, red wine contains negligible amounts of vitamin B6, and it is unlikely that these micronutrients alone can explain the beneficial effect. Glucuronides of (+)-catechin and (-)-epicatechin and their methylated analogues were identified in plasma from the red wine group post-intervention (Table 4.4). However, the amounts present could not be quantified and are clearly not sufficient to make a significant contribution to the increased levels of phenolics detected in plasma after red wine consumption (Table 4.3). The presence of the flavan-3-ol metabolites is, however, in line with the findings of a previous study in which following consumption of a single serving of 120 ml of red wine by human volunteers, (+)-catechin was detected in plasma after treatment with β -glucuronidase and arylsulphatase (Bell *et al.*, 2000). Although the red wine used in the present study contained a substantial amount of anthocyanins, particularly malvidin-3-glucoside (Table 4.2), no anthocyanins were detected in plasma despite a thorough search using HPLC-MS-MS. This is in keeping with the findings of other human studies where reported urinary recoveries of anthocyanins after consumption of red wine or anthocyanin-rich produce, typically range from 0.05 to 0.11 % of intake (Frank *et al.*, 2003; McGhie *et al.*, 2003). Similarly, very low plasma anthocyanin concentrations have been reported in humans (Cao & Prior, 1999) and frequently the levels are below the limits of detection (Felgines *et al.*, 2002; Wu *et al.*, 2002). It is unclear at this juncture whether anthocyanins enter the circulatory system in trace amounts or whether they are absorbed in more substantial quantities and rapidly removed from the bloodstream. Likewise, although the red wine contained a high level of gallic acid, despite a thorough search no gallic acid or metabolites of gallic acid were detected in the plasma of volunteers who consumed red wine.

4.7 Conclusion

In summary, a moderate consumption, over a two week period, of a red wine, rich in gallic acid, flavan-3-ols and anthocyanins, significantly increased the concentration of total phenolics in plasma of human volunteers. This small increase may be due to the trace levels of glucuronide and methylated metabolites of flavan-3-ol monomers that were detected in plasma post red wine consumption. There were significant reductions in conjugated dienes and TBARS and a significant and modest increase in HDL cholesterol in the red wine supplemented group. The findings from this study support the protective effects of red wine reported from previous epidemiological studies and current medical opinion that moderate daily intake of red wine may reduce the risk of developing CHD.

CHAPTER 5 THE ABSORPTION, METABOLISM AND EXCRETION OF FLAVAN-3-OLS AND PROCYANIDINS FOLLOWING THE INGESTION OF A TANNIN-RICH GRAPE SEED EXTRACT BY RATS..... 132

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Chapter 5 The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a tannin-rich grape seed extract by rats

5.1 Introduction

Flavan-3-ols are a complex subclass of flavonoids encompassing the simple monomers (+)-catechin and its isomer (-)-epicatechin, and the oligomeric and polymeric procyanidins, commonly known as condensed tannins (Figure 5.1). The procyanidins are formed from the condensation of monomeric units, between 2-5 units for oligomers and over 5 units for polymers. Procyanidins differ in their position and configuration of their monomeric linkages, with the dimers B₁, B₂, B₃ and B₄ being detected most frequently. Besides forming complexes with other flavan-3-ols, the monomeric flavan-3-ols are hydroxylated to form the gallo catechins, and also undergo esterification with gallic acid (Crozier, 2003).

Flavan-3-ols are widely dispersed in the human diet and are one of the most abundant dietary flavonoids with red wine, tea, berries, apples and chocolate providing the richest food sources (Sanoner *et al.*, 1999; Foo *et al.*, 2000; Auger *et al.*, 2004; Del Rio *et al.*, 2004). Although there are no accurate estimates of flavan-3-ol intake levels, it has been speculated that consumption ranges between 0.1-0.5 g/d, (De Pascual-Teresa *et al.*, 2000a; Scalbert & Williamson, 2000). The data of Arts *et al.* (2001a) imply that flavan-3-ols may be one of the more effective groups of dietary phenolics in reducing the risk of coronary heart disease. In keeping with this possibility (+)-catechin and procyanidins have been shown reduce platelet aggregation (Ruf *et al.*, 1995) and to act as powerful inhibitors of low-density lipoprotein (LDL) oxidation in vitro (Teissedre *et al.*, 1996; Steinberg *et al.*, 2003) and ex vivo (Kondo *et al.*, 1996), events believed to play a crucial role in the preventing the onset of atherogenesis (Steinberg *et al.*, 1989). It has also been demonstrated that consumption of a procyanidin-rich grape seed extract (GSE) reduces the incidence of cataracts in the eyes of hereditary cataractous (ICR/f) rats (Yamakoshi *et al.*, 2002). (+)-Catechin and (-)-epicatechin are absorbed in humans and animals appearing in plasma and urine primarily as glucuronidated, methylated and sulphated metabolites following the ingestion of chocolate (Baba *et al.*, 2001; Wang *et*

al. 2000; Rein *et al.*, 2000), black and green tea (Warden *et al.*, 2001; Yang *et al.*, 1998; Piskula and Terao 1998) and red wine (Bell *et al.*, 2000; Donovan *et al.*, 1999). There is, never-the-less, conflicting evidence on the absorption and metabolism of the oligomeric and polymeric flavan-3-ols in humans and animals. Koga *et al.* (1999) observed the presence of (+) catechin and (-)-epicatechin and an absence of dimers in the plasma of rats following ingestion of a GSE. Extending this study, Donovan *et al.* (2002) fed rats a GSE, (+)-catechin and procyanidin B₃ meals. While conjugated metabolites of (+)-catechin were detected in plasma and urine after both the (+)-catechin and GSE meals there was no evidence of absorption for the procyanidins. However, in another study (-)-epicatechin and (+)-catechin and trace amounts of procyanidin dimer B₂ were detected in sulphatase- and β -glucuronidase-treated human plasma collected 30 min after ingestion of a cocoa beverage rich in flavan-3-ol monomers and procyanidins (Holt *et al.*, 2002). In keeping with this report, it has been shown that after oral administration of B₂ to rats, the dimer is absorbed and excreted in urine with a portion of the procyanidin being converted to (-)-epicatechin, which undergoes post-ingestion conjugation and methylation (Baba *et al.*, 2002).

This chapter reports on a comprehensive study of the absorption, excretion and sequestration in body tissues of (+)-catechin, (-)-epicatechin, their metabolites and procyanidin dimers and a trimer following the oral intake of a GSE by rats. Liver, kidney, brain and GI tract, together with plasma, urine and faeces were collected at several time points up to 24 h post-ingestion of the extract. Samples were extracted and analysed by HPLC-tandem mass spectrometry for the identification of flavan-3-ols and metabolites.

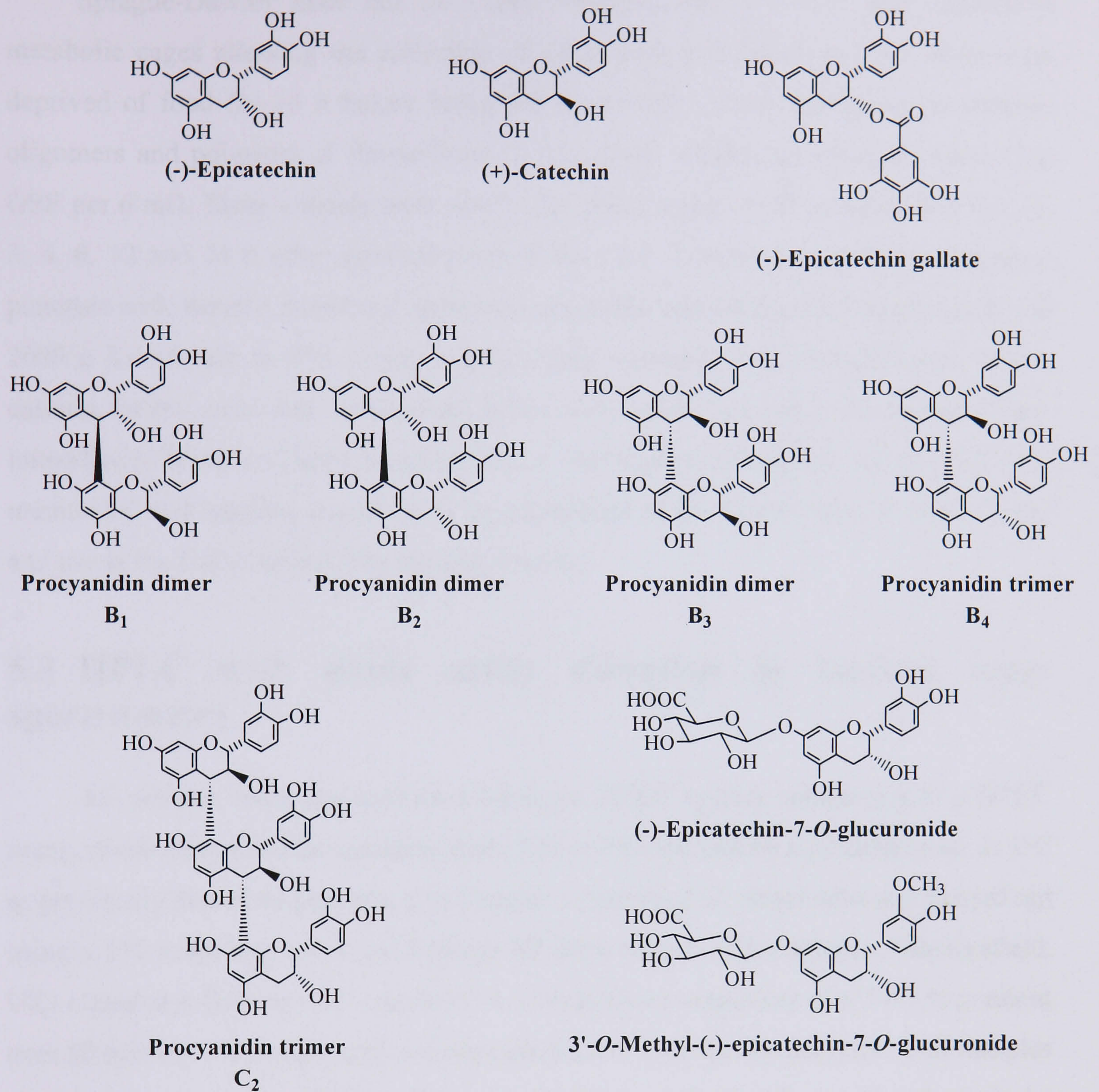


Figure 5.1 Some common flavan-3-ol structures.

5.2 Animal and sample preparation

Sprague-Dawley male rats ($n = 24$), weighing $250 \text{ g} \pm 5 \text{ g}$, were housed in metabolic cages allowing the collection of 24 h urine and faecal samples. Rats were deprived of food for 16 h before being fed by gavage a GSE containing monomers, oligomers and polymers of flavan-3-ols (1 g/kg body weight) dissolved in water (1 g GSE per 6 ml). Three animals were terminally anaesthetised with pentobarbital 0, 1, 2, 3, 4, 6, 12 and 24 h after administration of the GSE. Blood was removed by cardiac puncture with heparin moistened syringes and plasma was obtained by centrifugation at 2000 g for 10 min at 4°C . Liver, kidney, brain, stomach, duodenum/jejunum, ileum, caecum, colon, urine and faeces were collected at each time point. All samples were immediately frozen in liquid N_2 and stored at -80°C prior to analysis. All animals were maintained and handled according to the guidelines of the Committee on Animal Care and use at the University of Montpellier, France.

5.3 HPLC with diode array detection & tandem mass spectrometry

All samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, diode array detector scanning from 250 to 700 nm, and an autosampler set at 4°C as previously described previously in Chapter 2 (section 2.6). Separation was carried out using a $250 \times 4.6 \text{ mm}$ i.d. $4 \mu\text{m}$ Synergi RP-Max column (Phenomenex, Macclesfield, UK) eluted at a flow rate of 1 mL/min . A mobile phase consisting of a 5-40 % gradient over 60 min of 1 % formic acid and acetonitrile was used for the analysis of all samples except urine for which a 5-20 %, 60 min gradient was utilised. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 mL was directed to a LCQ Deca XP ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, USA). Analysis was carried out in negative ion mode operating in full scan mode from 100-2000 amu. Selective reaction monitoring (SRM) was used where compounds could not be identified using the full scan mode. With enhanced specificity and significantly reduced chemical noise this method was particularly useful for the identification and quantification of trace levels of procyanidins in urine. The conditions used were as outlined above except that the mass

spectrometer was in selected reaction monitoring mode. When screening for the presence of dimers the parent ion was m/z 577, collision energy 35 % and product ions were scanned from m/z 220-577. In the case of trimers, the parent ion was m/z 865 and product ions were scanned from m/z 350-865.

5.4 Identification of grape seed phenolics

In total 13 phenolic compounds were identified in the GSE by HPLC-MS-MS. The data obtained are illustrated in Figure 5.2 and summarized below and in Table 5.2. Where reference compounds were not available identifications were facilitated by previous analyses of flavan 3-ols in grapes seeds and GSE (Table 5.1).

Peak 1 (tR = 6.2 min, λ_{\max} = 280 nm) had a [M-H]⁻ at m/z 865 with MS² yielding two major fragment ions at m/z 695 and 577. Based on the mass spectral data, this compound is a procyanidin trimer. Its elution prior to procyanidin dimer B₃ (peak 2) (Santos-Buelga *et al.*, 1995) indicates that it is probably C₂, a known component of grape seeds (Romeyer *et al.*, 1986).

Peak 2 (tR = 12.1 min, λ_{\max} = 280 nm) had a [M-H]⁻ at m/z 577 with MS² yielding three fragment ions at m/z 425, 407 and 289. This mass spectrum and co chromatography with an authentic standard established that peak 2 is procyanidin dimer B₁, which has previously been detected in grape seeds (Ricardo da Silva *et al.*, 1991).

Peak 3 (tR = 13 min, λ_{\max} = 280 nm) also had a [M-H]⁻ at m/z 577 which fragmented to produce MS² ions at m/z 425, 407 and 289. Its elution prior to (+)-catechin (Sun *et al.*, 1999) and co-chromatography with an authentic standard demonstrates that peak 3 is procyanidin dimer B₃.

Peak 4 (tR = 14.3 min, λ_{\max} = 280 nm) had a [M-H]⁻ at m/z 289 and MS² produced fragment ions at m/z 245 and 205. This mass spectrum and co chromatography with an authentic standard identifies peak 4 as (+)-catechin.

Peak 5 (tR = 15 min, λ_{\max} = 280 nm) produced a [M-H]⁻ at m/z 1153 which on MS² yielded ions at m/z 865 and 577. Although a reference compound was not available, the elution of this peak close to (+)-catechin and the mass spectral data

indicate that it may be a non-galloylated procyanidin tetramer, the presence of which has previously reported in a GSE.

Peak 6 (tR = 15.5 min, λ_{\max} = 280 nm) had a [M-H]⁻ at m/z 865 with MS² producing three fragment ions at m/z 695, 577 and 407. Based on the mass spectral data, this compound is a procyanidin trimer. Its elution between (+)-catechin (peak 4) and the dimer B₄ (peak 7) indicates that it may be epicatechin-(4 β -8)-epicatechin-(4 β -8)-catechin previously identified in a GSE by Santos-Buelga *et al.* (1995).

Peak 7 (tR = 16.2 min, λ_{\max} = 280 nm) had a [M-H]⁻ at m/z 577 which on MS² yielded three ions at m/z 425, 407 and 289. This mass spectrum and co-chromatography with an authentic standard identifies peak 7 as procyanidin dimer B₄, a known component of grape seeds (De Pascual-Teresa *et al.*, 2000b).

Peak 8 (tR = 17 min, λ_{\max} = 280 nm) produced a [M-H]⁻ at m/z 577 which produced MS² fragments at m/z 425, 407 and 289. The mass spectral data indicated the presence of a procyanidin dimer and co-chromatography with an authentic standard identified it as the B₂ dimer.

Peak 9 (tR = 18.8 min, λ_{\max} = 280 nm) had a [M-H]⁻ at m/z 289 which on MS² yielded two ions at m/z 245 and 205. This mass spectrum and co-chromatography with an authentic standard established that peak 9 is (-)-epicatechin.

Peak 10 (tR = 20.7 min, λ_{\max} = 280 nm) produced a [M-H]⁻ at m/z 865, which yielded MS² fragments at m/z 695, 577 and 407. Based on the mass spectral data this compound is procyanidin trimer.

Peak 11 (tR = 21.3 min, λ_{\max} = 275 nm) had a [M-H]⁻ at m/z 1153, and MS² yielded ions at m/z 865 and 577. This is in keeping with peak 11 being a procyanidin tetramer.

Peak 12 (tR = 22.2 min, λ_{\max} = 275 nm) had a [M-H]⁻ at m/z 729, with MS² ions at m/z 577, 407 and 289. These ions are similar to those observed with the procyanidin dimers in peaks 8 and 9 with the m/z 577 fragment resulting from a 154 amu loss from the [M-H]⁻ which is indicative of the cleavage of a gallate unit. On the basis of the MS² spectrum, this compound is, therefore, a gallated procyanidin dimer. Although a reference compound was not available, the elution order of this peak after (-)-epicatechin (peak 9) and a procyanidin trimer (peak 10) along with the mass spectral data indicate that it is either B₁-3-*O*-gallate or B₂-3'-*O*-gallate both of which have been detected in grape seeds (Santos-Buelga *et al.*, 1995).

Peak 13 (tR = 27.2 min, $\lambda_{\text{max}} = 275$ nm) had a [M-H]⁻ at *m/z* 441 with MS² producing a fragment at *m/z* 289. This mass spectrum and co-chromatography with an authentic standard established that peak 3 is (-)-epicatechin-3-*O*-gallate. The levels of the 13 flavan-3-ols that were ingested when each rat was fed the GSE are presented in Table 5.2 together with the summarised analytical data. On a per kg body weight basis each rat ingested 23.2 mg of (+)-catechin, 14.4 mg of (-)-epicatechin, 28.4 mg of procyanidin dimers, 5.2 mg of trimers and 2.8 mg of tetramers. In addition, each rat also ingested a substantial but undetermined quantity of high molecular weight polymeric procyanidins whose presence in the GSE is evident from the increasing baseline and the broad unresolved band of components that elute across the HPLC-A280 chromatogram illustrated in Figure 5.2.

Table 5.1 Flavan-3-ols isolated from grape seeds.

Peak order	Compound
1	Catechin-(4 α -8)-catechin-(4 α -8)-catechin (C ₂)
2	Catechin-(4 α -8)-catechin (B ₃)
3	Epicatechin-(4 β -8)-catechin (B ₁)
4	(+)-Catechin
5	Epicatechin-(4 β -8)-epicatechin-(4 β -8)-catechin
6	Catechin-(4 α -8)-epicatechin (B ₄)
7	Catechin-(4 α -8)-catechin-(4 α -8)-epicatechin
8	Epicatechin-(4 β -6)-epicatechin-(4 β -8)-catechin
9	Catechin-(4 α ,6)-catechin (B ₆)
10	Epicatechin-(4 β -6)-epicatechin-(4 β -8)-epicatechin
11	Epicatechin-(4 β -8)-epicatechin (B ₂)
12	Epicatechin-(4 β -8)-epicatechin-3- <i>O</i> -gallate-(4 β -8)-catechin
13	Epicatechin-3- <i>O</i> -gallate-(4 β -8)-epicatechin (B ₂ -3- <i>O</i> -gallate)
14	(-)-Epicatechin
15	Catechin-(4 α -6)- epicatechin-3- <i>O</i> -gallate (B ₄ -3'- <i>O</i> -gallate)
16	Epicatechin-(4 β -8)-epicatechin-(4 β -6)-catechin
17	Epicatechin-3- <i>O</i> -gallate-(4 β -8)-catechin (B ₁ -3- <i>O</i> -gallate)
18	Epicatechin-(4 β -8)-epicatechin-3- <i>O</i> -gallate (B ₂ -3'- <i>O</i> -gallate)
19	Epicatechin-(4 β -6)-catechin (B ₇)
20	Epicatechin-(4 β -8)-epicatechin-(4 β -8)-epicatechin (C ₁)
21	Epicatechin-(4 β -8)-epicatechin-(4 β -8)-epicatechin-(4 β -8)-epicatechin
22	(-)-Epicatechin-3- <i>O</i> -gallate
23	Epicatechin-3- <i>O</i> -gallate-(4 β -6)- catechin (B ₇ -3- <i>O</i> -gallate)
24	Epicatechin-3- <i>O</i> -gallate-(4 β -8)-epicatechin-3- <i>O</i> -gallate (B ₂ -3,3'- <i>O</i> -digallate)
25	Epicatechin-(4 β -8)-epicatechin-(4 β -8)-epicatechin-3- <i>O</i> -gallate
26	Epicatechin-(4 β -8)- epicatechin-3- <i>O</i> -gallate-(4 β -8)-epicatechin-3- <i>O</i> -gallate
27	Epicatechin-(4 β -6)-epicatechin (B ₅)

Adapted from Santos-Buelga *et al.* (1995).

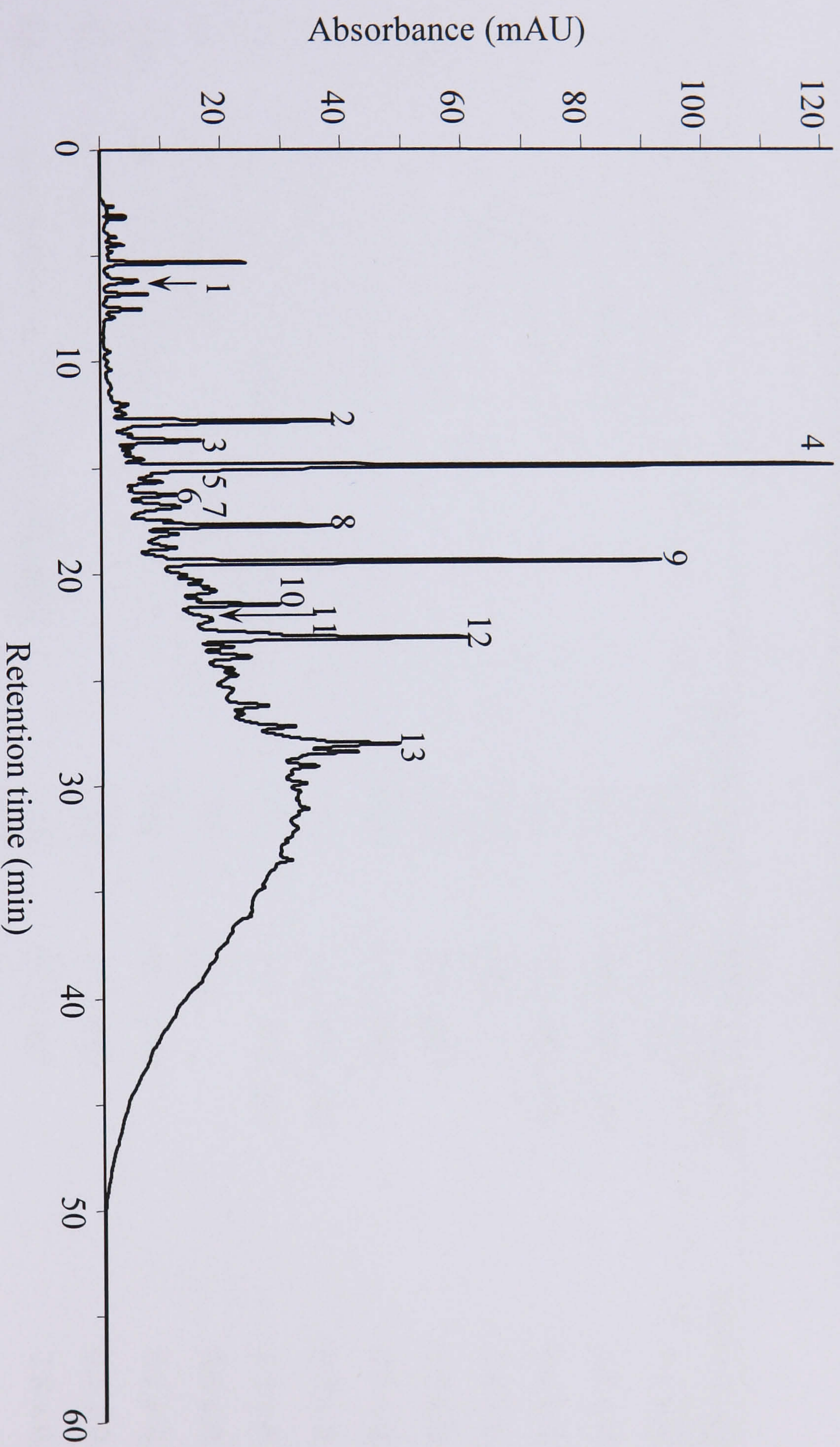


Figure 5.2 Reversed phase HPLC of a GSE using a 60 min 5-40% gradient of acetonitrile in 1% aqueous formic acid with detection at 280 nm. (For MS² data and identification and quantification of peaks 1-13, see Table 5.2).

Table 5.2 HPLC and mass spectral characteristics of flavan-3-ols in a GSE and amounts ingested per rat.

Peak	R _t (min)	Compound	[M-H] ⁻ (<i>m/z</i>)	MS ² ions (<i>m/z</i>)	Amount ingested
1	6.2	procyanidin trimer C ₂	865	577, 289	0.3 ± 0.0
2	12.1	procyanidin dimer B ₁	577	425, 407, 289	1.5 ± 0.2
3	13.1	procyanidin dimer B ₃	577	425, 407, 289	0.6 ± 0.2
4	14.3	(+)-catechin	289	245,	5.8 ± 0.2
5	15.0	procyanidin tetramer	1153	577, 289	0.2 ± 0.0
6	15.5	procyanidin trimer	865	577, 289	0.3 ± 0.1
7	16.1	procyanidin dimer B ₄	577	425, 407, 289	0.7 ± 0.1
8	16.9	procyanidin dimer B ₂	577	425, 407, 289	1.5 ± 0.2
9	18.7	(-)-epicatechin	289	245,	3.6 ± 0.1
10	20.6	procyanidin trimer	865	577, 289	0.7 ± 0.1
11	21.3	procyanidin tetramer	1153	577, 289	0.5 ± 0.2
12	22.2	procyanidin B ₁ -3- or B ₂ -3'- <i>O</i> -gallate	729	577, 407	2.8 ± 0.1
13	27.2	(-)-epicatechin-3- <i>O</i> -gallate	441	289	0.7 ± 0.2

Identifications based on retention times (R_t), and full scan negative ionization MS-MS data. Peak numbers and R_t values refers to peaks in Figure 5.2. [M-H]⁻-negative charged molecular ion. Mean amount ingested per rat expressed as mg ± standard error, where *n* = 3.

5.5 Identification of flavan-3-ol metabolites in body tissues and fluids

To reduce the sample weight to manageable proportions for HPLC analysis and obtain cleaner mass spectra, samples from three individual rats were combined and extracted according to the method of Mullen *et al.* (2002b) described in detail in Chapter 2 (section 2.7.2). The total weight of each organ and the amount of each organ used in the extraction method are summarised in Appendix II. Proteins in 1 mL volumes of plasma were precipitated and phenolics extracted as described in detail in Chapter 2 (section 2.7.1) and in Mullen *et al.* (2002b). Using a 5-40 % acetonitrile gradient HPLC with MS-MS in the full scan mode four flavan-3-ol metabolites were detected in the GI tract, plasma, liver, and kidneys. The results of these analyses are summarized below and in Table 5.3.

Peak M-1 (tR = 11.6 min, $\lambda_{\text{max}} = 280$ nm) and *M-2* (tR = 14.5 min, $\lambda_{\text{max}} = 280$ nm) both had a [M-H]⁻ at *m/z* 465 which yielded MS² fragments at *m/z* 289 and 245 corresponding to (+)-catechin/(-)-epicatechin. The [M-H]-176 loss is in keeping with the cleavage of a glucuronyl unit. The MS fragmentation pattern, together with the HPLC elution order, indicates that the earlier eluting *M-1* peak is a (+)-catechin glucuronide while *M-2* is an (-)-epicatechin glucuronide.

Peak M-3 (tR = 17.7 min, $\lambda_{\text{max}} = 280$ nm) and *M-4* (tR = 20.7 min, $\lambda_{\text{max}} = 280$ nm) also had identical mass spectra with a [M-H]⁻ at *m/z* 479 which on loss of 176 amu (cleavage of a glucuronyl unit) yielded a MS² ion at *m/z* 303. These fragments are 14 amu higher than the equivalent ions in the spectra of *M-1* and *M-2*, indicating the presence of methylated flavan-3-ol glucuronides. *M-3* is therefore probably a methyl-catechin glucuronide and the later eluting *M-4* a methyl-epicatechin glucuronide. Without reference compounds it was not possible to determine the positions on the flavan-3-ol skeleton of the methyl and glucuronic acid substituents. The most likely candidates for *M-2* and *M-4* are (-)-epicatechin-7-*O* glucuronide and 3'-*O*-methyl(-)-epicatechin-7-*O*-glucuronide which have been identified in rat plasma and urine after ingestion of (-)-epicatechin (Natsume *et al.*, 2003). (+) Catechin is converted to 3'-*O*-methyl-(+)-catechin in rats (Nakamura and Tonogai, 2003), so *M-3* may be a 3'-*O*-methyl-(+)-catechin glucuronide. However, no (+)-catechin glucuronides have been

structurally elucidated so it is not possible to speculate on the position of the glucuronide moiety in *M-1* and *M-3*.

Urine contained impurities that interfered with the analyses when HPLC was carried out with a 5-40 % gradient of acetonitrile. This was overcome with the use of a 5-20 % gradient which enhanced resolution and revealed the presence of the four previously detected metabolites (*M-1*, *M-2*, *M-3* and *M-4* at retention times of 14.1, 19.0, 26.0 and 30.2 min, respectively) together with four additional urinary metabolites, *U-1*, *U-2*, *U-3* and *U-4*, which were not present in other samples (Table 5.3).

Peak U-1 (tR = 24.6 min, λ_{\max} = 280 nm) and *U-2* (tR = 27.9 min, λ_{\max} = 280 nm) both had a [M-H]⁻ at *m/z* 369 yielding a MS² ion at *m/z* 289. The [M-H]-80 loss is in keeping with the cleavage of a SO₃ unit. The MS fragmentation pattern, together with the HPLC elution order, indicates that the earlier eluting *U-1* peak is a (+)-catechin sulphate while *U-2* is an (-)-epicatechin sulphate.

Peaks U-3 (tR = 29.2 min, λ_{\max} = 280 nm) and *U-4* (tR = 38.1, min, λ_{\max} = 280 nm) had a [M-H]⁻ at *m/z* 383 which on loss of 80 amu (cleavage of a SO₃ unit) yielded a MS² ion at *m/z* 303. These fragments are 14 amu higher than the equivalent ions in the spectra of *U-1* and *U-2*, indicating the presence of methylated flavan-3-ol glucuronides. Based on the fact that 3'-*O*-methylation of flavan-3-ols predominates in rats *U-3* is therefore probably a 3'-*O* methyl-(+)-catechin sulphate and the later eluting *U-4*, a 3'-*O*-methyl-(-)-epicatechin sulphate. This is the first direct evidence for the occurrence of sulphated flavan-3-ol metabolites in rats and as yet the position of the sulphate moiety remained undetermined.

Table 5.3 HPLC and mass spectral properties of flavan-3-ol metabolites detected in rats after oral ingestion of a GSE.

Peak	R _f (min)	Metabolite	[M-H] ⁻ (<i>m/z</i>)	MS ² ions (<i>m/z</i>)	Location	Tentative identification
M-1	11.6*	(+)-catechin glucuronide	465	289 [C] ([M-H] ⁻ -GlcUA)	P, U, D/J, I, L, K	
M-2	14.5*	(-)-epicatechin glucuronide	465	289 [EC] ([M-H] ⁻ -GlcUA)	P, U, D/J, I, L, K	(-)-epicatechin-7- <i>O</i> -glucuronide
M-3	17.7*	methyl-catechin glucuronide	479	303 [C] ([M-H] ⁻ -GlcUA)	P, U, D/J, I, L, K	3'- <i>O</i> -methyl-(+)-catechin-glucuronide
M-4	20.7*	methyl-(-)-epicatechin glucuronide	479	303 [EC] ([M-H] ⁻ -GlcUA)	P, U, D/J, I, L, K	3'- <i>O</i> -methyl-(-)-epicatechin-7- <i>O</i> -glucuronide
U-1	24.6 [§]	(+)-catechin sulfate	369	289 [C] ([M-H] ⁻ -SO ₃)	U	
U-2	27.9 [§]	(-)-epicatechin sulfate	369	289 [EC] ([M-H] ⁻ -SO ₃)	U	
U-3	29.2 [§]	methyl-(+)-catechin sulfate	383	303 [MC] ([M-H] ⁻ -SO ₃)	U	
U-4	38.1 [§]	methyl-(-)-epicatechin sulfate	383	303 [ME] ([M-H] ⁻ -SO ₃)	U	

*5-40 % 60 min HPLC gradient; [§]5-20 %, 60 min HPLC gradient. [M-H]⁻-negatively charged molecular ion; C-(+)-catechin; EC-(-)-epicatechin; GlcUA-glucuronyl unit; MC-methyl-catechin; ME-methyl-epicatechin; P-plasma; U-urine, D/J-duodenum/jejunum; I-ileum; L-liver; K-kidneys.

5.5.1 Plasma flavan-3-ol metabolites

(+)-Catechin glucuronide (*M-1*), (-)-epicatechin glucuronide (*M-2*), methyl-(+)-catechin glucuronide (*M-3*) and methyl-(-)-epicatechin glucuronide (*M-4*) were detected in plasma (Table 5.3). This is shown in the HPLC traces in Figure 5.3 which illustrate data obtained with plasma collected 4 h after GSE ingestion. The A280 nm trace contains a number of impurities in addition to the four flavan-3-ol metabolites and it is evident that *M-3* is a shoulder on a contaminant peak. None-the-less full scan MS² spectra, as summarized in Table 5.3, were obtained for all four metabolites which were quantified by using the enhanced selectivity obtained by monitoring the response at *m/z* 465 for *M-1* and *M-2* and *m/z* 479 for *M-3* and *M-4* (Figure 5.3). Unmetabolised (+)-catechin and (-)-epicatechin were not present in the plasma samples in detectable quantities and neither were procyanidin dimers, trimers and tetramers. The plasma pharmacokinetic profiles based on HPLC-MS-SRM analyses are illustrated in Figure 5.4a. Three h after GSE ingestion (+)-catechin glucuronide and (-)-epicatechin glucuronide attained peak plasma concentration of 10.3 ± 0.7 and 9.9 ± 0.1 $\mu\text{g/ml}$, respectively which corresponds to 36.0 ± 2.5 and 34.4 ± 0.2 $\mu\text{moles/L}$. The glucuronide derivatives of methyl-(+)-catechin and methyl-(-)-epicatechin also peaked after 3 h at 5.5 ± 0.1 and 3.3 ± 0.2 $\mu\text{g/ml}$, 14 respectively. There was a ca. 20 % reduction in the concentrations of all four plasma flavan-3-ol metabolites at 4 h after which the levels declined more rapidly (Fig.5.4a). Assuming ca. 12 ml of plasma per rat, the peak plasma concentrations of the two (+)-catechin metabolites corresponds to ca. 3 % of the (+)-catechin ingested and the (-)-epicatechin metabolites equate with ca. 4 % of intake.

5.5.2 Plasma antioxidant capacity

The antioxidant capacity of plasma was measured in the ABTS⁺ antioxidant assay (Miller *et al.*, 1993) as described previously in Chapter 2 (section 2.3.5). The antioxidant capacity of the plasma increased significantly ($P < 0.05$) from baseline 0.88 ± 0.0 to 0.97 ± 0.0 (10.4 %) and 1.06 ± 0.0 (21.5 %) mmol/L trolox equivalents at 2 and 4 h post ingestion of the GSE, respectively. Results are illustrated in Figure 5.4b.

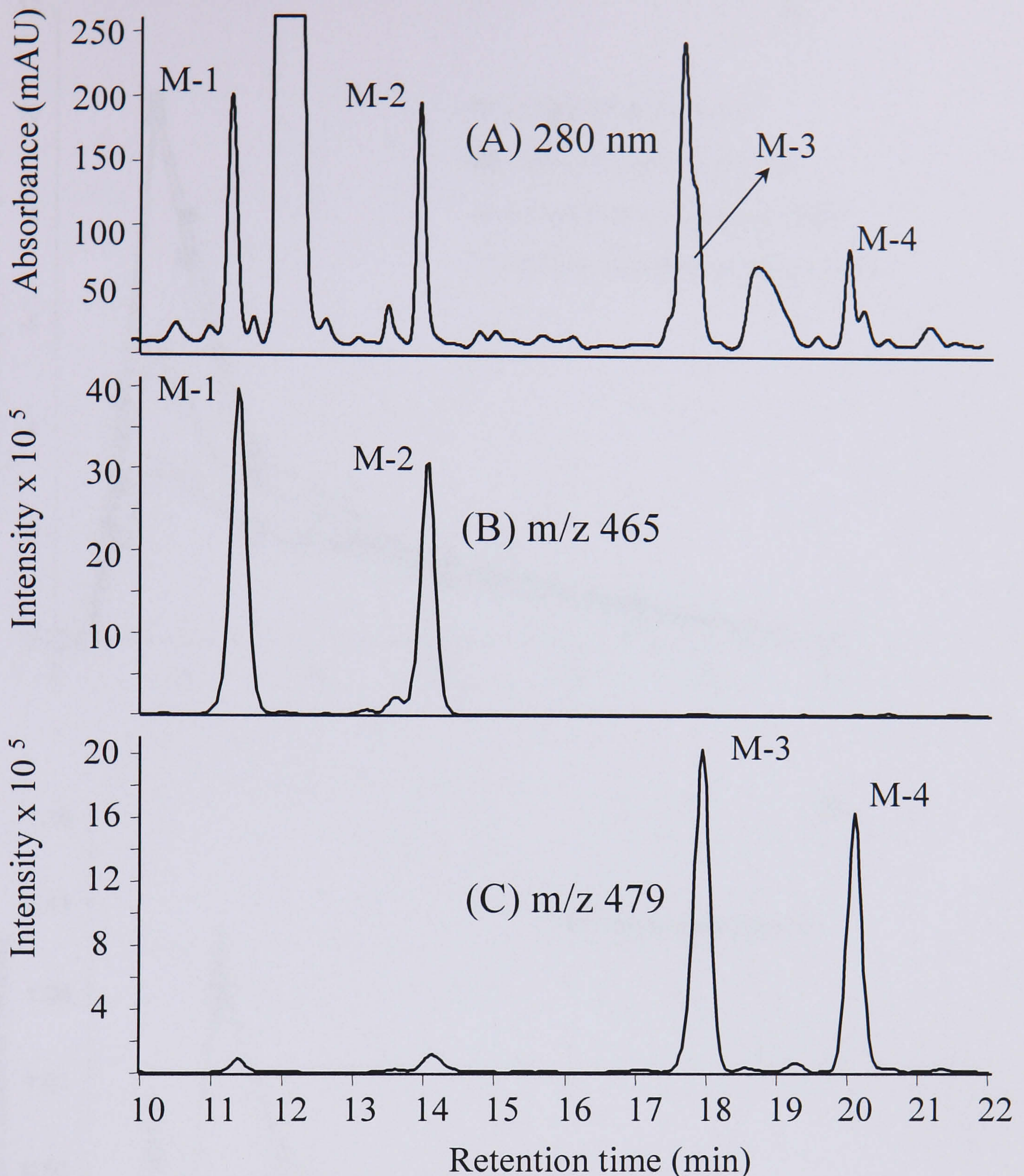


Figure 5.3 Reversed phase HPLC analysis, with diode array and full scan MS² detection, of the flavan-3-ol metabolites (+)-catechin glucuronide (*M-1*), (-)-epicatechin glucuronide (*M-2*), methyl-(+)-catechin glucuronide (*M-3*), methyl-(-)-epicatechin glucuronide (*M-4*) in rat plasma collected 4 h after the ingestion of a GSE grape seed extract. Extract analysed using a 60 min 5-40 % gradient of acetonitrile in 1 % aqueous formic acid with detection at (A) 280 nm, (B) *m/z* 465 and (C) *m/z* 479. (For MS² data, see Table 5.3).

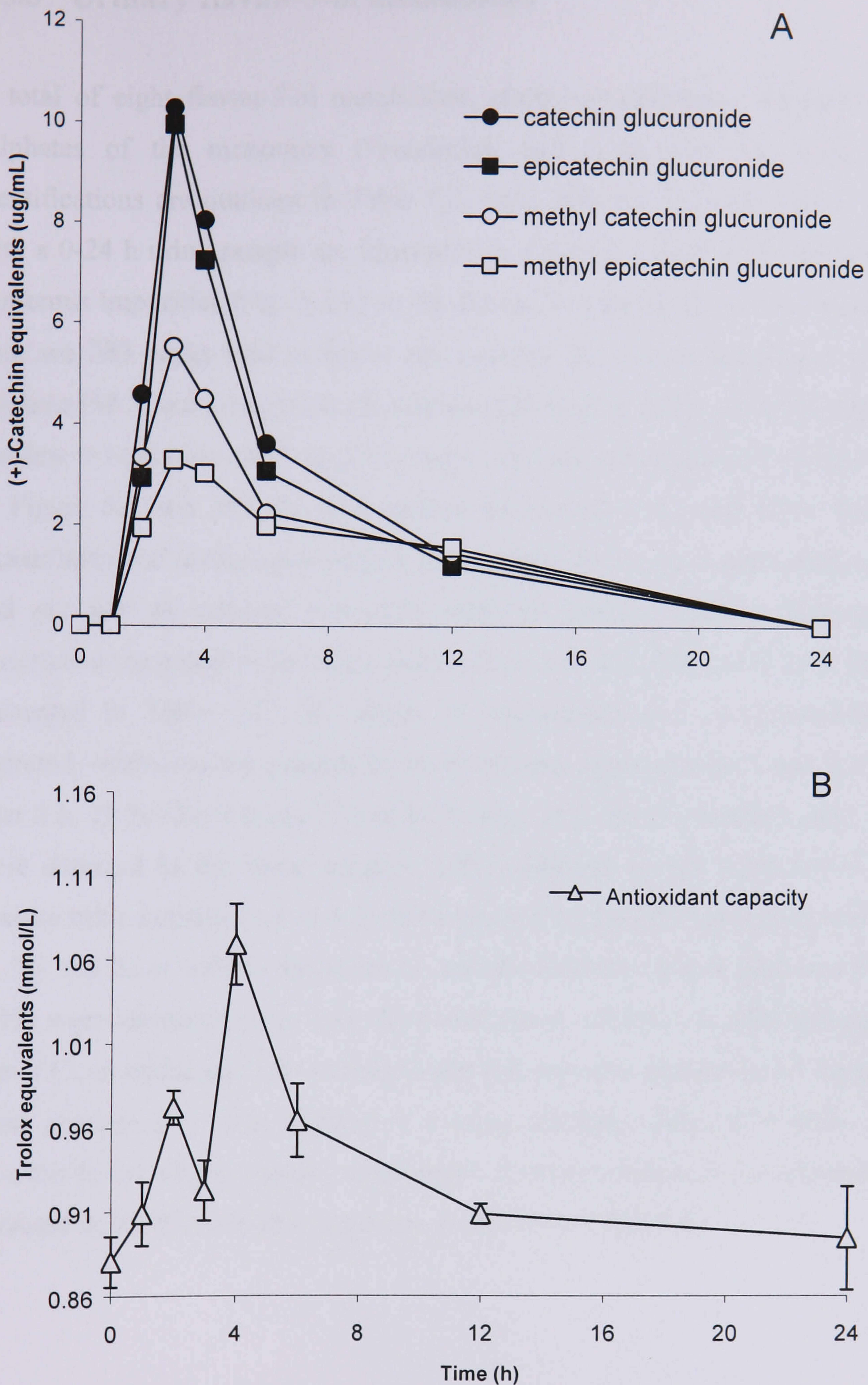


Figure 5.4 (A) Pharmacokinetic profile of flavan-3-ol metabolites detected in rat plasma collected over a 0-24 h period after the ingestion of a GSE. Results are expressed as μg (+)-catechin equivalents/ml \pm standard error, where $n = 3$ (triplicate analysis of same sample). In all instances standard error bars are smaller than symbols. (B) Plasma antioxidant capacity expressed as mmol/L trolox equivalents \pm standard error, where $n=3$ (triplicate analysis of same sample).

5.5.3 Urinary flavan-3-ol metabolites

A total of eight flavan-3-ol metabolites, methyl-glucuronides, sulphates and methyl-sulphates of the monomers (+)-catechin and (-)-epicatechin, whose LC-MS-MS identifications are outlined in Table 5.3, were detected in urine. HPLC data obtained with a 0-24 h urine sample are illustrated in Figure 5.5. The A280 nm trace contained numerous impurities (Fig. 5.5A) so the flavan-3-ol metabolites were quantified by MS with m/z 383 being used to detect and quantify the methyl derivatives of (+)-catechin sulphate (*M-3*) and (-)-epicatechin sulphate (*M-4*) (Fig. 5.5B) while m/z 369 was used to monitor (+)-catechin sulphate (*U-1*) and (-)-epicatechin sulphate (*U-2*) and as illustrated in Figure 5.5 m/z 383 for their methyl derivatives (*U-3* and *U-4*). The flavan-3-ol glucuronide and methyl-glucuronide metabolites, *M-1* to *M-4* were analysed at m/z 465 and m/z 479 as outlined previously with the plasma samples. Information on the cumulative excretion of the eight metabolites over 0-2, 0-4 and 0-24 h time periods is presented in Table 5.4. The levels of (+)-catechin and (-)-epicatechin metabolites excreted, relative to the quantity of the monomers ingested were 1 and 2 % respectively after 2 h, 13 % after 4 h and 27 and 36 % after 24 h. No (+)-catechin and (-)-epicatechin were detected in the urine samples. HPLC-MS-MS in the SRM mode was used to analyse urine collected up to 4 h after ingestion of the GSE and procyanidin dimers B_1 , B_2 , B_3 and B_4 as well as the trimer C_2 and the unknown trimer (that was peak 10 in the GSE) were identified (Fig. 5.6). Also analysis at m/z 865 revealed the presence of the trimer C_1 an additional later eluting trimer that was also present in the ingested GSE. All these procyanidins were detected in low μg amounts (Table 5.5) which is much less than the levels of the urinary metabolites of (+)-catechin and (-)-epicatechin that were excreted in the first 4 h after ingestion of the GSE (Table 5.4).

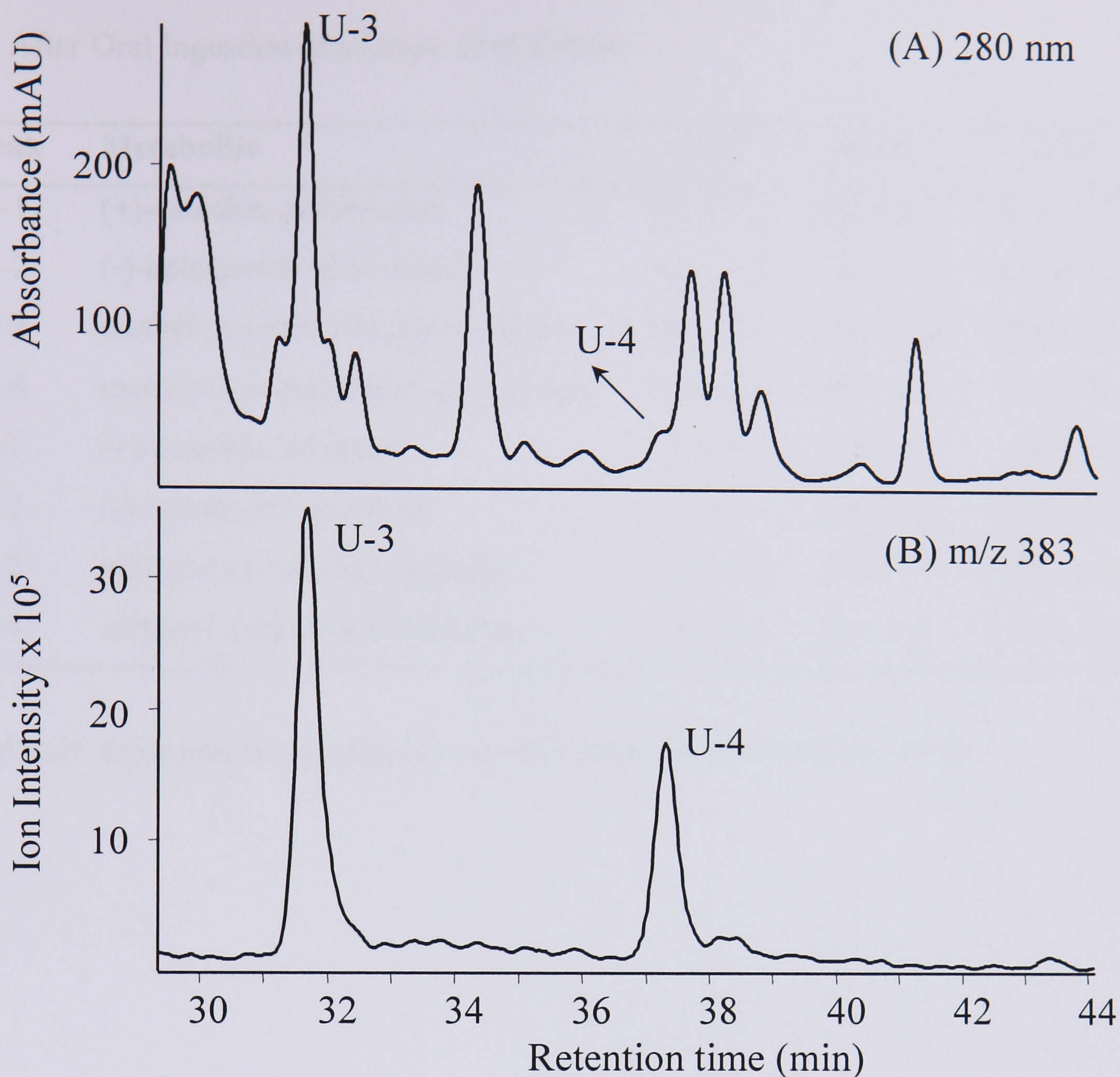


Figure 5.5 Reversed phase HPLC analysis, with diode array and full scan MS² detection, of methyl-(+)-catechin sulphate (*U-3*) and methyl-(-)-epicatechin sulphate (*M-4*) in rat urine collected 4 h after the ingestion of a GSE. Extract analysed using a 60 min 5-20 % gradient of acetonitrile in 1 % aqueous formic acid with detection at (A) 280 nm, (B) *m/z* 383. (For MS² data, see Table 5.3).

Table 5.4 Cumulative Excretion of Flavan-3-ol Metabolites in Rat Urine 0-2, 0-4 and 0-24 After Oral Ingestion of a Grape Seed Extract.

Peak	Metabolite	0-2 h	0-4 h	0-24 h
M-1	(+)-catechin glucuronide	40 ± 0.2	324 ± 8.2	814 ± 49
M-2	(-)-epicatechin glucuronide	11 ± 2.2	201 ± 1.0	635 ± 23
M-3	methyl-(+)-catechin glucuronide	66 ± 7.7	332 ± 9.0	534 ± 25
M-4	methyl-(+)-epicatechin glucuronide	6.3 ± 0.2	193 ± 0.7	391 ± 48
U-1	(+)-catechin sulphate	2.5 ± 0.1	27 ± 0.4	78 ± 6.1
U-2	(-)-epicatechin sulphate	n.d.	39 ± 0.2	139 ± 13
U-3	methyl-(+)-catechin sulphate	2.3 ± 0.2	45 ± 11	120 ± 1.1
U-4	methyl-(-)-epicatechin sulphate	1.4 ± 0.1	29 ± 1.4	152 ± 1.3

Results are expressed as μg excreted per rat \pm standard error, where. $n = 3$.

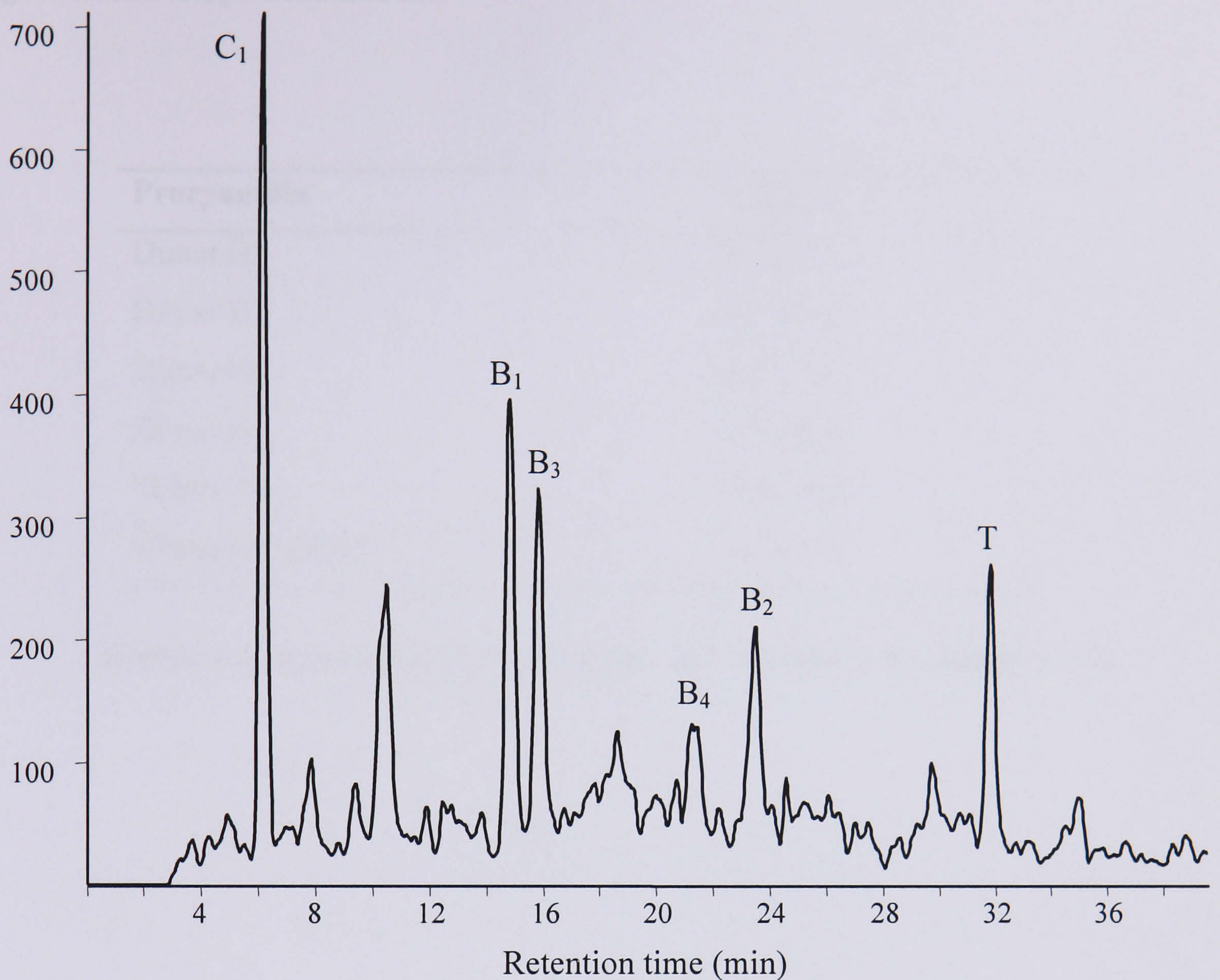


Figure 5.6 Reversed phase HPLC analysis, with diode array and MS² detection in the selected reaction monitoring mode, of procyanidin dimers (A) and trimers (B) in rat urine collected 4 h after the ingestion of a GSE grape seed extract. Extract analysed using a 60 min 5-40 % gradient of acetonitrile in 1% aqueous formic acid with detection at (A) m/z 577 (B) m/z 865.

Table 5.5 Cumulative Excretion of Procyanidin Dimers in Rat Urine 0-4 h After Oral Ingestion of a Grape Seed Extract.

Procyanidin	Amount
Dimer B ₁	7.5 ± 0.3
Dimer B ₂	4.2 ± 0.2
Dimer B ₃	6.4 ± 0.1
Dimer B ₄	3.0 ± 0.4
Trimer C ₁	13.0 ± 0.1
Unknown trimer	5.1 ± 0.4

Results are expressed as μg excreted per rat \pm standard error, where. $n = 3$.

5.5.4 Flavan-3-ols and metabolites in the GI tract

The levels of flavan-3-ols and their metabolites that were detected in the various sections of the GI tract are presented in Table 5.6. Although the amounts fluctuated somewhat from time point to time point, presumably because of rat-to-rat variations and variable recoveries, which, precludes a detailed analysis of the data, certain trends are none-the-less apparent. One hour after GSE supplementation (+)-catechin, (-)-epicatechin and other flavan-3-ols were found the GI tract, the highest quantities were present in the stomach with low levels in the duodenum/jejunum and ileum. Trace quantities of metabolites, detected in the duodenum/jejunum and ileum 1 h after the ingestion of the GSE, were identified as glucuronides of the monomers (+)-catechin and (-)-epicatechin and their methylated analogs. The amounts present in these organs at 2 h corresponded to only 1.2 % and 2 %, respectively, of the (-)-epicatechin and (+)-catechin in the ingested GSE. Small amounts of flavan-3-ol metabolites were present in the duodenum/jejunum and ileum up to 6 h after ingestion after which they were not detected. No metabolites were detected in the stomach, caecum and colon at any time point throughout the 24 h collection period although relatively small quantities of unmetabolised flavan-3-ols, including the B₁, B₂, B₃ and B₄ dimers, were present up to 12 h after ingestion of the GSE. All the GSE flavan-3-ols were eliminated from the GI tract 24 h after ingestion.

Table 5.6 Flavan-3-ols and their metabolites detected in the GIT of rats over a 24 h period after ingestion of a GSE. Data expressed as µg per organ ± standard error (n = 3)*

Organ	Time	C	EC	B ₁	B ₂	B ₃	B ₄	ECG	PCBG	CGlcUA	ECGlcUA	A	
												MeCGlcUA	MeECGlcUA
Stomach	1 h	1173 ± 43	926 ± 7.2	367 ± 7	549 ± 18	212 ± 4	152 ± 13	265 ± 1	866 ± 3	n.d.	n.d.	n.d.	n.d.
	2 h	1856 ± 99	1265 ± 94	1061 ± 3	782 ± 107	719 ± 6	578 ± 71	724 ± 3	1351 ± 11	n.d.	n.d.	n.d.	n.d.
	3 h	715 ± 89	635 ± 61	357 ± 2	329 ± 52	186 ± 1	140 ± 3	290 ± 13	594 ± 1	n.d.	n.d.	n.d.	n.d.
	4 h	1711 ± 98	1755 ± 96	1819 ± 9	1706 ± 7	749 ± 2	355 ± 8	1503 ± 89	1204 ± 187	n.d.	n.d.	n.d.	n.d.
	6 h	1468 ± 10	1120 ± 75	612 ± 3	517 ± 1	338.3	279 ± 10	694 ± 3	121 ± 64	n.d.	n.d.	n.d.	n.d.
	12 h	515 ± 53	313 ± 48	109 ± 11	103 ± 21	68 ± 4	56 ± 9	256 ± 21	266 ± 34	n.d.	n.d.	n.d.	n.d.
Duodenum/jejunum	24 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1 h	119 ± 1	73 ± 3	14 ± 1	22 ± 1	18 ± 1	11 ± 1	21 ± 8	21 ± 4	3 ± 0	n.d.	n.d.	n.d.
	2 h	35 ± 2	26 ± 1	42 ± 1	16 ± 6	7 ± 1	7 ± 1	26 ± 5	13 ± 3	7 ± 0	6 ± 1	n.d.	n.d.
	3 h	46 ± 1	29 ± 0	16 ± 1	6 ± 1	5 ± 1	5 ± 1	7 ± 1	n.d.	9 ± 1	n.d.	n.d.	n.d.
	4 h	71 ± 2	45 ± 1	6 ± 3	7 ± 0	9 ± 1	5 ± 0	12 ± 4	11 ± 1	1 ± 1	n.d.	n.d.	n.d.
	6 h	45 ± 18	30 ± 11	6 ± 3	4 ± 0	5 ± 0	3 ± 0	7 ± 2	n.d.	n.d.	3 ± 0	n.d.	2 ± 1
Ileum	12 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	24 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1 h	208 ± 2	156 ± 7	91 ± 3	73 ± 2	49 ± 2	31 ± 2	26 ± 4	97 ± 1	8 ± 1	8 ± 0	30 ± 1	33 ± 1
	2 h	196 ± 1	142 ± 8	82 ± 1	73 ± 6	44 ± 1	30 ± 1	21 ± 2	83 ± 4	8 ± 0	6 ± 1	21 ± 2	26 ± 0
	3 h	204 ± 4	141 ± 3	72 ± 1	30 ± 0	37 ± 3	69 ± 1	20 ± 3	79 ± 3	7 ± 4	n.d.	33 ± 2	36 ± 2
	4 h	271 ± 10	188 ± 3	96 ± 3	79 ± 1	41 ± 1	40 ± 4	41 ± 25	117 ± 3	n.d.	n.d.	15 ± 2	n.d.
Caecum	6 h	178 ± 8	121 ± 6	79 ± 1	60 ± 2	41 ± 1	29 ± 2	34 ± 10	96 ± 2	11 ± 1	n.d.	46 ± 4	23 ± 2
	12 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	24 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2 h	58 ± 3	66 ± 7	29 ± 1	18 ± 2	17 ± 1	44 ± 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	3 h	122 ± 1	245 ± 3	156 ± 6	40 ± 2	23 ± 1	239 ± 12	n.d.	47 ± 1	n.d.	n.d.	n.d.	n.d.
Colon	4 h	437 ± 81	271 ± 20	339 ± 3	307 ± 25	56 ± 5	88 ± 3	n.d.	396 ± 4	n.d.	n.d.	n.d.	n.d.
	6 h	761 ± 6	428 ± 45	443 ± 2	394 ± 39	137 ± 6	188 ± 7	n.d.	489 ± 37	n.d.	n.d.	n.d.	n.d.
	12 h	n.d.	n.d.	67 ± 4	n.d.	11 ± 1	64 ± 9	n.d.	105 ± 36	n.d.	n.d.	n.d.	n.d.
	24 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
glucuronide; ECglcUA - (-)-epicatechin glucuronide; MeCGlcUA - methyl-(+)-catechin glucuronide;	3 h	78 ± 1	106 ± 5	56 ± 1	80 ± 2	35 ± 1	150 ± 40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	4 h	129 ± 4	69 ± 1	66 ± 1	63 ± 1	16 ± 0	21 ± 1	n.d.	95 ± 1	n.d.	n.d.	n.d.	n.d.
	6 h	120 ± 3	79 ± 19	66 ± 9	69 ± 9	22 ± 1	32 ± 1	n.d.	76 ± 10	n.d.	n.d.	n.d.	n.d.
	12 h	n.d.	36 ± 2	93 ± 0	92 ± 3	20 ± 1	32 ± 3	n.d.	84 ± 3	n.d.	n.d.	n.d.	n.d.
	24 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

*C - (+)-catechin; Ec - (-)-epicatechin; B1, B2, B3, B4 - procyanidin dimers; ECG - (-)-epicatechin gallate; PCBG - procyanidin B₁-3- or B₂-3'-O-gallate; CGlcUA - (+)-catechin glucuronide; ECglcUA - (-)-epicatechin glucuronide; MeCGlcUA - methyl-(+)-catechin glucuronide; MeECGlcUA - methyl-(-)-epicatechin glucuronide.

5.5.5 Analysis of flavan-3-ol metabolites in the the liver, kidneys and brain

HPLC-MS-MS analyses, as described above, identified glucuronidated and methylated metabolites of (-)-epicatechin and (+)-catechin in liver extracts collected 1 and 4 h after ingestion of the GSE. The methylated conjugates were the major metabolites (Table 5.7). None of the metabolites were present in subsequent liver samples collected 6, 12 and 24 after ingestion. The levels of metabolites detected in the kidney were low reaching a maximum by 4 h post-ingestion with 134 and 28 μg per total catechin and epicatechin ingested, respectively. In the liver, a number of compounds were present from 1 h after the grape seed extract. In the present study no metabolites could be detected in the brain using either full scan MS or SRM for glucuronides, methyl glucuronides or sulphates at any time point following ingestion of the GSE.

5.5.6 Metabolites identified in the faeces

HPLC-MS-MS with SRM detected trace non-quantifiable amounts of (+)-catechin glucuronide, (-)-epicatechin glucuronide and their methyl derivatives in the occasional faecal sample collected over a 24 h period after GSE ingestion. They were, however, not detected in the majority of samples that were analysed.

Table 5.7 Levels of flavan-3-ol metabolites in liver and kidney of rats over a 24 h period after the ingestion of a GSE.

Organ	Time (h)	CGlcUA	ECGlcUA	MeCGlacUA	MeECGlcUA
Liver	1	4.3 ± 01	2.5 ± 2.0	57 ± 1.3	21 ± 0.1
	2	n.a.	n.a.	n.a.	n.a.
	3	n.a.	n.a.	n.a.	n.a.
	4	2.3 ± 0.1	1.3 ± 1.0	45 ± 10	20 ± 0.9
	6	n.d.	n.d.	n.d.	n.d.
	12	n.d.	n.d.	n.d.	n.d.
	24	n.d.	n.d.	n.d.	n.d.
Kidney	1	n.d.	n.d.	n.d.	n.d.
	2	7.6 ± 0.1	4.1 ± 0.2	87 ± 0.1	9.1 ± 0.3
	3	2.7 ± 0.2	1.2 ± 0.3	8.4 ± 0.4	2.7 ± 0.4
	4	3.1 ± 0.2	2.1 ± 0	131 ± 1.0	26.3 ± 2.5
	6	2.7 ± 0.6	2.3 ± 0.1	58 ± 0.3	8.2 ± 0.3
	12	n.d.	n.d.	n.d.	n.d.
	24	n.d.	n.d.	n.d.	n.d.

Results are expressed as μg per organ \pm standard error, where $n = 3$. CGlcUA-(+) catechin glucuronide; ECGlcUA-(-)-epicatechin glucuronide; MeCGlcUA-methyl catechin glucuronide; MeECGlcUA-methyl-epicatechin glucuronide; n.a.—not analysed; n.d. - not detected.

5.6 Discussion

In the present study rats were fed a single acute dose of a flavan-3-ol rich GSE. The mean amounts fed to each rat by gavage were 5.8 mg of (+)-catechin, 3.6 mg of (-)-epicatechin, 4.3 mg of procyanidin dimers, 1.3 mg of trimers and 0.7 mg of tetramers (Table 5.2). This dose is equivalent to ca. 700 mg GSE based on a 1g/kg body weight basis for a 70 kg human. In terms of obtaining this amount in the diet, three glasses of the red wine used in this study (Chapter 4) would provide ca. 600-700 mg of grape derived polyphenols. This amount is unlikely to be consumed in a typical diet and other dietary sources are likely to provide additional sources. In addition, the GSE contained a substantial, but undetermined, quantity of high molecular weight polymeric procyanidins. Relatively high levels of the various components in the GSE remained in the stomach for 6 h, declined by 12 h and had disappeared after 24 h. Small amounts of GSE phenolics appeared in the duodenum/jejunum and to a greater extent the ileum after 1 h and in both instances had disappeared by 12 h. The phenolics were detected in the caecum 2 h after ingestion and only trace amounts remained after 12 h. Smaller amounts were found in the colon 3-12 h after gavage. The vast majority of compounds detected in the GI tract were the original GSE flavan-3-ols with only trace levels of four metabolites occurring in the duodenum/jejunum and ileum. The data presented in Table 5.6 do not show either a sizable increase in either (+)-catechin or (-)-epicatechin or a concomitant decrease in the relative amounts of dimers which supports the view that oligomeric proanthocyanidins are not depolymerised into monomeric flavan-3-ols to any extent, if at all, during passage through the stomach and GI tract (Donovan *et al.*, 2002; Rios *et al.*, 2002; Nakamura and Tonogai, 2003). HPLC retention data and MS² fragmentation patterns indicated that the trace levels of flavan-3-ol metabolites appearing in the GI tract were a (+)-catechin glucuronide and a methyl-(+)-catechin glucuronide together with the corresponding (-)-epicatechin derivatives which in view of earlier studies with rats (Natsume *et al.*, 2003) were tentatively identified as (-)-epicatechin-7-*O*-glucuronide and 3'-*O*-methyl(-)-epicatechin-7-*O*-glucuronide (Table 5.3). The fate of the GSE flavan-3-ols in the GI tract of rats is, therefore, very different to that of quercetin-4'-glucoside which undergoes very rapid conversion to a mixture of glucuronide, methyl and sulphated metabolites with only 25 % of the parent glycoside remaining after one hour (Mullen *et al.*, 2002b). In marked contrast to the GI tract

where the parent GSE flavan-3-ols were the major components, the circulatory system contained only the four previously mentioned flavan-3-ol metabolites in detectable quantities. Three hours after ingestion a peak plasma concentration of ca. 35 $\mu\text{mole/L}$ was attained with the (+)-catechin and (-)-epicatechin glucuronides with their methylated derivatives present at 2-3 fold lower levels (Figure 5.4). The peak plasma concentrations of the two (+)-catechin metabolites corresponds to ca. 3 % of the (+)-catechin ingested and the (-)-epicatechin metabolites equate with ca. 4 % of intake. There was a statistically significant increase in the plasma antioxidant capacity at 2 and 4 h post-ingestion of the GSE (Fig. 5.4b). However, this increase did not coincide with the peak plasma concentration of metabolites at 3 h. A previous study with rats has shown an increase in the plasma antioxidant capacity following ingestion of (-)-epicatechin and quercetin. The major circulatory compounds in the plasma were conjugated derivatives indicating that they may play a role in the antioxidant defences of plasma (Terao, 1999). The apparent discrepancy observed in the present study may simply have been due to inter-individual differences between each rat. The levels of the metabolites in the bloodstream were far greater than the amounts present in GI tract. This is in keeping with the methylation and glucuronidation of (+)-catechin and (-)-epicatechin occurring on the luminal side of the endoplasmic reticulum of the small intestine during transport into the blood stream (Kuhnle *et al.*, 2000; Donovan *et al.*, 2001). The small quantities of metabolites in the GI tract could be due to either low level efflux back into the lumen of the intestine or enterohepatic recirculation via the bile. Trace levels of the methylated and glucuronidated flavon-3-ol metabolites were also detected in liver and kidney extracts (Table 5.3) but they were excreted in much higher amounts in urine along with four sulphated metabolites, namely (+)-catechin sulphate, that were not present in either the GI tract, the liver and kidneys or the circulatory system. In contrast to other studies, no free (+)-catechin or (-)-epicatechin were detected outside the GI tract, although trace quantities of the procyanidin B₁, B₂, B₃ and B₄ dimers and the C₂ trimer were detected in urine (Figure 5.6, Table 5.5). The levels of (+)-catechin and (-)-epicatechin metabolites excreted relative to the quantity of the monomers ingested were 27 and 36 %, respectively, after 24 h. This is similar to the 37 % urinary excretion reported to occur after feeding (-)-epicatechin to rats (Donovan *et al.*, 2002). This provides further, albeit indirect, evidence that the procyanidin oligomers in the GSE were not depolymerised to monomers to any extent after

ingestion. If this had occurred the level of recovery of the monomeric metabolites in urine is likely to have been substantially higher than the 37 % obtained by Donovan *et al.* (2002) after feeding rats with (-)-epicatechin. The ca. 30 % recovery of the ingested (+)-catechin and (-)-epicatechin as metabolites in urine is well in excess of comparable figures of ca. 2-3 % and 0.1 % that are typically obtained with flavonols and anthocyanins, respectively (Prior *et al.*, 2003). However, this still leaves 70 % of the ingested monomer unaccounted for and although seemingly not absorbed to any extent, only miniscule small amounts of the procyanidins in the GSE reach the colon (Table 5.6) and are excreted in faeces. The most likely fate of these compounds is that they are converted to low molecular weight phenolic acids (Déprez *et al.*, 2000; Gonthier *et al.*, 2003a) most notably 3-hydroxyphenylpropionic acid (Ward *et al.*, 2004). These compounds were not analysed in the current study. They have a low extinction coefficient and a λ_{max} below 250 nm and as a result are not readily detected with a diode array detector and, in addition, they do not ionize readily when subjected to MS with an electrospray interface. Although HPLC-MS with an electrospray interface has been used to analyse phenolic acid (Gonthier *et al.*, 2003b) the method lacks the sensitivity it exhibits with many flavonoids so quantitative analyses has to be based on selected reaction monitoring rather than full scan MS (Gonthier *et al.*, 2003c). The method of choice of many investigators when analyzing putative phenolic acid catabolites is gas chromatography-mass spectrometry with electron impact ionization (Rechner *et al.*, 2002; Olthof *et al.*, 2003). Moreover, despite a thorough search by HPLC-MS-MS no metabolites could be identified in the brain in either full scan or SRM. El Mohsen *et al.* (2002) reported the presence of (-)-epicatechin glucuronide and 3-*O*-methyl epicatechin glucuronide in rat brain extracts following the ingestion of (-)-epicatechin. In this study rats were fed by gavage a dose of 100 mg/per kg body weight, which is ca. 20 mg (-)-epicatechin ingested per rat. This is vastly higher (ca. 5-fold) than the dose of 3.6 mg (-)-epicatechin ingested by each rat in the present study, and therefore it is likely that the reason we failed to detect any such metabolites in the brain was simply due to the differences in the dose supplemented.

5.7 Conclusion

In summary, the present investigation indicates that flavan-3-ol monomers, oligomers and polymers can be directly absorbed into the bloodstream while the monomers are metabolised into glucuronidated and methylated conjugates, which may contribute to an increase in the antioxidant capacity of plasma. This study provides further, albeit indirect, evidence that the procyanidin oligomers in the GSE were not depolymerised to monomers to any extent after ingestion. Their cardioprotective effects stem from the ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species. Further investigation of the metabolism of these phytochemicals is justified to extend structure-activity relationships to preventive and therapeutic nutritional strategies.

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Chapter 6 General Discussion

The research presented in this thesis has reported on a series of investigations undertaken in an effort to determine the antioxidant activity, protective effects and absorption of phenolic and polyphenolic compounds in red wine. This work has identified and quantified the major antioxidant phenolics in red wine and has shown that some of these phenolics, namely the flavan-3-ols are bioavailable in humans following a moderate and regular consumption of red wine which may contribute to some of the protective effects observed. (+)-Catechin, (-)-epicatechin, procyanidin dimers (B₁, B₂, B₃ and B₄) and trimers (C₂ and an unknown trimer) are directly absorbed into the bloodstream from the GI tract in rats. Conjugated derivatives of the monomers are present in detectable amounts in the plasma, urine, liver and kidney and to a lesser extent in the proximal small intestine. The oligomers do not appear to be depolymerised in the GI tract releasing monomers however their metabolic fate remains undetermined.

6.1 Identification of the major antioxidants in red wine

In the present investigation red wines were analysed for their phenolic content and antioxidant activity. The combination of three different techniques HPLC, MS² and HPLC with on-line detection of antioxidant activity enabled the identification of the major phenolics in red wines together with their individual contribution to the total antioxidant activity (Chapter 3). Extensive research has focused on the identification of the active components in red wine; however the isolation of these compounds has been met with little success as existing techniques have been unable to fully separate and isolate single phenolic compounds in such complex mixtures. Many studies have focused on separating phenolics in red wine using solid phase and liquid-liquid extraction procedures. However, this approach lacks specificity due to the differences in polarity between different phenolics within a group and as such previous studies have reported inconsistent findings. Large-scale preparative HPLC was initially used to separate wine into 60 aliquots, although it did not allow for the complete separation of red wine components the results indicate that increasing antioxidant activity was highly and significantly correlated with increasing total phenolic and catechin content of each

wine fraction. Recently sensitive and reliable on-line methods of analysing radical scavenging activity have been developed (Dapkevicius *et al.*, 2001, Koleva *et al.*, 2001). Such methods require a stable model free radical system such as 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS⁺) with radical scavenging activity assessed in comparison to the water-soluble synthetic vitamin E derivative Trolox. On-line assessment of antioxidant activity allows complex mixtures to be separated by HPLC and the antioxidant contribution of individual components can be evaluated. In summary the present study has demonstrated for the first time the identification and quantification of the major phenolic contributors to the total antioxidant activity in red wine. Red wine is a rich source of antioxidants and gallic acid, (+)-catechin, (-)-epicatechin and procyanidin dimers B1 and B2 contributed to the antioxidant potential. The findings, however, should be taken within the context of this study and are not representative of red wines in general as variations in grape type, vintage and vinification methods are known to influence the levels of phenolics.

6.2 Protective effects of red wine

In this study daily moderate consumption of an antioxidant rich red wine for 2-weeks in healthy volunteers was associated with a small but significant increase in the plasma total phenolic concentration. Trace levels of flavan-3-ol metabolites, mainly glucuronides and methyl glucuronides of (+)-catechin and (-)-epicatechin were detected in the plasma of the red wine group indicating that these phenolics are absorbed from the GI tract (Chapter 4). The flavan-3-ols; (+)-catechin, (-)-epicatechin, procyanidin dimers B1 and B2 were identified as the major *in vitro* antioxidants in this red wine (Chapter 3). The findings from the present study confirm and extend previous reports that some phenolic compounds in red wine are absorbed and metabolised in the human body. These metabolites may contribute to protective effects such as raising HDL cholesterol concentrations and enhancing the resistance of LDL to withstand oxidative modification. It is possible that other phenolics and/or metabolites were present in the plasma that were undetected or may have been present in levels which were below the limits of detection. The possible health effects of red wine are therefore likely to be due

to the cumulative effects of phenolic compounds (Serafini *et al.*, 1998) and alcohol working in synergy (Duthie *et al.*, 1998).

6.3 Absorption, metabolism & excretion of flavan-3-ols in rats

In the present investigation glucuronidated, methylated and sulphated metabolites of (+)-catechin and (-)-epicatechin were exclusively detected in the plasma and urine, with only trace amounts of metabolites occurring in the duodenum/jejunum and ileum of rats following the ingestion of a GSE. (-)-Epicatechin has been shown to be modified on absorption during transfer across the small intestine (Kuhnle *et al.*, 2000). UDP-glucuronosyl transferase has been found in the mucosa of the upper half of the small intestine, caecum and upper half of the large intestine (Piskula & Terao, 1998) confirming that conjugation of flavan-3-ols can take place in the intestine. The fate of GSE flavan-3-ols in the GI tract are in contrast with the findings of Mullen *et al.* (2002), whereby quercetin-4'-glucoside was very rapidly converted to a mixture of glucuronide, methyl and sulphated metabolites with only 25 % of the parent glycoside remaining after one hour. Methylation and glucuronidation of (+)-catechin and (-)-epicatechin occurs on the luminal side of the endoplasmic reticulum of the small intestine during transport into the blood stream (Donovan *et al.*, 2001). Therefore the small quantities of metabolites in the GI tract in the present study indicate either low level efflux back into the lumen of the intestine or enterohepatic recirculation via the bile. Trace quantities of the procyanidin B₁, B₂, B₃ and B₄ dimers and the C₂ trimer were detected in urine suggesting that these compounds have limited bioavailability. The levels of (+)-catechin and (-)-epicatechin metabolites, excreted relative to the quantity of the monomers ingested, were 27 and 36 %, respectively after 24 h in agreement with Donovan *et al.* (2002). This provides further, albeit indirect, evidence that the procyanidin oligomers in the GSE were not depolymerised to monomeric flavan-3-ols to any extent, if at all, during passage through the stomach and GI tract.

6.4 Future Research

Research into the alleged health benefits of phenolic and polyphenolic compounds continues to have considerable potential however many questions remain to be answered. Extending the work in this thesis ileostomy studies would provide valuable information on the absorption and/or modification of phenolics occurring in the small intestine following red wine consumption. This information could then be related to the levels of phenolics detected in plasma and urine giving an indication of the extent of metabolism and sequestration within the human body. The fate of compounds escaping digestion in the stomach and small intestine is little understood and future work should investigate the role of colonic bacteria in this regard. Incubation of red wine phenolics with human faecal samples and subsequent analysis of the metabolites by LC-MS² would provide further insight into the nature of these compounds. Moreover, the biological importance of the conjugated derivatives and microbial metabolites of polyphenols should be evaluated and future *in vitro* studies should focus on the activity of these compounds instead of the parent compound.

Finally, any health benefit arising from red wine must be considered within the context of the adverse effects of excessive alcohol consumption, and should not be actively promoted. A balanced diet rich in fruits and vegetables and low in saturated fat is recommended for good health supplemented by moderate consumption of red wine, green tea and dark chocolate, according to taste.

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Appendices

Appendix I:

Information Sheet (Chapter 4)

Title of study:

The effect of red wine consumption on the susceptibility of plasma and low-density lipoprotein (LDL) to oxidative modification in healthy volunteers.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read this information very carefully and discuss with friends and family if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide to take part in this research study, you should understand enough about the risks and benefits to make an informed judgement. This process is known as informed consent. Once you understand about the study, you will be asked to sign a consent form if you wish to participate.

What is the purpose of this study?

A number of large studies have shown that a moderate alcohol intake may be protective against the death from coronary heart disease (CHD). There is evidence that red wine can offer a greater protection than white wine, beer or spirits. The protective effects have been attributed to substances known as phenolic compounds contained in red wine. It is believed that compounds in red wine act as antioxidants. Antioxidants protect the body from damaging oxidation reactions caused by 'free radicals'. The oxidation of low-density lipoproteins (a protein which carries cholesterol in the blood) is one such damaging reaction. When LDL becomes oxidised or damaged by 'free radicals' it can result in a series of steps that are believed to be involved in the development of atherosclerosis which can lead to CHD. Phenolic compounds in red wine are mainly from grapes and, in particular, the skins and seeds which are removed during the procedure to make white wine. There are large variations in the phenolic contents of

different wines depending on the grape, variety, climatic conditions in which the grapes are grown and the wine-making techniques.

The purpose of this study is to find out if the consumption of red wines that have been shown to be rich in antioxidant phenolic compounds can influence indicators of oxidation in the blood.

What will it involve?

You will be involved in the study for approximately 4 weeks and we hope to start the study in June 2001. Prior to starting the study we will ask you to complete a questionnaire called a food frequency questionnaire so we can assess your usual intake of antioxidant rich foods such as fruits, vegetables, tea and wine. During the study you will be asked to follow your usual eating habits but to avoid phenolic rich foods/beverages and all alcohol. You will be required to give fasting blood samples before and after the wine-drinking period. You will also be required to fast overnight before giving blood. This means not having anything to eat or drink from about 8 pm the evening before. The blood samples will be taken by a qualified nurse in the morning.

Can you tell me more about the wine?

Moderate intake of wine has been shown in a number of large studies to be protective against CHD. In fact, a consumption of a moderate amount of wine is recommended as part of a healthy, balanced diet. In this study, volunteers will be asked to drink 2-3 glasses of red wine every day for two weeks. The wine used in this study will be commercially available.

What are the benefits in taking part in this study?

CHD is a major cause of death in Scotland. Diet is believed to play an important role in the development of this disease. This study aims to increase understanding of the manner in which dietary constituents affects our overall dietary intake in relation to heart disease risk. The findings of the study will be published in the scientific and medical literature so that understanding of the ways in which diet can influence the risk of CHD can be increased.

Are there any risks involved?

Taking blood samples can cause minor bruising. Very rarely it may cause inflammation of the vein and possible infection. A qualified and experienced nurse will be employed to take the blood samples and they will make every effort to avoid this happening. Drinking 2-3 glasses of wine will cause drowsiness and may impair your judgement and therefore, you should not drive, cycle or operate mechanical instruments afterwards. To take part in this study, it is important that you are healthy and not suffering from any illnesses such as CHD, cancer or diabetes or not taking any medication (other than the contraceptive pill). As alcohol consumption is not recommended for pregnant women, you should not take part in the study if you are pregnant. Other exclusion criteria for the study are: taking antioxidant/nutrition supplements and excessive alcohol consumption which means drinking more than 21 unites of alcohol per week for men and drinking more than 14 units for women (1 unit=half pint beer, small glass of wine/sherry, one pub measure of spirits).

How inconvenient will the study be to me?

Giving blood samples may be inconvenient to you. You will be required to come to the Department of Human Nutrition on two occasions to give a blood sample. You will also be required to fast overnight before giving blood. This means not having anything to eat or drink from about 8 pm the evening before. The blood sample will be taken by a qualified nurse in the morning.

Confidentiality

Although information will be stored by computer, each subject will be entered as a number rather than by name and will not be identifiable. This is in accordance with the data protection act.

Do I have to take part in this study?

Your decision to take part in this study is entirely voluntary. You may leave the study at any time. If you have any questions about the study you may contact Dr Siobhan Higgins, University Department of Human Nutrition, University of Glasgow, Yorkhill NHS Trust Hospital on: (0141) 201 0768.

Statement of Informed Consent

The effect of red wine consumption on the susceptibility of plasma and low-density lipoprotein to (LDL) oxidative modification in healthy volunteers.

I, (Name).....

Of (Address).....

.....

.....

agree to take part in this research project described above.

Dr/Mr/Mrs/Miss/Ms.....has explained to me what I have to do, how it might affect me and the purpose of the research project.

Signature of subject:..... Date:.....

Signature of witness:..... Date:.....

**Red wine Intervention Study
Food Intake Record**

An example of food intake recorded over 3 days of the week (Chapter 4).

	Time	Food description	Estimated portion size (see handout)
Wed 2/05/01	07.30	Rice krispies/ semi-skimmed milk	1B
	10.30	Banana, glass water	
	12.30	2x wholemeal bread Houmous Diet Irn-Bru	9B
	18.30	Mushroom and mascarpone pasta Pepsi Cola	9B
Thurs 3/05/01	08.30	Rice krispies/ semi-skimmed milk	1B
	12.00	Banana, glass water	
	15.30	Diet Irn-Bru	
	18.00	6x meat free quorn goujons	4B
		2x potato waffles Houmous Pepsi cola	6B
Sun 6/05/01	09.45	Rice krispies/ semi-skimmed milk	1B
	12.00	1x brown bread, spread, honey banana, bean burger meal (no tomatoes), French fries, diet coke	
	14.00	Ice-cream, glass ginger beer	
	19.00	Macaroni cheese, potatoes glass coke	9B,10B

Appendix II Total weight of each rat organ (Chapter 5)

Rat	Time (h)	St	D/J	II	Ca	Co	K	B	L	P*	I†
1	0	1.5	3.0	4.1	6.1	2.7	1.9	1.7	7.3	3.0	0.8
		1.9	5.2	5.8	4.3	4.1	2.1	1.9	7.9	3.0	0.5
2	0	2.1 (5.5)	4.5 (12.6)	4.6 (14.4)	4.0 (14.4)	2.1 (8.8)	2.0 (5.9)	2.0 (5.6)	7.3 (22.5)	4.0 (10.0)	0.7 (2.0)
		5.2	5.1	4.8	5.3	2.6	1.9	2.0	7.2	3.0	
3	1	3.9	4.3	5.2	3.9	3.6	1.9	1.8	7.3	3.0	
		3.9 (13)	4.4 (13.8)	5.3 (15.3)	6.0 (15.2)	5.4 (11.6)	1.8 (5.7)	1.9 (5.7)	6.9 (21.3)	3.0 (9.0)	
4	1	5.5	7.0	6.9	6.8	5.8	2.1	1.9	8.9	4.0	0.9
		5.5	4.9	4.9	4.1	5.4	2.0	1.9	7.6	4.0	0.3
5	2	5.9 (16.9)	6.5 (14.3)	6.5 (18.3)	7.2 (18.1)	6.4 (17.5)	2.1 (6.2)	1.9 (5.7)	9.0 (25.6)	4.0 (12.0)	1.2 (2.4)
		1.9	4.4	4.8	5.1	3.4	1.9	1.9	8.4	5.0	
6	3	3.6	5.3	6.2	4.2	3.8	2.1	1.9	9.4	5.0	0.7
		4.3 (9.9)	4.1 (13.7)	5.5 (16.5)	4.9 (14.3)	4.3 (11.5)	2.1 (6.1)	1.9 (5.7)	8.7 (26.5)	5.0 (15.0)	0.8 (1.5)
7	4	4.7	4.8	5.7	5.2	4.3	1.9	1.9	8.2	5.0	1.1
		5.7	4.1	5.2	3.7	1.8	1.9	1.8	7.8	4.0	3.1
8	4	6.6 (16.9)	6.0 (14.9)	8.4 (19.3)	5.9 (14.8)	3.6 (9.7)	2.0 (5.9)	1.7 (5.4)	8.3 (24.3)	4.0 (13.0)	2.1 (6.3)
		5.7	4.4	5.7	6.3	2.4	2.1	2.0	8.8	4.0	2.0
9	6	2.3	3.5	4.6	4.9	2.7	2.0	1.8	7.7	4.0	0.8 (2.8)
		2.6 (10.6)	4.0 (11.8)	4.0 (14.3)	4.5 (15.6)	3.5 (8.5)	2.0 (6.1)	1.9 (5.7)	8.0 (24.5)	4.0 (12.0)	
10	12	1.7	4.0	4.0	6.1	4.9	1.9	1.9	8.2	5.0	0.6
		2.3	2.9	3.6	5.2	1.8	2.2	1.8	7.7	5.0	1.2
11	12	1.8 (5.7)	4.1 (10.9)	4.2 (11.7)	5.1 (16.4)	3.4 (10.1)	1.9 (6.0)	1.9 (5.6)	8.3 (24.1)	5.0 (15.0)	1.7 (3.5)
		1.8	2.6	2.9	3.0	1.9	2.1	2.0	6.5	4.0	0.8
12	24	2.0	3.0	2.9	1.8	1.3	1.9	1.7	7.2	4.0	1.9
		1.8 (5.6)	3.1 (8.6)	3.7 (9.4)	2.9 (7.7)	2.3 (5.5)	1.9 (5.9)	1.9 (5.6)	7.9 (21.6)	3.0 (11.0)	1.1 (3.8)

Total weight of each organ per rat and in parenthesis the sum of 3 rats. Results expressed in grams and mL*. St-stomach, D/J-duodenum/jejunum, Il-ileum, Ca-caecum, Co-colon, K-kidney, B-brain, L-liver, P-plasma, F-faeces.

Amount of each sample used in the extraction procedure.

Rat	Time (h)	St	D/J	II	Ca	Co	K	B	L	P.	F
1	0	0.8	1.5	1.1	0.9	1.2	0.9	0.9	3.6	1.0	0.8
		0.9	2.9	1.6	0.9	1.1	1.0	1.1	3.0	0.8	0.5
2		1.2 (3.0)	2.5 (6.9)	1.3 (3.9)	0.9 (2.8)	0.8 (3.2)	1.0 (3.0)	1.0 (3.1)	2.7 (9.3)	0.7 (2.5)	0.7 (2.0)
		2.4	3.0	0.9	1.3	0.9	1.0	1.3	1.3	1.3	0.7
3	1	1.9	1.8	1.9	1.0	0.8	0.9	1.1	1.9	0.9	
		1.8 (6.1)	2.2 (7.0)	2.1 (4.9)	1.7 (4.1)	1.9 (3.7)	0.9 (2.9)	1.2 (3.5)	3.0 (6.2)	0.9 (2.5)	
4	2	2.2	3.0	2.1	1.5	1.1	1.2	0.9	2.0	1.8	0.9
		2.2	2.1	16	0.7	1.5	1.0	0.8	2.1	1.6	0.3
5		1.7 (6.1)	2.6 (7.7)	1.3 (5.1)	1.9 (4.1)	2.2 (4.8)	1.0 (3.3)	0.7 (2.4)	3.6 (7.7)	1.6 (5.0)	1.2 (2.4)
		1.5	2.4	1.8	0.9	1.1	1.0	1.0	2.6	1.7	
6	3	1.2	2.0	2.0	1.2	1.3	1.0	0.8	2.7	1.7	0.7
		1.4 (4.1)	1.9 (6.4)	1.9 (5.7)	1.5 (3.7)	0.8 (3.2)	0.9 (3.0)	0.8 (2.6)	2.7 (7.9)	1.3 (4.7)	0.8 (1.5)
7	4	1.1	1.4	1.3	1.0	1.6	0.9	0.9	1.2	2.5	1.1
		1.0	1.5	1.4	1.3	1.0	0.9	1.1	1.9	1.9	3.1
8		1.2 (3.3)	2.3 (5.1)	2.3 (4.9)	0.8 (3.1)	1.4 (4.0)	1.0 (2.9)	1.0 (3.1)	2.5 (5.7)	1.9 (6.3)	2.1 (6.3)
		1.5	2.4	2.4	2.4	1.0	1.0	1.5	0.9	1.2	2.0
9	6	1.0	1.8	1.7	1.3	1.1	1.0	0.9	2.3	1.1	0.8 (2.8)
		1.0	1.8	1.7	1.3	1.1	1.0	0.9	2.3	1.1	0.8 (2.8)
10		0.9 (3.4)	1.3 (5.5)	1.6 (5.7)	0.9 (4.7)	1.5 (3.6)	1.0 (3.0)	0.9 (3.3)	2.5 (5.8)	1.4 (3.7)	
		0.9	1.3	1.9	1.2	1.5	0.9	0.8	3.2	1.8	0.6
11	12	1.0	1.2	1.7	1.5	1.0	1.1	0.7	2.2	1.8	1.2
		0.9 (2.8)	1.7 (4.2)	1.9 (5.4)	1.9 (4.6)	1.3 (3.8)	0.9 (2.9)	0.9 (2.5)	3.5 (8.9)	1.6 (5.2)	1.7 (3.5)
12	24	0.9	1.2	1.6	1.4	0.8	1.0	1.3	2.0	1.5	0.8
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
13		0.8 (2.6)	0.7 (3.1)	1.8 (4.4)	0.3 (3.7)	1.3 (3.1)	1.0 (2.9)	0.8 (2.9)	1.9 (7.3)	0.8 (3.3)	1.1 (3.8)
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
14	24	0.9	1.2	1.6	1.4	0.8	1.0	1.3	2.0	1.5	0.8
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
15		0.8 (2.6)	0.7 (3.1)	1.8 (4.4)	0.3 (3.7)	1.3 (3.1)	1.0 (2.9)	0.8 (2.9)	1.9 (7.3)	0.8 (3.3)	1.1 (3.8)
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
16	6	1.5	2.4	2.4	2.4	1.0	1.0	1.5	0.9	1.2	2.0
		1.0	1.8	1.7	1.3	1.1	1.0	0.9	2.3	1.1	0.8 (2.8)
17		0.9 (3.4)	1.3 (5.5)	1.6 (5.7)	0.9 (4.7)	1.5 (3.6)	1.0 (3.0)	0.9 (3.3)	2.5 (5.8)	1.4 (3.7)	
		0.9	1.3	1.9	1.2	1.5	0.9	0.8	3.2	1.8	0.6
18	12	1.0	1.2	1.7	1.5	1.0	1.1	0.7	2.2	1.8	1.2
		0.9 (2.8)	1.7 (4.2)	1.9 (5.4)	1.9 (4.6)	1.3 (3.8)	0.9 (2.9)	0.9 (2.5)	3.5 (8.9)	1.6 (5.2)	1.7 (3.5)
19	24	0.9	1.2	1.6	1.4	0.8	1.0	1.3	2.0	1.5	0.8
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
20		0.8 (2.6)	0.7 (3.1)	1.8 (4.4)	0.3 (3.7)	1.3 (3.1)	1.0 (2.9)	0.8 (2.9)	1.9 (7.3)	0.8 (3.3)	1.1 (3.8)
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
21	24	0.9	1.2	1.6	1.4	0.8	1.0	1.3	2.0	1.5	0.8
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
22		0.8 (2.6)	0.7 (3.1)	1.8 (4.4)	0.3 (3.7)	1.3 (3.1)	1.0 (2.9)	0.8 (2.9)	1.9 (7.3)	0.8 (3.3)	1.1 (3.8)
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
23	24	0.9	1.2	1.6	1.4	0.8	1.0	1.3	2.0	1.5	0.8
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9
24		0.8 (2.6)	0.7 (3.1)	1.8 (4.4)	0.3 (3.7)	1.3 (3.1)	1.0 (2.9)	0.8 (2.9)	1.9 (7.3)	0.8 (3.3)	1.1 (3.8)
		0.8	1.2	1.0	0.9	0.9	0.9	0.9	3.4	1.0	1.9

Results expressed in grams and mL* per rat and in parenthesis the sum of 3 rats. St-stomach, D/J-duodenum/jejunum, II-ileum, Ca-caecum, Co-colon, K-kidney, B-brain, L-liver, P-plasma, F-faeces.