Phenolic Antioxidants in Red Wine: Content and Activity

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Abstract

The moderate consumption of alcoholic beverages has been associated with protection against the development of coronary heart disease (CHD). While alcohol alone can help prevent CHD through a number of mechanisms, red wine appears to offer protection above and beyond that attributable to alcohol alone. Red wine is a complex fluid containing grape, yeast and wood-derived phenolic compounds, the majority of which have been recognised as potent antioxidants. Phenolics are secondary plant metabolites, characterised by having more than one aromatic ring with at least one hydroxyl group attached to it. Limited information is available on the content and activity of the major phenolic compounds in red wines. The aim of this project is to investigate the relationship between the phenolic content of wines and their antioxidant activity, and to identify the major phenolic contributors to the antioxidant activity of wine.

Many of the phenolic compounds in wine are present in very low levels compared with other wine constituents such as sugars, alcohols and organic acids. With the increasing interest in the nutritional properties of red wines, a range of reversed-phase high performance liquid chromatography methods were developed which provided accurate information on the identity and concentration of the major phenolics in red wines. Methods were developed for the analysis of flavonoids and non-flavonoids. The flavonoid family includes the flavonols, myricetin, quercetin, kaempferol and isorhamnetin, which exist as both aglycones and as sugar conjugates; the flavan-3-ols (+)-catechin and (-)-epicatechin; and the anthocyanins such as malvidin-3-glucoside. The non-flavonoids encompass gallic acid; hydroxycinnamates, including *p*-coumaric, caffeic and caftaric acids; and the stilbenes, *trans*-resveratrol, *cis*-resveratrol and *trans*-resveratrol-*O*-β-glucoside. The approach taken was to develop a number of short selective isocratic runs, specific to the compound(s) of interest, rather than the general long gradient approach used widely in the literature. Solvents, columns, detector systems were all

optimised for each family of phenolics analysed. A range of detector systems was employed including UV absorbance, fluorescence and mass spectroscopy.

These sensitive and selective methods were applied to the analysis two batches of bottled wines. The relationship between the antioxidant activity, based on the reduction in Fremy's radical, vasodilation activity (batch I only), and phenolic content was investigated. Wines were selected to provide a range of origins, grape varieties and vinification methods. Batch I wines were sourced mainly from the Old World, while those in batch II were predominately from the New World. The total phenolic content was determined by the Folin-Ciocalteu colorimetric assay and by the cumulative measurements obtained by HPLC. Total anthocyanins were determined using a spectral assay. While the wines exhibited a wide range in values in all parameters, with both batches the total phenol content, determined by both the Folin-Ciocalteu assay and HPLC, was very closely correlated with the ESR-derived antioxidant activity. Likewise a strong correlation was noted between the phenolic content and the vasodilation activity of Batch I wines.

The antioxidant activity of Batch I wines was significantly correlated with the gallic acid, total stilbene and total flavan-3-ol content. Similarly with Batch II wines, gallic acid and total flavan-3-ols, along with polymeric pigments were significantly correlated with the ESR-derived antioxidant activity.

Batch I and II had significantly different phenolic profiles although in both cases the flavan-3-ols and anthocyanins were quantitatively the major skin-derived phenolics present. However, in thirteen of the sixteen Batch I wines the major phenolics present were the flavan-3-ols compared with only five of the twenty-two Batch II wines. This discrepancy may be attributed to the viticultural practices of the Southern Hemisphere where many of these wines originated. The climate allows the grapes to be fully ripe prior to picking and hence have a greater anthocyanin content.

In a further effort to identify the major determinants of the antioxidant activity in wine, the extraction of phenolics during vinification was studied. Four wines were followed during days 2-9 of vinification. Individual phenolic compounds were quantified by HPLC and the ESR-derived antioxidant activity was determined. The extraction of the phenolics was found to be influenced by vinification procedure, grape quality and grape variety. However it was observed that although after nine days the fermenting wines reached a Folin-Ciocalteu total phenolic content comparable to a bottled wine, the antioxidant activity was significantly lower than a finished wine. This suggests that the antioxidant activity of a wine may be due to the presence of the larger complexes and condensation products that appear as it ages.

A novel fractionation approach was employed to further investigate the identity of the major antioxidants in red wines. Wine phenolics were separated using a preparative HPLC column and fractionated into sixty aliquots. The ESR-derived antioxidant activity of each fraction was determined along with the identity of the major phenolics in each fraction. Fractions 5 and 6 were noted to have a significantly higher antioxidant activity compared with the other fractions. These fractions contained two major compounds. While one compound remains unknown, the second compound has been identified as a procyanidin, possibly B_3 or B_4, however further work is necessary to elucidate the exact structure of the compound.

These results suggest that the catechin based condensed tannins, namely the procyanidins, may be responsible for the antioxidant activity of red wines. This offers the possibility of producing wines with increased levels of procyanidins, which may, if absorbed, offer enhanced health benefits.

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Special thanks to my family for their continual love and support, and last but not least, to Jules for putting life into perspective.

This is for you.

Author's Declaration

The composition of this thesis and the work described within it was carried out entirely by myself unless otherwise cited or acknowledged. Its contents have not previously been submitted for any other degree. The research for this thesis was carried out between October 1997 and September 2000.

Signed.....

Jennifer Burns

October 2000

Definitions

AAPH	2,2´-azobis(2-amidinopropane)dihydrochloride
ABTS	2,2´-azobis-(3-ethylbenzothiazoline-6-sulphonic acid)
ACN	Acetonitrile
a.m.u.	Atomic mass unit
CHD	Coronary heart disease
CHS	Chalcone synthase
CCRC	cumulative concentration-dependent response curves
cAMP	cyclic Adenosine monophosphate
cGMP	cyclic Guanosine monophosphate
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ESR	Electron-spin resonance
FRAP	Ferric reducing antioxidant potential
GAE	Gallic acid equivalents
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
LDL	Low-density lipoprotein
MeOH	Methanol
MS	Mass spectrometry
n.d.	Not detected
NMR	Nuclear magnetic resonance
NO	Nitric oxide
OD	Optical density
PAL	Phenylalanine-ammonia lyase
PDA	Photo diode-array
PE	Phenylephrine
PVPP	Polyvinylpolypyrrolidone
SD	Standard deviation
SEM	Standard error of the mean
SS	Stilbene synthase
TEAC	Trolox equivalent antioxidant capacity
TFA	Trifluroacetic acid
TLC	Thin-layer chromatography
UV	ultra-violet
λ max	Wavelength of maximum absorption

Aims of Study

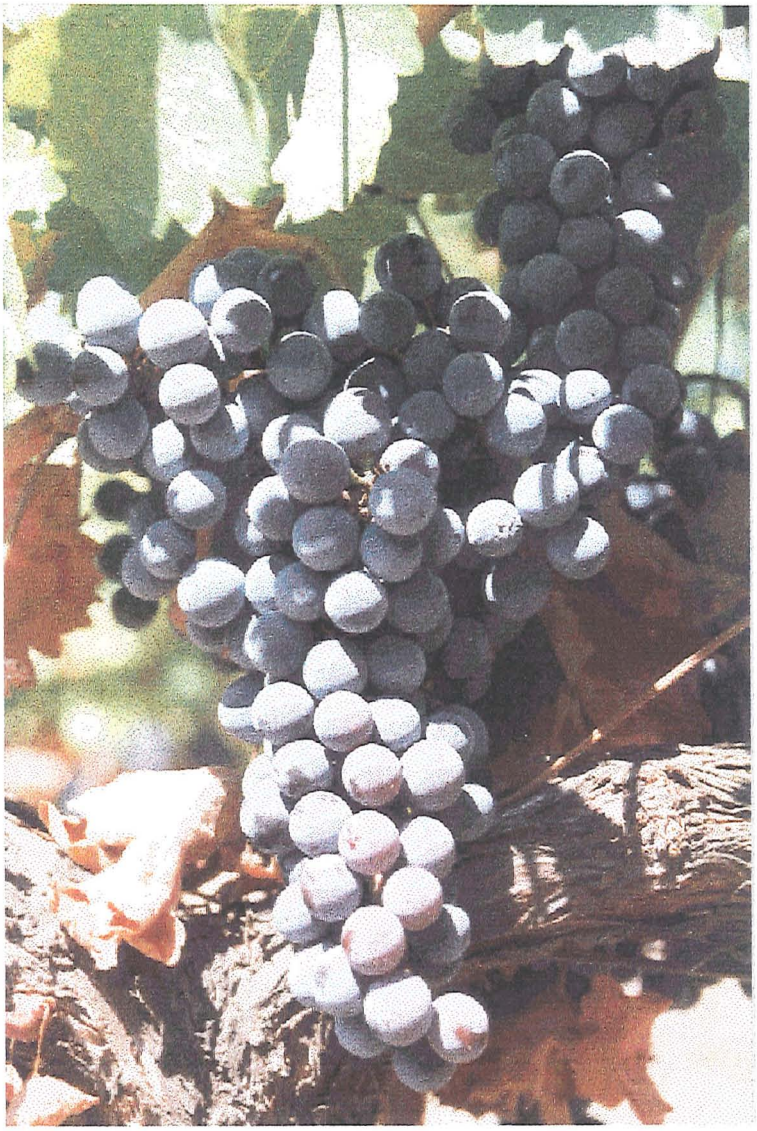
Wine has been a part of the diet of many people for thousands of years. Recent evidence has suggested that the consumption of red wine can protect against the development of coronary heart disease. This protection is attributed to the phenolic content of red wine. The identity of the major contributors to this protective effect has remained in question.

This study set out to achieve the following aims

1. To develop sensitive and selective high performance liquid chromatography methods to allow the accurate quantification of a range of phenolics in grapes, musts and wines.

2. To quantify the levels of the major phenolics in finished red wines and investigate the relationship between the phenolic content, ESR-derived antioxidant capacity and vasodilation capacity of red wine.

3. To determine the major contributors to the antioxidant activity of wine by investigating the extraction of phenolics during vinification and by fractionating wine.



"Nothing more excellent than wine was ever granted man by God."

Plato

Chapter 1 Introduction

Grapevine belongs to the botanical family *Vitaceae*, comprising 12 or 14 genus, the exact number of which remains under discussion. It is the *Vitis* genus and its 700 species that are of interest to the winemaker, particularly *Vitis vinifera*. *Vitis vinifera* originated south of the Black Sea in Transcaucasis, where it was also cultivated. The first recorded mention of vine growing is found in the Bible (Genesis IX, xviii). After weathering the storms, Noah landed the Ark on the slopes of Mount Ararat, in modern day Turkey. Venturing out he "began to be a husbandman, and he planted a vineyard: and he drank of the wine, and was drunken." Just to settle his nerves naturally. This over indulgence appeared to have done no harm and he was reported to live for another 200 years. The Bible is littered with references to wine in all its guises, as an intoxicant, a gift and also for its ability to appear miraculously at a party. Historically it has been associated with celebrating and raising, if not soothing, the spirit. Indeed Hippocrates, the father of medicine, used wine as an integral part of his remedies, recommending it as a diuretic and as a general antiseptic.

From its first reported origins in the region south of the Caucasus Mountains, vine growing and wine making spread down into Egypt. Although not enjoyed by the general public, the royal priests made use of its mystical and healing properties. A number of items of pottery remain which depict vine growing and the effects of imbibing the product. As a result of the extensive travels of the Phoenicians and the Romans, grape growing and winemaking spread swiftly through mainland Europe. Through the 16th, 17th and 18th centuries *V. vinifera* was transported to South America, South Africa, Australia and New Zealand. Its introduction to the New World was due to the travels of Catholic missionaries who required a steady supply of wine for their religious ceremonies (Johnston, 1989). In modern times wine has had mixed fortunes. In the Middle Ages the puritans viewed it with suspicion, and even in the early twentieth century prohibition in the USA outlawed its consumption. However in recent years wine has increased in popularity, due in part to the publicity

concerning its beneficial effects on health. The increasing popularity of red wine has been attributed to a phenomenon called the "French Paradox". A multicultural study of dietary fat highlighted the apparent ability of red wine consumption to offer a form of protection against the development of coronary heart disease (St Leger et al., 1997).

Wines come in many guises. They can be red, pink or white, and range from light to robust in style. Many of these characteristics are due to the presence of phenolic compounds. It is the action of these phenolics that is purported to be responsible for the proposed health benefits of red wine.

1.1 Introduction to phenolics

Phenolics are secondary plant metabolites, characterized by having at least one aromatic ring with more than one hydroxyl group attached. With in excess of 8000 phenolic structures reported, they are widely dispersed throughout the plant kingdom. The nature and distribution of phenolics can vary depending on the plant tissue, with many of the phenolics synthesized from carbohydrates via the shikimate pathway (Fig. 1.1 and Table 1.1). This is the pathway responsible for the biosynthesis of the aromatic amino acids (Strack, 1997). These have been termed the 'essential' amino acids as the shikimate pathway is limited to plants and micro-organisms and these compounds must form part of a mammalian diet.

Phenolics range from simple, low molecular weight, single aromatic-ringed compounds to the large and complex tannins (Lairon and Amiot, 1999). They can be classified by the number and arrangement of their carbon atoms (Fig. 1.2). They are reactive compounds and wide ranges of derivatives can be formed. They are commonly found conjugated to sugars, organic acids, aliphatic and aromatic groups and other phenolics. Phenolics can be classified into two groups, the flavonoids and the non-flavonoids.

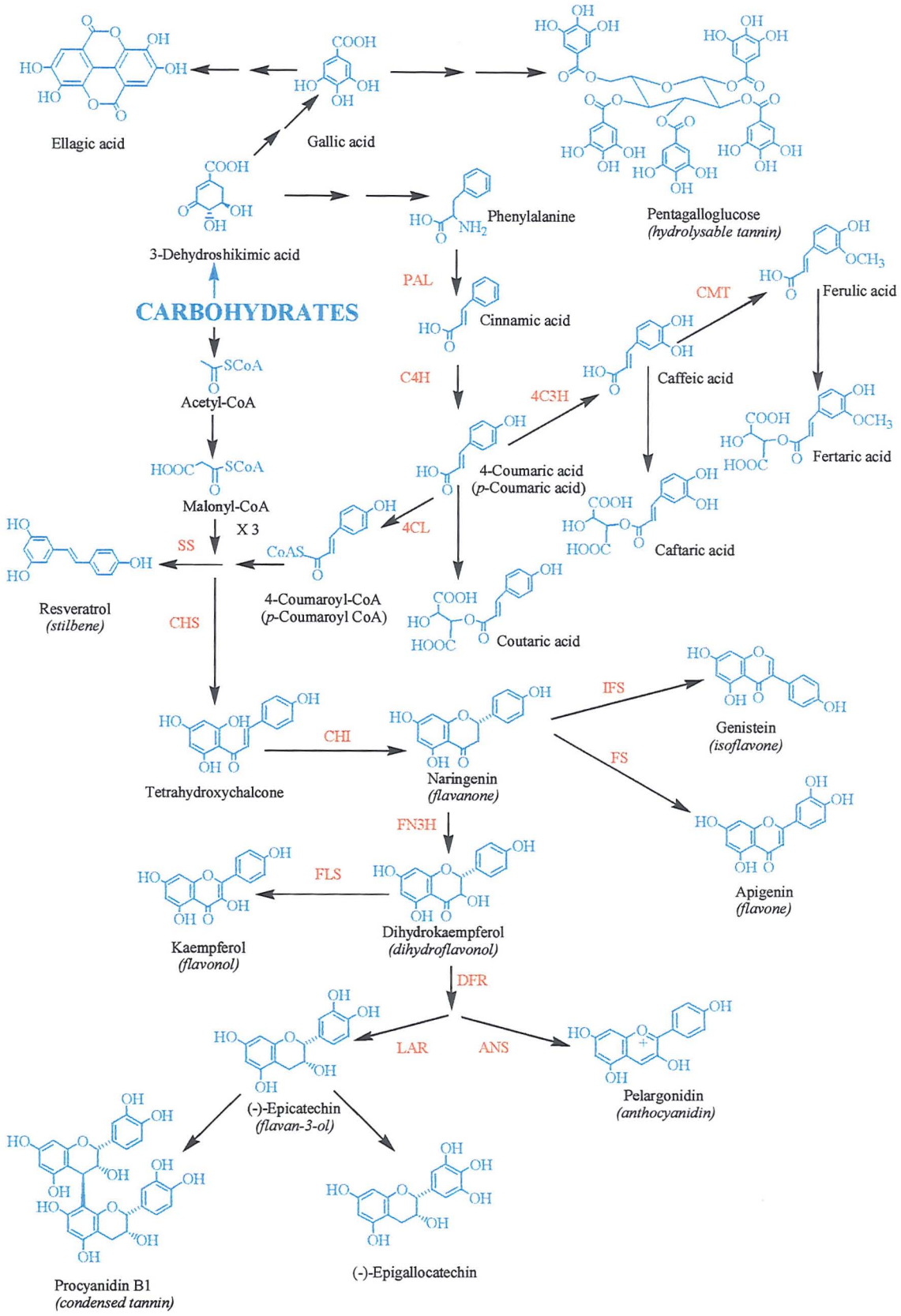


Figure 1.1 Summary of the phenolic biosynthetic pathway

Table 1.1. Enzyme acronyms for biosynthetic pathway

Acronym	Enzyme
PAL	Phenylalanine ammonia-lyase
C4H	Cinnamate 4-hydroxylase
4C3H	4-Coumarate 3-hydroxylase
CMT	Caffeate methyl transferase
4CL	4-Coumarate:CoA ligase
SS	Stilbene synthase
CHS	Chalcone synthase
CHI	Chalcone isomerase
IFS	Isoflavone synthase
FS	Flavone synthase
FN3H	Flavanone 3-hydroxylase
FLS	Flavonol synthase
DFR	Dihydroflavonol 4-reductase
ANS	Anthocyanidin 4-reductase
LAR	Leucoanthocyanidin 4-reductase

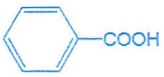
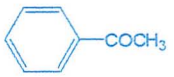
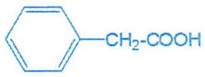
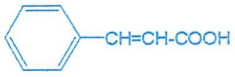
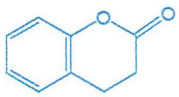
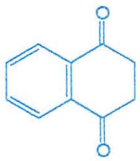
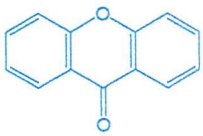
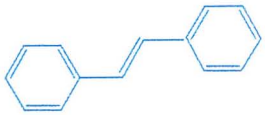
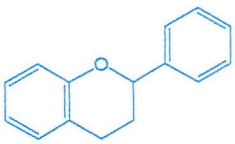
Carbon No.	Skeleton	Compound Class	Compound Example	Basic Structure
7	C_6-C_1	Phenolic acids	Gallic acid	
8	C_6-C_2	Acetophenones	Xanthoxylin	
8	C_6-C_2	Phenylacetic acid	*p*-Hydroxy-phenylacetic acid	
9	C_6-C_3	Hydroxycinnamic acids	Caffeic acid	
9	C_6-C_3	Coumarins	Esuletin	
10	C_6-C_4	Naphthoquinones	Juglone	
13	C_6-C_1-C_6	Xanthones	Gentisin	
14	C_6-C_2-C_6	Stilbenes	Resveratrol	
15	C_6-C_3-C_6	Flavonoids	Quercetin	

Figure 1.2 Examples of phenolic structures

1.1.1 Flavonoids

Flavonoids are found ubiquitously in plants, particularly in high concentrations in the leaf and fruit epidermis, principally as sugar conjugates. As secondary plant metabolites, flavonoids have many important and varied roles. These include UV protection, pigmentation, stimulation of nodules and disease resistance (see Koes et al., 1994).

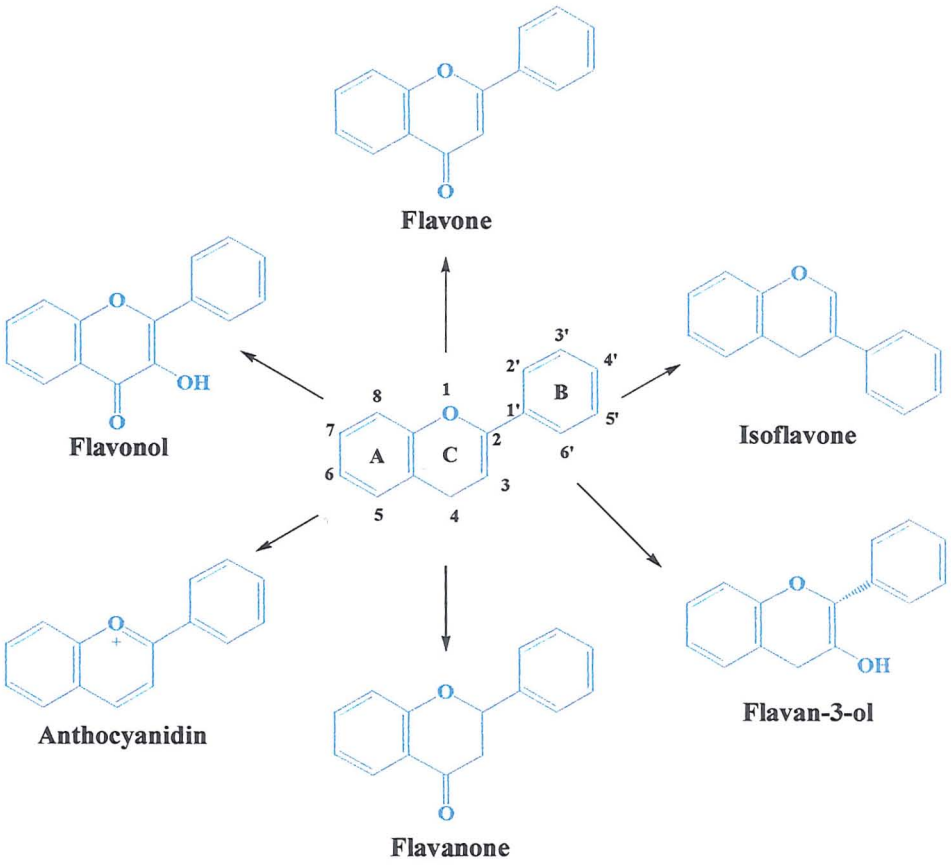


Figure 1.3. Generic structure of the flavonoids

The term flavonoid primarily encompasses the flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins (Fig. 1.3). Although chemically distinct, each group shares a common 2-phenyl-benzo-γ-pyrane nucleus. The basic flavonoid skeleton is derived from two sources. The B-ring and part of the C-ring are derived from the shikimate pathway, while the A-ring is formed from the head to tail condensation and cyclisation of three

acetate units from malonyl-CoA. Due to the extensive substitution of the three rings a large number of flavonoids can be found in nature. Hydrogenation, hydroxylation, methylation, sulphation, acetylation and glycosylation have all been widely reported (Heller and Forkman, 1992).

1.1.1.1 Flavonols

Of all the flavonoids, flavonols are perhaps the most commonly found. With the exception of fungi and algae, they are widely dispersed throughout the plant kingdom. The distribution and structural variations of flavonols are extensive and have been well-documented (Wollenweber, 1992; Williams and Harborne, 1992). In plant cells flavonols such as quercetin and kaempferol are most commonly found as *O*-glycosides, thereby increasing their water solubility. Conjugation occurs most commonly at the 3 position of the C ring although 5, 7, 4′, 3′ and 5′ substitutions also occur (Herrman, 1976). Although found in most plant tissues, they are particularly prevalent in fruit. Within the fruit tissue flavonols are generally found in the skin where they act as UV protectants (Koes et al., 1994). Flavonols have also been shown to play a role in pollen germination by regulating the growth of pollen tubes in the stigma (Mo et al., 1992).

1.1.1.2 Flavan-3-ols

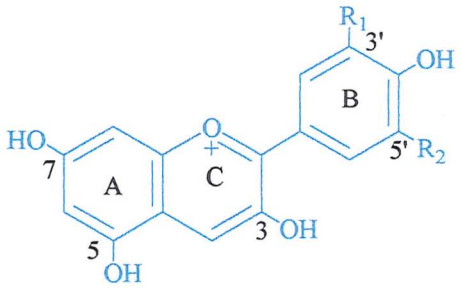
This is the most complex subclass of flavonoids ranging from the simple monomers (+)-catechin and its isomer (-)-epicatechin, to the oligomeric and polymeric proanthocyanidins, also known as condensed tannins. In addition to forming complexes with other flavan-3-ols, they are hydroxylated to form the gallocatechins, and also undergo esterification with gallic acid. In addition methylation, prenylation and *O*-glycosylation reactions have all been reported (Porter, 1992).

Many of the flavan-3-ols are astringent and are involved in the taste and texture of foods and beverages. As a family the flavan-3-ols have been historically interesting due to their role in the tanning industry. This is the

formation of leather from the interaction of the protein in animal hides with polyphenolic compounds of plant origin (Bohm, 1998).

1.1.1.3 Anthocyanins

Anthocyanins are widely dispersed throughout the plant kingdom, being particularly evident in fruit and flower tissue where they are responsible for red, blue and purple colours. In addition they are also found in leaves, stems, seeds and root tissue (Strack and Wray, 1992). As well as having an important role to play in the attraction of pollinating insects they are also implicated in the protection of plants against excessive light by shading of the leaf mesophyll cells (Bohm, 1998).



Anthocyanidin	R_1	R_2	Colour
Pelargonidin	H	H	Orange/red
Cyanidin	OH	H	Red
Delphinidin	OH	OH	Pink
Peonidin	OCH_3	H	Bluish purple
Petunidin	OCH_3	OH	Purple
Malvidin	OCH_3	OCH_3	Reddish-purple

Figure 1.4. Structures of the anthocyanidins in red wine

The term anthocyanidin refers to the aglycone structure (Fig. 1.4) however these compounds are invariably found conjugated, primarily to sugars, but also to hydroxycinnamates and organic acids such as malic and acetic acids. Although conjugation can take place on carbons 3, 5, 7, 3′ and 5′ it occurs

most often on C3. Malvidin-3-glucoside is the major anthocyanin in red wines, with cyanidin-3-sophoroside particularly prevalent in raspberries.

1.1.1.4 Flavones

Flavones have a very close structural relationship to the flavonols and they tend to be dealt with together. Although flavones have A and C ring substitutions, they lack oxygenation at C3. Chemically flavonols can be classified as 3-hydroxyflavones. Once again a huge range 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. They have been reported in celery, parsley and other herbs. In addition polymethoxylated flavones have been found in citrus (Kühnau, 1979)

1.1.1.5 Flavanones

The flavanones are the first flavonoid products of the biosynthetic pathway. They are characterized by the absence of the C2-C3 double bond and the presence of a chiral center at C2. In the majority of naturally occurring flavanones the C2 phenyl group is in the α-configuration. The flavanone structure is highly reactive and they have been reported to undergo hydroxylation, glycosylation and *O*-methylation reactions. Flavanones are common dietary components as they are found particularly in citrus fruits. The most common flavanone is naringin, which can be extracted from grapefruit peel (Kühnau, 1979). In citrus fruits flavanones are commonly found conjugated to rutinosides that have no flavour, however when conjugated with neohesperidose they are highly bitter compounds as evidenced by the taste of grapefruits and lemons (Bohm, 1998).

1.1.1.6 Isoflavones

Isoflavones are derived via the flavonoid biosynthetic pathway and can be converted into a wide variety of different isoflavonoids. This family of compounds is characterised by having the B ring attached at the C3 of the phenylchromane structure. They are abundantly dispersed throughout the plant kingdom, but the principal source is *Fabaceae* (*Leguminosae*) (Bohm, 1998). The soya derived iosflavones, genistein and daidzein, are reported to have phytoestrogen activity and may offer protection against hormone-dependent cancers such as breast cancer and prostate cancer (Wiseman, 2000). Phytoestrongens are diet-derived compounds which have a similar structure and/or activity as estrogen.

Isoflavone itself has not been reported naturally, and the most common flavones are the dihydroxyflavone, daidzein and the trihydroxyflavone genistein. As with the rest of the flavonoid family they undergo hydroxylation and methylation reactions, in addition to prenylation (Wollenweber, 1992).

1.1.2 Non-flavonoids

The main non-flavonoids are derivatives of benzoic and cinnamic acids, and their respective aldehydes. Along with other phenylpropanes they are synthesised from phenylalanine (Fig. 1.1). They are susceptible to substitution of the aromatic ring and also to oxidative modification of their side-chains, giving rise to a large number of related compounds.

1.1.2.1 Hydroxybenzoates

Although gallic acid was named by a Frenchman, its name derives from the French word galle, not Gaul. Galle is the French term for Gall, a swelling in the tissue of a plant after attack by parasitic insects. The gall contains carbohydrate and other nutrients that support the growing insect larvae. In some species the polyphenol component of the gall consists of up to 70% gallic acid esters (Gross, 1992).

It is generally accepted that benzoic acids are derived from the corresponding cinnamic acid by the removal of an acetate unit from the aliphatic side chain. It would therefore be expected that gallic acid (3,4,5-trihydroxybenzoic acid) would be formed from trihydroxycinnamic acid, however this compound has not been found in nature. Two alternative pathways have been proposed (Fig. 1.5). The first example (A) shows that the initial phenolic pathway has been adapted by the addition of a side chain degradation step. The second pathway (B) involves the direct aromatization of 3-dehydroxyshikimic acid. Evidence suggests that both the pathways exist in plants depending on age and species.

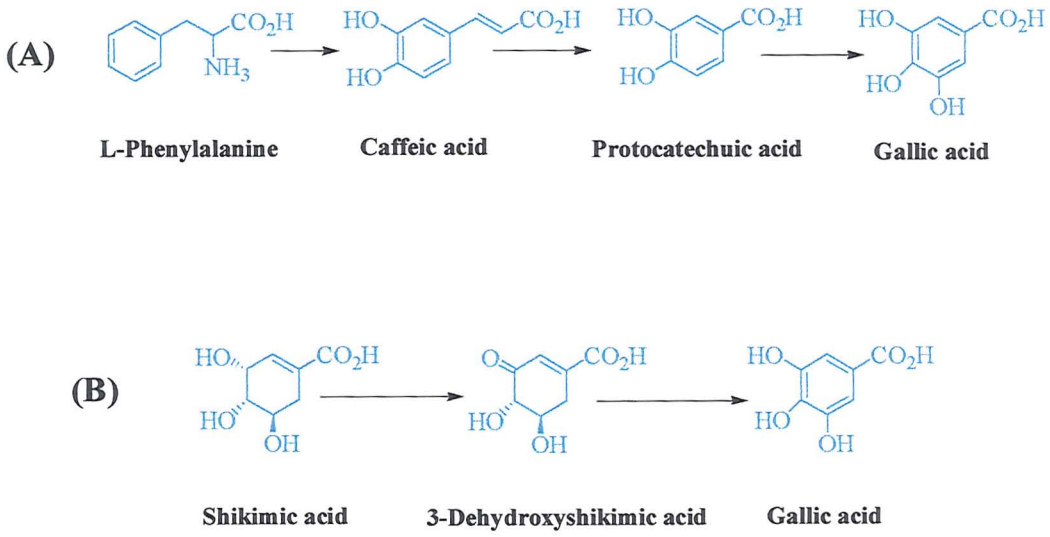


Figure 1.5. Proposed pathways for the biosynthesis of gallic acid

1.1.2.2 Hydroxycinnamates

Cinnamic acid is produced by the deamination of the amino acid phenylalanine by phenylalanine ammonia lyase (PAL), a reaction believed to be common to all plants. *p*-Coumaric acid is then produced by hydroxylation of cinnamic acid. The most common hydroxycinnamates are caffeic, *p*-

coumaric, ferulic and sinapic acids and are produced by a series of hydroxylation and methylation reactions. In addition to being found in their free form hydroxycinnamates are also found esterified to sugars, organic acids and choline (Strack, 1997).

1.1.2.3 Stilbenes

Members of the stilbene family are known to be phytoalexins, compounds produced by a plant in response to attack. *Trans*-resveratrol is synthesised from a branch of the same pathway as the flavonoids. The action of stilbene synthase (SS) enables the condensation of *p*-coumaric acid with three units of malonyl Co.A (Fig. 1.1). Stilbene synthase and chalcone synthase (CHS) have been shown to be structurally very similar and it is believed that they are both members of a family of polyketide enzymes (Soleas et. al., 1997b). However they differ in one major respect, while CHS is constitutively present in tissues, SS is inducible only by a range of stresses including UV-radiation, trauma and infection.

1.1.2.4 Other families

The families described above include the major structural classes found in wine and other foods and beverages. These are only a small fraction of the over 8000 phenolic structures known (Bravo, 1998). Other minor families include the acetophones, coumarins, chalcones, xanthones and lignins (Fig. 1.2).

1.1.3 Dietary sources and intake of phenolics

The ubiquitous nature of phenolic distribution in plants ensures that they are found in significant levels in the diet. Limited information is available, however, on the levels of phenolics, particularly non-flavonoids, in many foods and beverages. A wide range of methods has been used to quantify food phenolics but few studies have examined more than one food group. This inconsistency in analysis and the inaccurate methodology used in older studies

have combined to leave the estimated dietary intake of phenolics open to question. Estimates are available for the total dietary intake of flavonoids. They range from as little as 23 mg/day (Hertog et al., 1993b) to over 1000 mg/day (Kuhnau, 1976). The major dietary sources are red wine, tea, onions, apples and citrus fruit.

1.1.3.1 Beverages

Depending on the country in question, either tea or red wine provides the greatest proportion of beverage-derived flavonoid intake. Red wine contains between 1000 and 4000 mg/L phenolics compared with < 1000 mg/L for white wine. The majority of wine phenolics are derived from the anthocyanins and the proanthocyanidins (Burns et al., 2000). Likewise tea contains mainly catechins and proanthocyanidins, providing between 70 and 210 mg/g dry weight total flavonoids for black and green tea respectively (Lairon and Amiot, 1999).

Distilled spirits also contain phenols derived from fruit or from ageing in oak barrels (Goldberg et al., 1999). A survey of the concentration of six polyphenols in distilled spirits reported that 23 single malt whiskies contained an average of 14 mg/L total phenols, with 10 mg/L provided by ellagic acid.

1.1.3.2 Berries

The major phenolics in berries are the anthocyanins. Many fruits can be identified on the basis of their anthocyanin profile. While raspberries contain primarily cyanidin glycosides (100-600 mg/kg), pelargonidin conjugates predominate in strawberries at around 150-350 mg/kg (Clifford, 2000a).

Grapes contain stilbenes, particularly *trans*-resveratrol and its glucoside. They are found within the skin in levels of approximately 2.0 and 7.0 μg/g fresh weight.

1.1.3.3 Citrus

Citrus fruit is characterised by its content of flavanone glycosides and polymethoxylated flavones. The major sugar conjugates are 7'-*O*-rutinosides (lemons and limes) and neohesperidosides (grapefruits).

Flavanone intake has not been considered in many of the epidemiological studies undertaken (Tomás-Barberán and Clifford, 2000a). However the flavanone intake from a glass of orange juice (200 mL) or an average orange has been estimated at 25-60 mg and 125-375 mg respectively.

1.1.3.4 Other fruits

Fruits are known to contain high levels of flavonol glycosides and anthocyanins within their skin. The red colour of many fruits, with the exception of tomatoes, is due to anthocyanins and as such they will be major contributors to the dietary intake of phenolics (Kuhnau, 1976).

Apples are reported to contribute around 7% of the total daily flavonoid intake (Hertog et al., 1993b), however this method quantified only the flavonols. Apples also contain chlorogenic acid (50-300 mg/kg), procyanidin/catechin compounds (100-400 mg Procyandidn B2 /kg) and phloridzin (Bremner et al., 2000).

Tomatoes also occur widely in the diet. In addition to containing flavonol glycosides, mainly in the form of rutin, the hydroxycinnamate chlorogenic acid and the flavanone naringenin, have also been reported (Paganga et al., 1999). Tomatoes contain flavonols at levels between 1.3 ± 0.1 and 22.2 ± 0.8 μg/g fresh weight depending on variety and country of origin (Stewart et al., 2000).

1.1.3.5 Vegetables

Flavonols, particularly in the form of glycosides are widely found in leafy vegetables. Lettuce was reported to contain between 10 and 900 μg quercetin/g for round and Lollo Rosso varieties (Crozier et al., 1997b). The major phenolic compounds in lettuce have been identified and include caftaric and chlorogenic acids, and the flavonols quercetin-3-glucuronide, quercetin-3-glucoside, quercetin-3-(6-malonylglucoside) and quercetin-3-(6-malonylglucoside) 7-glucoside (Ferreres et al., 1997).

According to Hertog et al. (1993 b) over 48% of the daily total flavonol intake is from onions. Onions principally contain flavonol glycosides, particularly quercetin-3,4′-diglucoside, quercetin-4′-glucoside and lower amounts of quercetin-7,4′-glucoside, quercetin-3-glucoside, quercetin-7-glucoside and isorhamnetin-4′-glucoside. On average 956 mg/kg quercetin-3,4′-diglucoside and 340 mg/kg of quercetin-4′-glucoside are found in yellow onions, making them an invaluable source of dietary flavonol glucosides (Aziz et al., 1998).

Leafy vegetables contain many other phenolics (Clifford, 2000a). Lettuce has been reported to be high in the hydroxycinnamate dicaffeoyltartaric acid (5-15 mg/100 g). Likewise spinach is a major source of conjugated *p*-coumaric acid (30-35 mg/100g). Chlorogenic acid is present in the flesh of aubergine at very high levels (9477 ± 2078 mg/kg) and the skin has been shown to contain conjugates of the anthocyanidin delphinidin (Paganga et al., 1999).

1.1.3.6 Other sources

As with other fruit derived products, olive oil contains phenolic compounds including vanillic acid, ferulic acid and the flavones luteolin and apigenin. In general, levels of phenolic compounds increase with fruit ripeness. The total content of the phenolics analysed in Picudo olive oil reached around 670 mg/kg oil (Brenes et al., 1999).

Chocolate, made from cocoa beans, contains high levels of the flavan-3-ols (+)-catechin and (-)-epicatechin, with dark chocolate containing 107.5 and 502.5 mg/kg respectively (Arts et al., 2000).

1.2 Function of phenolics in plants

The term 'secondary plant metabolite' was initially coined to describe the many thousands of plant products that appeared to have no essential role in plant physiological processes. Many of the phenolic compounds found in plants fall into this category. However, although they have no primary role in plant function they have been implicated in stress defence, including UV protection, pigmentation, disease resistance and nodule production (reviewed by Koes et al., 1994; Pierpoint, 2000).

1.2.1 UV protection

Plants acquire their energy through photosynthesis, which necessitates UV exposure and can result in irreparable damage to DNA and changes in plant growth, development and morphology. Plants have developed a range of measures to combat these dangers including the up-regulation of the genes of the phenylpropanoid pathway (Jansen et al., 1998). This results in the accumulation of phenolic compounds, particularly the flavonols and hydroxycinnamates which are strongly absorbing in the UV region of 280 to 340 nm. Flavonols and hydroxycinnamates are generally located in the upper epidermal tissue where they contribute to preventing all but 10% of incident UV radiation from being transmitted further (De Lucia et al., 1992).

Mutants of *Arabidopsis thaliana*, *tt5* and *fah1*, which lack the enzymes chalcone synthase (CHS) and ferulic acid hydroxylase (FAH) respectively have been employed to study the relative protection offered by flavonoids and hydroxycinnamates (Landry et al., 1995). Although the *fah1* mutant retained its ability to accumulate flavonoids, it was unable to synthesize sinapic acid esters. This mutant showed more physiological damage by UV exposure than either the *tt5* mutant or the wildtype.

1.2.2 Pigmentation

The colour of plant leaves and flowers is more than just nature's cosmetics. Pigmentation is a potent signal, functioning as either an attractant or a deterrent. It can act as a beacon for pollinating insects, a warning against eating potentially toxic tissue, or even as an encouragement to eat and scatter seeds (Brouillard and Dangles, 1992).

The most important phenolic pigments are the flavonoids, in particular the yellow aurones and chalcones, and the red/purple anthocyanins. Other colourless phenolics may have a role to play in co-pigmentation by forming complexes with anthocyanins and metal ions. These complexes stabilise the anthocyanins and greatly increase the range of colours (Haslam, 1998).

1.2.3 Disease defence

A number of phenolic compounds have been ascribed a role in plant defences against microbial attack. Plants are susceptible to attack from viruses, bacteria, pathogenic fungi, herbivores and also other plants. In a defensive response they produce a range of compounds known as phytoalexins which are toxic to the attacking pathogen. Phytoalexins have been defined as low molecular weight compounds that are synthesised by a plant after exposure to microbes. It was originally specified that the compounds had to undergo *de novo* synthesis, i.e. they could not be present prior to infection and could not be synthesised from existing compounds. In recent years the term phytoalexin has been used to describe compounds produced as a result of other stresses including temperature shock, UV radiation and wounding (Bohm, 1998).

Common phytoalexins include terpenoids, stilbenes, polyacetates and flavonoids. In general the phytoalexin produced is plant specific, i.e. a particular chemical is synthesised rather than a gamut of compounds. For instance *Leguminosaea* are reported to synthesise isoflavonoids in response to infection (Koes et al., 1994), whereas the stilbene *trans*-resveratrol is known

to be produced in vine stems, leaves and grape tissue in response to attack by *Botrytis cinerea* (Soleas et al., 1997b).

1.2.4 Others

Flavonoids are involved in the symbiotic relationship between legumes and soil rhizobia. Rhizobia infect the roots of the host plant and produce a root nodule where they fix atmospheric nitrogen. Flavonoids, synthesised in root cells of the host plant are released into the soil where they come into contact with the rhizobia. They induce the expression of the genes responsible for nodulation (Schlaman et al., 1992). The different flavonoids released by plants are recognised by different bacteria in a manner that has not been fully explained.

The flavonoid content of a host plant has long been known to influence plant-eating insects (Harborne and Grayer, 1992). The biting factor of the larvae of silk worms in Mulburry was found to be quercetin-3-glucoside. Examples of feeding attractants have been found in many different flavonoid classes, with the active ingredient tending to be a glucoside. Flavonoids can also act as feeding deterrents in relatively low concentrations. Indeed it has been demonstrated that a particular compound can act as an attractant or a deterrent depending on the insect.

Phloridzin, a dihydrochalcone glucoside, has been shown to be a probing stimulant to the aphids *Aphis pomi* and *Rhapalosiphu insertum*, but a deterrent to the pea aphid. The proanthocyanidins are also well known feeding deterrents, evident at dietary concentrations of 0.5% for the larvae of *Pieris bassical*.

1.3 Grape and wine phenolics

Red wine is a complex fluid containing grape, yeast and even oak derived products (Table 1.2). Many of these compounds are found only in low levels and have little influence on the organoleptic properties of red wines although

they may be important in terms of the aroma and texture of wine. The major contributors to taste are those compounds found in higher quantities, water, ethanol, sugars, organic acids, glycerol and phenolics in red wines.

Table 1.2. Phenol composition for typical white and red wines

Phenol Class[b]	Source[c]	White Wine		Red Wine	
		Young	Aged	Young	Aged
Non-flavonoids, total		175	160-260	235	240-500
Cinnamates, deriv	G,D	154	130	165	150
Low vol. benzene deriv	D,Y,G,E	10	15	50	60
Tyrosol	Y	19	10	15	15
Volatile phenols	Y,D,E	1	5	5	15
Hydrolysable tannins	E	0	0-100	0	0-260
Macromolec. complexes					
Protein-tannin	G,D,E	10	5	5	10
Flavonoids, total		30	25	1060	705
Catechins	G	25	15	200	150
Flavonols	G,D	tr	tr	50	10
Anthocyanins	G	0	0	200	20
Soluble tannins, deriv	G,D	5	10	550	450
Other flavonoid, deriv	G,D,E,Y	?	?	60?	75?
Total Phenols		215	190-290	1300	955-1215

Data expressed as mg/L GAE[a] [a]GAE = Gallic acid equivalent [b]Deriv = derivative [c] G = grapes, D = degradation product, Y = yeast, E = environment. Adapted from Soleas et al., 1997c.

Although red wine does contain wood and yeast derived phenolics, the greater majority is grape derived. The phenolic profiles of grapes and wines differ due to the vinification processes undertaken, which are discussed in greater detail below. Although the chemical reactions taking place during vinification can affect the nature and extent of conjugation and polymerization of phenolics, similar major phenolics are common to both grape and wine.

The most common flavonoids in red wine are the flavonols, flavan-3-ols and the anthocyanins, with the flavonols and anthocyanins deriving from grape skins, and the flavan-3-ols mainly from the seeds and stems (Fig. 1.6).

1.3.1 Flavonols

The first report of flavonols in grapevine tissues comes from the 1870's when quercetin was isolated from grape leaves (see Singleton and Esau, 1969). However the early history of flavonol research was littered with vague characterisation and misidentification of compounds. Confusion arose over the identification of sugar groups conjugated to flavonols. Early reports of quercitrin (quercetin-3-rhamnoside) should correctly be described as quercetin glycoside.

Wines contain grape-derived flavonols, and in both cases the major flavonol aglycones are quercetin, myricetin, kaempferol and isorhamnetin (Burns et al., 2000). Flavonols are found mainly as sugar conjugates (Table 1.3), which aids their solubility and subsequent transport and storage within a tissue. They occur as *O*-glycosides and glucuronides, and in grapes and wines are typically bound to glucose (Fig. 1.7).

Paper chromatography was one of the original methods used to identify grape and wine flavonols (Ribéreau-Gayon, 1964). In addition to myricetin-3-glucosides, quercetin-3-glucosides and kaempferol-3-glucosides, quercetin-3-glucuronide was identified in red grapes.

The more advanced technique of HPLC later enabled the identification of the flavonol profile of Cinsault grape skins (Cheynier and Rigaud, 1986). Identification was by UV spectroscopy, mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy and thin layer chromatography (TLC) of the sugars released after acid hydrolysis. This technique revealed that kaempferol-3-glucoside co-eluted with kaempferol-3-galactoside casting doubt on previous identification.

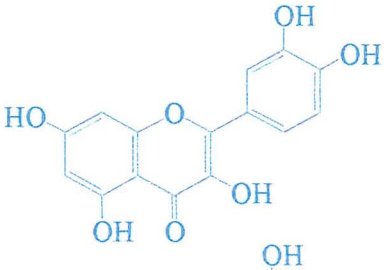
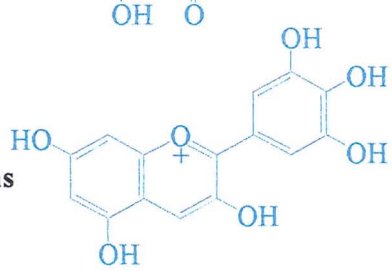
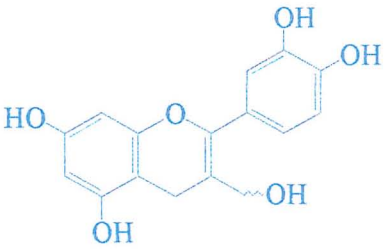
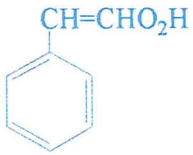
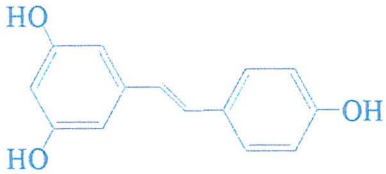
General Type	General Structure	Example
Flavonoids		
Flavonols		quercetin myricetin kaempferol isorhamnetin
Anthocyanidins		cyanidin delphinidin petunidin peonidin malvidin
Flavan-3-ols		(+)-catechin (-)-epicatechin epigallocatechin procyanidins (condensed tannins)
Non-Flavonoids		
Hydroxybenzoates		gallic acid ellagic acid hydrolysable tannins
Hydroxycinnamates		*p*-coumaric acid caffeic acid ferulic acid sinapic acid
Stilbenes		*trans*-resveratrol *cis*-resveratrol *trans*-resveratrol glucoside

Figure 1.6. The major classes of phenolics in wine

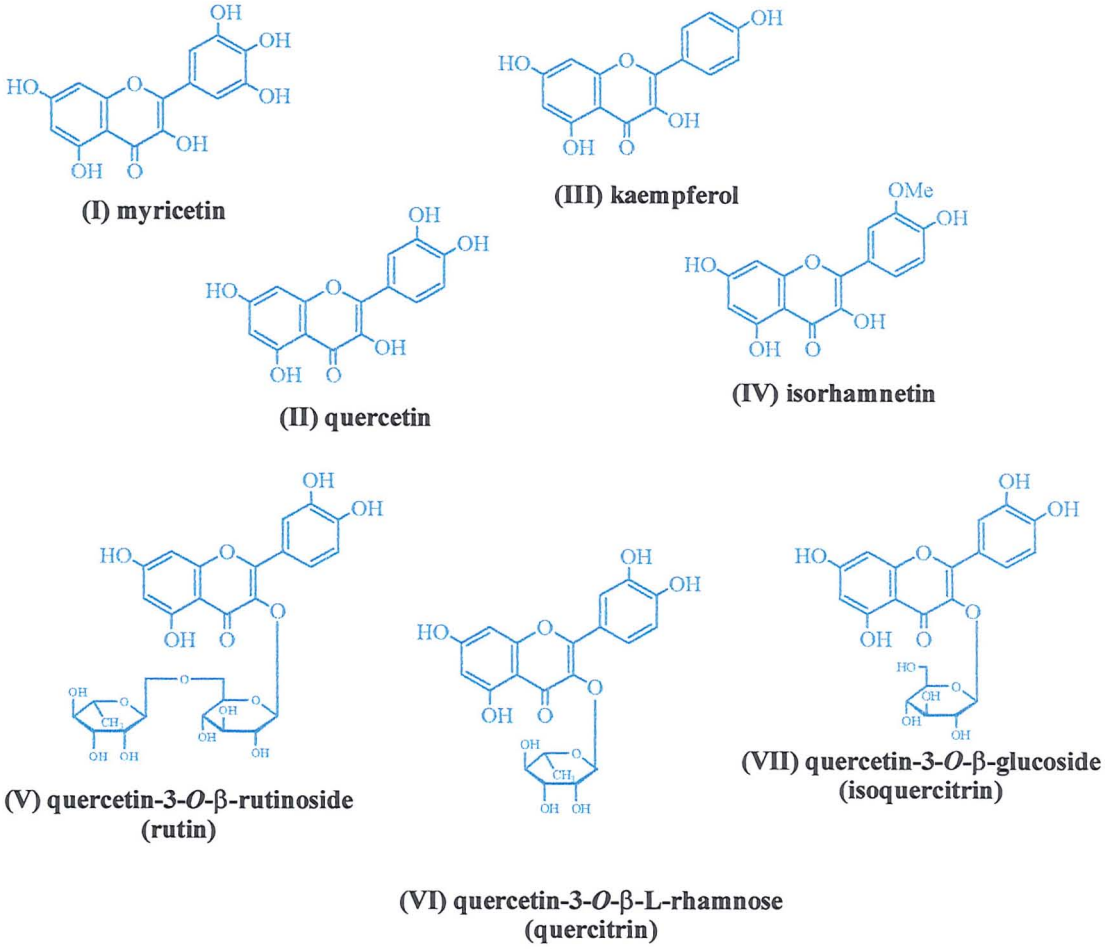


Figure 1.7. Structures of the major flavonol aglycones and conjugates in red wine

NMR confirmed quercetin-3-glucuronide while myricetin- and kaempferol-3-glucuronide were identified only by their elution just prior to the corresponding glucosides. Isorhamnetin-3-glucoside was also identified by NMR, MS of the aglycone and TLC of the sugar group. Three diglucosides were tentatively identified as quercetin-3-glucosylgalactoside, quercetin-3-glucosylxyloside and kaempferol-3-glucosylarabinoside. No quantitative data was included as part of this report.

The flavonol content of grape leaves and stems have also been investigated. Quercetin-3-rhamnogalactoside, quercetin-3-glucuronoside, quercetin-3-glucoside, quercetin-3-rhamnoside and quercetin-3-rutinoside were identified

along with the aglycones myricetin, quercetin and kaempferol in the leaves of Moroccan *Vitis vinifera* cultivars (Hmamouchi et al., 1996). In addition the 3-glucosides of myricetin, quercetin and kaempferol and the 3-glucuronosides of myricetin and quercetin were identified in the stems of grape vines (Souquet et al., 2000).

Table 1.3. Flavonols reported in Black grapes and Red wines

Flavonols reported	Tissue	Reference
Myricetin-3-glucoside; Quercetin-3-glucoside, Kaempferol-3-glucoside; Quercetin-3-glucuronide	Grape and wine	Ribereau-Gayon, 1964
Kaempferol-3-glucoside; Kaempferol-3-galactoside; Quercetin-3-glucoside; Myricetin-3 glucoside; Kaemperol-3-glucoside; Isorhamnetin-3-glucoside; Quercetin-3-glucosylgalactoside; Quercetin-glucosylxyloside; Kaempferol-3-glucosylarabinoside	Cinsaut grape skin	Cheynier and Rigaud, 1986
Quercetin-3-rhamnogalactoside; Quercetin-3-glucuronide; Quercetin-3-glucoside; Quercetin-3-rhamnoside; Quercetin-3-rutinoside; Myricetin, Quercetin, Kaempferol	Grape vine leaves	Hmamouchi et al., 1996
Quercetin-3-glucuronide; Kaempferol-3-glucuronide; Myricetin-3-glucoside; Quercetin-3-glucoside; Kaempferol-3-glucoside	Grape vine stems	Souquet et al., 2000

Although a number of flavonol glycosides have been identified in grape tissue, regular quantification can be difficult due to a lack of available standards. In general only the aglycone is quantified, in some instances after acid hydrolysis to cleave the sugar groups. Free quercetin was analysed in over 870 wines and found to vary dramatically depending on region, variety and climate (Goldberg et al., 1998b). Levels of quercetin ranged from 0.5 ± 0.3 to 10.8 ± 1.3 mg/L in Cabernet Sauvignon from Central Europe and Shiraz from Australia respectively. By analysing only the free aglycones simple and misleadingly low estimates are obtained as most of the flavonols in wine conjugated to sugar moieties.

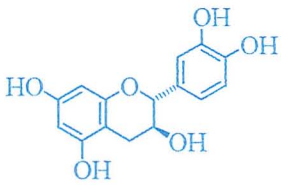
This is evident in the higher values seen in a survey of the free and conjugated myricetin and quercetin content of red wine obtained before and after acid hydrolysis where levels of total flavonols were found to vary between 4.6 to 41.6 mg/L (McDonald et al., 1998). Highest levels were found in wines from thick-skinned Cabernet Sauvignon grapes. In particular it was noted that wines made in hot, sunny climates had elevated levels of myricetin and quercetin. Vinification technique was also observed to be of importance. The more intensive the extraction process, the greater the eventual content of myricetin and quercetin in the wines.

1.3.2 Flavan-3-ols

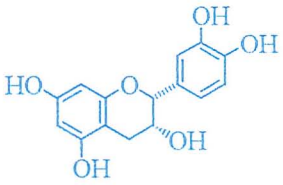
The highest concentration of flavan-3-ols in grape clusters is found in the seeds, although high levels are also found in the stem tissue. However it is the grape skins that are the primary source of wine flavan-3-ols due to the ease of transfer of phenolics from skins into wines. Information on the levels of flavan-3-ols in wines has been limited and contradictory. It is most likely that the low flavan-3-ol levels reported in the early literature are due to inadequate extraction techniques and poor methodology. A recent report (Goldberg et al., 1998a) surveyed the content of (+)-catechin and (-)-epicatechin in over 800 red wines from different wine producing regions. They presented results broken down by country/region and variety. Levels of (+)-catechin ranged from an average of 22 ± 3.2 mg/L in 24 Australian Shiraz wines and to a high of 208 ± 18.4 mg/L in 56 wines from Burgundy made with Pinot Noir grapes. Concentrations of (-)-epicatechin showed similar variations, but were generally lower than (+)-catechin. (-)-Epicatechin ranged from a low of 15 ± 1.6 mg/L in 12 wines from the Spanish region of Rioja, to a high of only 88 ± 11.6 mg/L found in 10 Canadian wines made from Pinot Noir. It appears as if, independent of environmental factors, Pinot Noir grapes are constitutively higher in catechins. It was hypothesised that this may be a consequence of the thin pale skin of the variety. It is necessary to extract the grapes for longer to get an acceptable colour. This extended maceration may also encourage the increased extraction of catechins.

In grapes the gallate esters are found in only very low levels that decrease as the grape ripens, while the gallocatechins are found in even lower levels. In wines it has been reported that, unlike the gallate esters and gallocatechins, the flavan-3-ol monomers remain relatively stable (Singleton, 1982). This however is in contradiction to reports of flavan-3-ol condensation reactions with catechin monomers and flavonols (Haslam, 1998)

Catechin monomers

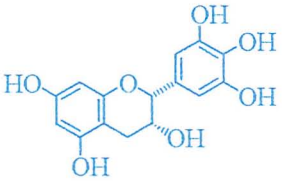


(VIII) (+)-catechin

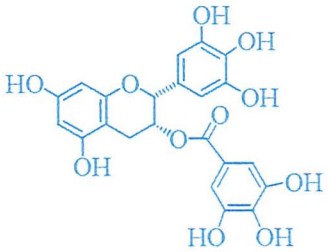


(IX) (-)-epicatechin

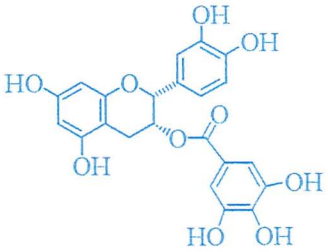
Gallocatechins and gallate esters



(X) epigallocatechin

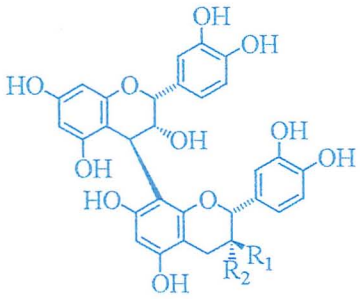


(XI) epigallocatechin gallate

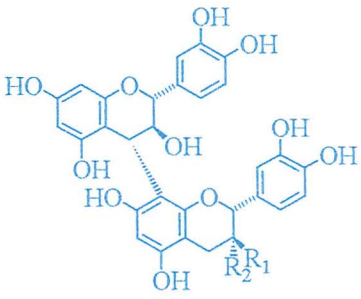


(XII) epicatechin gallate

Procyanidins



(XIII) Procyanidin-B_1 R_1 = OH R_2 = H
(XIV) Procyanidin-B_2 R_1 = H R_2 = OH



(XV) Procyanidin-B_3 R_1 = OH R_2 = H
(XV1) Procyanidin-B_4 R_1 = H R_2 = OH

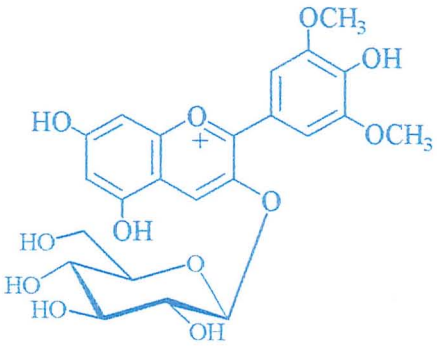
Figure 1.8. Structures of the main flavan-3-ols in red wine

Grapes and wines also contain larger more complex compounds, using the monomers as basic structural units, known as proanthocyanidins. Those derived from (+)-catechin and (-)-epicatechin are known as procyanidins, while those from the gallocatechins are described as prodelphinidins. The nomenclature is based on that of the anthocyanins. The most common procyanidins in grapes are the simple dimers B1, B2, B3 and B4 and the trimers C1 and T2. The flavan-3-ol contents of 95 French red wines were investigated (Carando et al., 1999b). In each wine the four dimers and 2 trimers mentioned above were reported, with mean levels of 557.9 mg/L. Concentrations of Procyanidin B1 were on average 25.4 mg/L, similar to a mean of 47.4 mg/L found with Procyanidin B2. However mean levels of 119.6 mg/L and 81.9 mg/L were reported for Procyanidins B3 and B4 respectively.

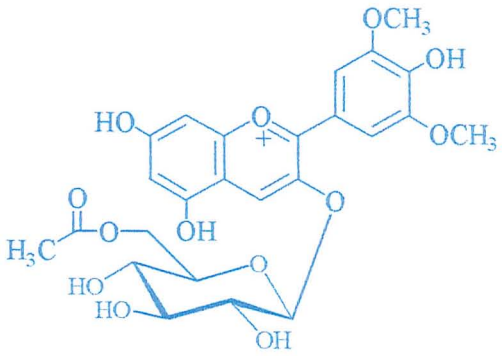
1.3.3 Anthocyanins

Anthocyanins are responsible for the colouring of black grapes and red wines. They are lacking in white grapes due to a biosynthetic blockage. They are principally found in the skins of grapes, although they have been reported in the flesh of some berries, a characteristic known as teinture. The amount and concentration of anthocyanins in red grapes will vary depending on the variety, maturity, climate, terrior and the fruit yield. The total anthocyanin content of red grapes ranges from about 300-7500 mg/kg fresh weight ripe berries (Mazza, 1995). The anthocyanins described in grape skins are predominately the 3-*O*-glucosides of delphinidin, cyanidin, petunidin, peonidin and the major compound malvidin, although coumaric, caffeic and acetic acid esters have also been reported. Although the types of anthocyanins are similar between different grape varieties, the relative amounts of individual compounds differ. For example, compared to other varieties Tempranillo was shown to have a higher percentage of the 3-*O*-glucosides of delphinidin, cyanidin and petunidin, and a lower percentage of malvidin-3-glucoside and malvidin-3-acetylglucoside (Mazza, 1995). Likewise it has been noted that Pinot Noir grapes contain no acylated anthocyanins. Features such as these have enabled anthocyanins to be used taxonomically and to

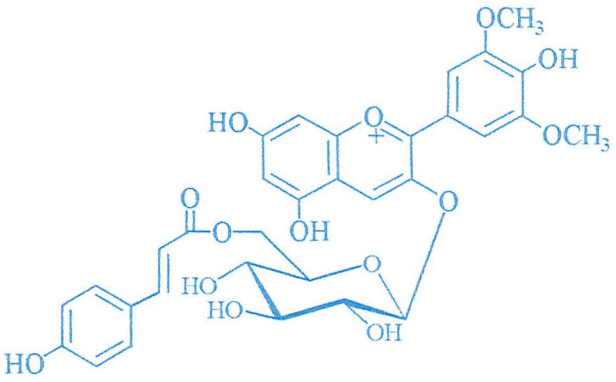
detect adulteration in wines. Anthocyanins are readily extracted from grape skins and provide the vibrant redish-purple tones of young red wines. The extent of anthocyanin extraction depends on fermentation temperature and duration, and the concentration of sulphur dioxide and alcohol.



(XVII) malvidin-3-*O*-glucoside



(XVIII) malvidin-3-*O*-(6-*O*-acetyl)glucoside



(XIX) malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside

Figure 1.9. Structures of the main anthocyanins in red wine

Indeed, although grape skins contain significant levels of petunidin, delphinidin and lesser amounts of cyanidin and peonidin in addition to malvidin, seven months into vinification the malvidin derived anthocyanins contribute 85% of total anthocyanins in wine.The major anthocyanin derivatives are malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-acetyl)glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside (Fig. 1.9).

As a wine ages the content of free anthocyanins decreases and levels of larger condensed polymeric pigments increase (Haslam, 1998). These pigments absorb around 420 nm, accounting for the maturing of the colour of a wine from bright red to orange/brown. The identity and chemical nature of many of these complex pigments remains unknown, although malvidin-catechin dimers have been reported in model wine solution and also wine (Revilla et al., 1999).

1.3.4 Hydroxybenzoates

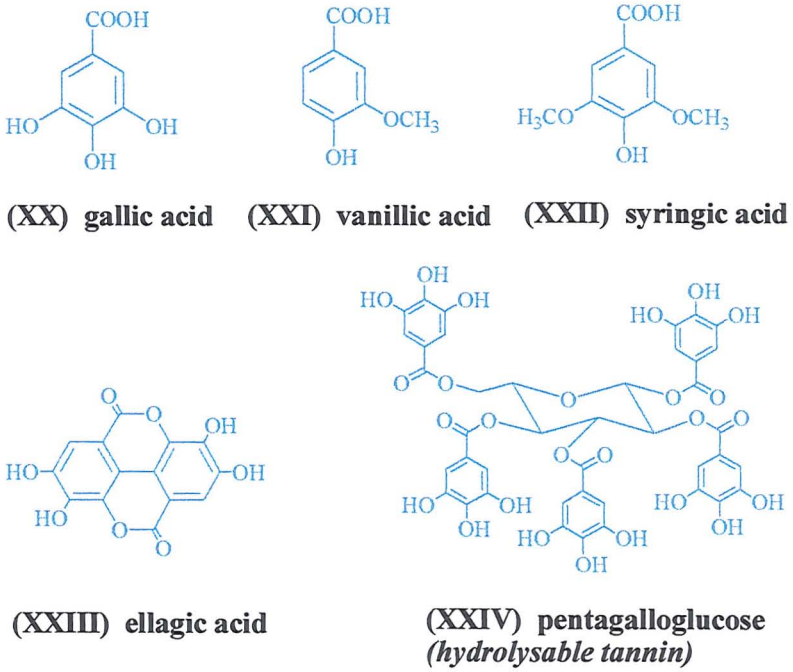
Wines contain grape and wood derived hydroxybenzoates. Few fruits and vegetables contain free hydroxybenzoates, the majority is found conjugated. In wine, the major hydroxybenzoates are gallic acid and its dimer ellagic acid, although vanillic acid and syringic acid have also been reported (Soleas et al., 1997a). Gallic acid is found in free run juices of white wines at levels of approximately 1.09 mg/L (Betes-Saura et al., 1996).

Increasing the length of skin fermentation has been shown to increase the extraction of gallic acid (Singleton and Trousdale, 1983). Red wines have been reported to contain higher levels of gallic acid than white wines (Frankel et al., 1995). Red wine contained on average greater than 20 mg/L of gallic acid (Soleas et al., 1997a).

If grape juices stand for an extended period of time a precipitate forms which has been identified as ellagic acid. Grape juices, made from black and white grapes, contain between 1.6 ± 0.3 and 23.1 ± 1.2 μg/mL of ellagic acid (Boyle and Hsu, 1990). It is believed that the ellagic acid is derived from the degradation of hydrolysable tannins as it stands during storage and prior to packaging.

Ellagic acid is extracted into wine from oak during maturation in barrels (Laszlavik et al., 1995). Wood variety and treatment can affect levels of ellagic acid in wines. Toasting wood increased the concentration of ellagic acid in wine seven fold with Hungarian *Quercus petrea* but only just over two fold with *Quercus robar*.

In wines gallic acid and ellagic acid are the common phenolic components of the hydrolysable tannins, a sugar core onto which gallic acid is esterified (Fig. 1.10). Hydrolysable tannins are based on gallic acid and its derivatives. The gallic groups are esterified around a sugar group. The sugar group can be either cyclic or linear, with further substitutions found involving ellagic acid and flavan-3-ols.



(XX) gallic acid **(XXI) vanillic acid** **(XXII) syringic acid**

(XXIII) ellagic acid

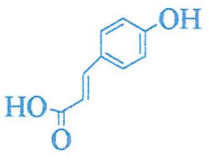
(XXIV) pentagalloglucose
(hydrolysable tannin)

Figure 1.10. Structures of the main hydroxybenzoates in red wine

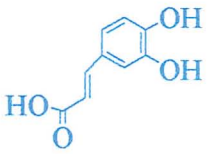
1.3.5 Hydroxycinnamates

The major non-flavonoids found in wines are the hydroxycinnamates, which are stored primarily in the cell vacuoles of the grape flesh and therefore are easily extracted on crushing. As they are flesh derived they are a major component of both red and white wines. The main hydroxycinnamates in wine are caffeic and *p*-coumaric acids, both of which are found as the *cis*- and *trans*-isomers. The major isomer is *trans* and unless otherwise stated all

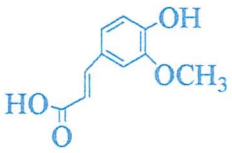
further references to the hydroxycinnamates will refer to the *trans*-isomer. Hydroxycinnamates are found in wines esterified to sugars, organic acids and various alcohols. In particular the hydroxycinnamates caffeic and *p*-coumaric acids in grapes and wines are esterified with tartrate (Fig. 1.11).



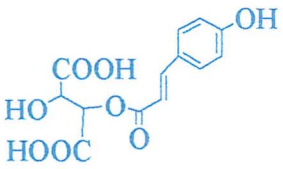
(XXV) *p*-coumaric acid



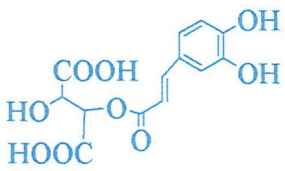
(XXVI) caffeic acid



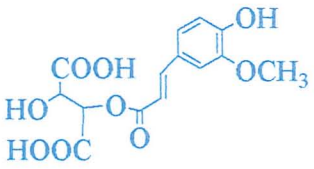
(XXVII) ferulic acid



(XXVIII) coutaric acid



(XXVIX) caftaric acid



(XXX) fertaric acid

Figure 1.11. Structures of the main hydroxycinnamates in red wine

In grapes and young wines *p*-coumaric and caffeic acids are generally found only as the tartrate esters, coutaric and caftaric acids respectively. Caftaric acid is found in grapes in higher levels to either coutaric or fertaric acid, 100 mg/L, 20 mg/L and 5 mg/L respectively, (Ong and Nagel, 1978). Caftaric acid is also the most abundant hydroxycinnamate in wines (Nagel et al., 1979). A survey of white Reisling wines from California, Washington and Alsace showed that caftaric acid varied from a low of 29 mg/L in musts from Alsace to a high of 267 mg/L in Californian wines (Nagel et al., 1979).

A more recent study used GC-MS to quantify *p*-coumaric acid and caffeic acids in Ontario wine (Soleas et al., 1997a). *p*-Coumaric acid was found in lower levels compared with caffeic acid. Twenty-one red wines from five varieties were studied. Caffeic acid varied from 3.15 to 12.95 mg/L, the highest levels in Cabernet Sauvignon. *p*-Coumaric acid was found in highest

levels in Gamy Noir (4.5 mg/L) and the lowest (2.61 mg/L) in Pinot Noir. No significant difference was observed between the levels of the hydroxycinnamates in red or white varieties.

A long gradient HPLC method was used to separate phenolics in twenty-one Cabernet Sauvignon wines, including caftaric and coutaric acids and the corresponding aglycones (Ritchey and Waterhouse, 1999). Caftaric acid arranged from 8.03 to 68.53 mg/L and coutaric acid from 4.29 to 42.63 mg/L. Total hydroxycinnamates reached a high of 158.11 mg/L compared with a low of 27.74 mg/L.

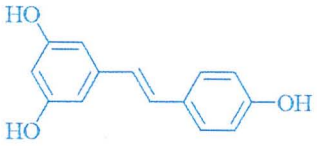
1.3.6 Stilbenes

Stilbenes have been reported in medicinal plants since the 1930's, but *trans*-resveratrol was first isolated from grapevines in 1976 (Langcake and Pryce). They reported that it was a major component of grapevine wood, and described it as a phytoalexin in leaves.

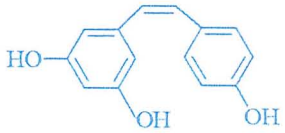
The first report of *trans*-resveratrol in grape skin was in 1988 (Creasy and Coffee, 1988). Its production was initiated in grape skin within a few hours of UV exposure. The stilbene content of grapes is dictated by three factors: cultivar, disease pressure and time (Creasy and Coffee, 1998). Analysis of the *trans*-resveratrol content of Californian table grapes, for instance, showed an almost 20-fold variation between different clones. Levels of 0.16 mg/kg have been reported in Crimson seedless grapes, compared with 3.0 mg/kg in Fantasia seedless. One of the major influences on stilbene levels in grapes is fungal infection. *Trans*-resveratrol is synthesised in response to infection and is present in toxic levels, approximately 1mM in skin cells. Such levels cannot be sustained and there is rapid turnover of *trans*-resveratrol. It can be either degraded by enzyme conversion or transported to woody tissue as a means of excretion.

Within the cells of the grape berry skins *trans*-resveratrol is principally as *trans*-resveratrol-*O*-β-glucoside, otherwise known as polydatin or piceid

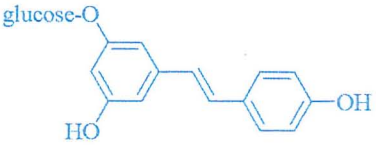
(Waterhouse and Lamuela-Raventós, 1994). Although the *cis* isomers of both resveratrol and the glucoside have been reported in wines, they have not been identified in grape tissue (Fig. 1.12).



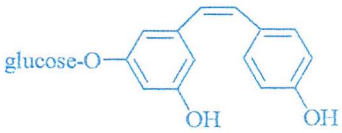
(XXX) *trans*-resveratrol



(XXXII) *cis*-resveratrol



(XXXIII) *trans*-resveratrol-*O*-β-glucoside



(XXXIV) *cis*-resveratrol-*O*-β-glucoside

Figure 1.12. Structures of the main stilbenes found in red wine

Although there has been an explosion of interest in the stilbene content of wines, few studies have investigated the content of grape juices, (Yasui et al., 1997; Soleas et al., 1995) and these looked only at levels of *trans*-resveratrol. Recently Romero-Pérez et al. (1999) reported that resveratrol glucosides were the major stilbene derivatives in grape juices. They found that red grape juice samples averaged 10-fold higher total stilbene levels than white juices. While red juice samples had on average 3.38 mg/L and 0.79 mg/L of *trans*- and *cis*-resveratrol glucosides respectively, white juice had average concentrations of 0.18 mg/L and 0.26 mg/L respectively.

Interest in the stilbene content of wines heightened when it became known in the 1980's that *trans*-resveratrol was believed to be the active component of a Chinese and Japanese herbal remedy, ko-jo-kon which was used for the treatment of atherosclerosis and inflammation (Kimura et al., 1985). It was hypothesised that the stilbenes may be the components in red wine responsible for the French Paradox. Although many studies have confirmed the presence of *cis*- and *trans*-resveratrol, their glucosides, and other related and unknown

stilbenes, they are generally found in lower levels than many other phenolics in red wine (Siemann and Creasy, 1992; Jeandet et al., 1993; Lamuela-Raventós and Waterhouse, 1993; Goldberg et al., 1995b, 1996a; Lamuela-Raventós et al., 1995; Ribeiro de Lima et al., 1999).

The most comprehensive study to date on the stilbene content of wines used direct-injection HPLC and UV detection to assay over 700 wines from most of the world's areas of wine production (Goldberg et al., 1996a). They found the levels of *cis*- and *trans*-resveratrol glucosides were similar. *Cis*-resveratrol ranged from an average of 200 ± 0.0 μg/L in fifteen Australian Cabernet Sauvignon wines, to 2442.6 ± 814 μg/L in eight South American Merlot. While *trans*-resveratrol ranged from 251.1 ± 75.3 μg/L, once again in the Australian Cabernet Sauvignon, to 2487.9 ± 311 μg/L in 26 wines from the Southern Rhone Valley.

Levels of *cis*- and *trans*-resveratrol were slightly higher than the corresponding glucosides. Concentrations of *cis*-resveratrol ranged from 251.1 ± 139.5 μg/L in 28 Californian Cabernet Sauvignon to 2784.7 ± 278.5 μg/L in 14 Pinot Noir wines from California. Likewise *trans*-resveratrol levels ranged from 730.4 ± 182.6 μg/L in the 28 Californian Cabernet Sauvignon wines to 3172.7 ± 503.6 μg/L in 20 Australian Shiraz wines. Patterns to the distribution of the stilbenes in wines are difficult to discern due to the combined effects of influences on the concentration of stilbenes in grapes and also the processes occurring during vinification.

1.4 Viticulture and vinification

Viticulture and vinification are the twin pillars of wine-making. Viticulture is the study of grape-growing, while vinification describes the art of turning grapes into wine.

1.4.1 Red wine, from grape to glass

With the large amounts of money to be made in the wine industry, grape growing is not left to chance. Vines are planted with particular care to the terrior, the combined influence of the vineyard soil and microclimate. Grape composition is influenced by the nutrients in the soil, access to water, sun exposure and temperature. When these factors are optimised the vines growing will be harvested for many decades.

Vines are trellised and pruned to ensure that individual grape bunches are sufficiently exposed to sunlight, and that an inefficient number of leaves are not being produced by the vine.

Grapes are picked, manually or mechanically, once a particular ripeness has been achieved, and are then sent to the winery. The methods used for red wine vinification vary depending on the country, region, winery, winemaker and even the vintage. Generally once the grapes are picked they are destemmed and crushed prior to alcoholic fermentation. Destemming, and the careful removal of contaminating leaves and stalks, limits the production of off flavours within the wines.

Fermentation describes the chemical conversion of sugar to ethanol and carbon dioxide due to the anaerobic metabolism of yeasts. Although fermentation can occur due to the natural yeasts present on the surface of the berry, it is increasingly common for specific yeasts to be used depending on the grape variety or the required characteristic of the finished wine. Sulphur dioxide can also be added as an antioxidant to protect the more delicate phenolics during fermentation. The increasing alcohol content during fermentation encourages the extraction of the phenolic compounds from the grape tissue into the wine. This extraction is mediated by maceration. The term maceration describes the steeping of a material in a liquid, without kneading, in order to separate out the softened parts of the material from the hardened ones. This process can be quick, lasting only a few days and producing a light wine, or up to several weeks. The temperature of the

fermentation and maceration process is carefully controlled and manipulated. Although fermentation is usually carried out in the presence of skins, they can be removed, pressed and the pressing wine added back into the free-run wine. If a wine is rich in tannins, full-bodied and sufficiently robust to survive, it can be barrelled. Cooperage is the maturation of wine in wood, principally oak, and can have a significant influence on the phenolic content of the resulting wine.

After cooperage the wine can be fined prior to blending. A fining agent is added to the wine and acts to coagulate or absorb any large particles and precipitate them out of solution leaving the wine clear. They can remove tannins, protein and phenolics and help prevent the formation of sediment in the bottle. Traditionally egg white was used, however nowadays a fine clay, bentonite, is commonly used. Although wineries fine and stabilise their wines for the general market place, it also to some degree strips the wine of the tannins that provide colour, texture and flavour to the wine.

1.4.2 Influence of viticulture on phenolic compounds

The profile and content of phenolic compounds in grapes will vary depending on a range of vineyard influences including genetics, climate, location and disease pressure.

1.4.2.1 Grape variety

The phenolic content of a grape has been used to confirm identity and detect any adulteration in wine. Grapes have identifiable, if not unique, phenolic profiles. Pinot Noir grapes are reported to have higher levels of (+)-catechin and (-)-epicatechin compared with other varieties (Goldberg et al., 1998a). Likewise Pinot Noir grapes do not contain acylated anthocyanins unlike other varieties (Mazza, 1995).

Flavonoids are found in the skin of the berry due to their role as UV protectors. A thick-skinned variety such as Cabernet Sauvignon has the

potential to release more polyphenols than a thin-skinned Pinot Noir grape (McDonald et al., 1998).

1.4.2.2 Sun exposure

High sunlight will encourage the synthesis and accumulation of polyphenols. Grapes grown in the shade have been shown to have 7-fold lower levels of flavonols than similar grapes grown in direct sunlight (Price et al., 1995).

1.4.2.3 Terrior

Nutrients and water are soil derived and can vary even between two adjacent plots. High nitrogen has been shown to suppress anthocyanin production with high potassium affecting berry pH thereby altering the stability of colour. Over irrigation can increase fruit yield, however it also increases tannin and anthocyanin levels (Nadal and Arola, 1995). An increase in fruit yield is also believed to be associated with a reduction in the quality of the grapes.

1.4.2.4 Disease pressure

Among the diseases that infect grapevines, one of the most studied has been the Nobel Rot, *Botrytis cinerea*. This is the fungal infection that is responsible for the desiccation of the Sauvignon blanc, Semillon and Muscadelle grapes found famously in the Sauterne region of France. The damp climate of this region promotes the growth and dispersal of the fungus. Phytoalexin stilbenes, particularly the resveratrol compounds, are produced in response to fungal infection (Langcake and Pryce, 1976). However it has been reported that severely infected grapes have a lower concentration of *trans*-resveratrol than those which are not infected, or only mildly infected (Jeandet et al., 1995). It is hypothesised that the fungus produces a laccase-like enzyme that is able to degrade the stilbene.

1.4.3 Influence of vinification on phenolic compounds

The phenolic compounds present in the skins, flesh, seeds and stems of grapes are extracted into wine during vinification. Different technologies and treatments can influence the extraction. The phenolic profile of grapes, free-run juice, young wines and old wines, are all very different as a result of these processes.

1.4.3.1 Length of maceration

The longer the maceration, the greater the opportunity for compounds to leach out of the grape tissue. Increasing pomace contact results in significant compositional changes in wines (Singleton and Trousdale, 1983). The concentration of catechins and procyanidins was observed to increase over 14 days (Kovac et al., 1992). Anthocyanins are rapidly extracted into the wine, reaching a maximum by day 3 and decreasing thereafter (Nagel and Wulf, 1979). The falling levels of anthocyanins are due to the formation of complexes with other phenolics as opposed to their degradation or the absence of extraction. Levels of flavonoids increase to a greater extent than levels of the non-flavonoids (Ramey et al., 1986). However the concentration of gallic acid in a wine increases with skin contact. It is likely that this is due to extraction from grape skin, although it may also be the results of the hydrolysis of gallate esters (Singleton and Trousdale, 1983). Likewise maceration on skins for 7 days increases the extraction of resveratrol into red wines up to 13-fold when compared with the free run juice (Jeandet et al., 1995).

1.4.3.2 Presence of stems and seeds

The stems of grapevines and grape seeds contain phenolic compounds, particularly catechins, which are extracted into wine. Comparing wines made with and without destemming it was noted that the presence of stems contributed to a higher concentration of catechins and proanthocyanidins in

wine (Kovac et al., 1992). It has been reported that nearly all of the catechins and proanthocyanidins in stems are extracted into wine (Sun et al., 1999).

Adding progressively more seeds to fermenting wines increased the content of catechins and proanthocyanidins by 606 mg/L for each 60 g of seeds added per Kg grapes (Kovac et al., 1992). In a separate study the seed proanthocyanidins that were extracted into wines were found to be oligomeric, rather than polymeric (Sun et al., 1999).

1.4.3.3 Temperature

Thermovinification makes use of a particularly high temperature for a short period prior to fermentation. It is reported to increase the extraction of tannins and stabilise wine colour. However increasing the temperature to 70 °C for 1 h had no effect on the extraction of phenolics from grape seeds (Oszmianski et al., 1986). Indeed there was a greater release when the temperature reached only 35 °C. The concentration of anthocyanins in wine was shown to increase after thermovinification, but this was not sustained and levels decreased. However the final concentration of anthocyanins in a wine was dependent on the temperature during vinification (Gao et al., 1997).

Temperature is closely controlled throughout every stage of vinification as it can affect the phenolic concentration of a wine. At higher temperatures the extraction of phenolics, over 25 h, from Chardonnay skins was found to proceed at a greater rate (Ramey et al., 1986).

Phenolic compounds differ in their response to vinification temperatures. While syringic and *p*-coumaric acids were found in greater levels after vinification at 14 °C compared with 19 °C, the opposite was observed with ellagic and sinapic acids (Ramos et al., 1999).

1.4.3.4 Enzymes

The grape must, yeasts and lactic acid bacteria contain β-glucosidase among other enzymes. As many of the grape-derived phenolic compounds are present as sugar conjugates, it can be expected that the presence of β-glucosidase would have a significant influence on the phenolic profile of a wine.

The effect of commercially available enzyme preparations on the phenolic content of Pinot Noir and Cabernet Sauvignon wines was investigated (Wightman et al., 1997). Significant differences were observed between the preparations, and their effects on the two grape varieties. Three of the enzyme treated Pinot Noir wines had less malvidin conjugates than the control, however only two of the Cabernet Sauvignon treatments showed a similar effect. Quercetin glucuronide was also cleaved to the aglycone.

Esterase activity was observed with the formation of caffeic acid from caftaric acid. Enzyme activity has also been attributed to the reduction in the levels of resveratrol glucosides and the concomitant increase in the concentration of the aglycone, observed during fermentation (Mattivi et al., 1995; Vrhovsek et al., 1997).

Pectolytic enzymes can be added to a fermenting wine. They are able to breakdown sugars as well as help degrade tissues. Their presence increases the extraction of all phenolics (Pardo et al., 1999). In addition they aid the initiation of fermentation, make pressing easier and increase the alcohol content of a wine.

1.4.3.5 Ageing

Wines have been traditionally aged in wooden casks, particularly oak. The breakdown of lignans in wood leads to an increase in levels of benzoic acid, cinnamaldehyde and benzaldehyde derivatives (Soleas et al., 1997c). During

ageing polymeric anthocyanin pigments are formed from the condensation of anthocyanins with other phenolics (Haslam, 1998).

1.4.3.6 Others

The influence of fining agents gelatin and polyvinylpolypyrrolidone (PVPP) on the phenolic compounds of wines has been investigated. PVPP forms hydrogen bonds with compounds and precipitates them, so the greater the number of hydroxyl groups the more likely a compound is to be removed. *Cis* and *trans*-resveratrol were precipitated by PVPP, but there was no effect on the levels of the glucosides (Vrhovsek et al., 1997).

The addition of sulphur dioxide to a wine was shown to have little effect on the extraction of grape seed phenolics into wine, unless accompanied by additional alcohol (Oszmianski et al., 1986).

The presence of yeasts has been reported to be responsible for the falling concentration of anthocyanins during the early stages of fermentation (Mayen et al., 1994). The yeasts are speculated to retain the anthocyanins and similar observations have been made with other phenolics (Ramey et al., 1986).

1.5 Role of phenolics and disease prevention

Phenolic compounds were implicated in the protection against and prevention of diseases in humans before the active ingredients had been identified. Flavonoids were initially named vitamin P. Extracts of Hungarian pepper were noted to decrease the permeability of capillaries (Rusznyák and Szent-Györgyi, 1936). The active substances were identified as flavan or flavonol glycosides and named vitamin P. It was believed that vitamin P was required along with ascorbic acid to prevent the development of scurvy. However it was subsequently observed that flavonoids were not essential for human health and thus could not be described as vitamins. Although not essential elements in the diet their presence can have many beneficial effects.

Phenolic compounds are ubiquitously found throughout the plant kingdom. As such they are found in many plant-derived traditional remedies although their mode of action remains unknown in many cases. Their antioxidant activity is well established but a wide range of other activities has also been observed.

1.5.1 Anti-carcinogenic

The relationship between dietary phenolic intake and the development of cancer at various sites has been investigated (Knekt et al., 1997; Garcia-Closas et al., 1998, 1999). A cohort study of nearly 10 000 Finnish men and women compared their intake of flavonols and their risk of cancer over a period of 24 years (Knekt el al., 1997). An inverse relationship was observed between dietary flavonol intake and the development of cancer at all sites. This relationship was primarily attributed to a protection against lung cancer. In a recent Spanish study the risk of lung cancer was not observed to be attenuated by flavonol intake (Garcia-Closas et al., 1998). This study investigated only 103 cases (all women) and 206 controls, matched by age and residence. However a further case-controlled study with 354 cases of gastric cancer found that the intake of kaempferol offered protection (Garcia-Closas et al., 1999). High quercetin intake was noted to be associated with a lower risk of stomach cancer.

Although the epidemiological evidence for the prevention of cancer from dietary phenolics is contradictory, *in vitro* studies suggest that polyphenols can exert their anti-carcinogenic activity at various stages of tumor development (Stavric, 1994). Recent work using transgenic HTLV-1 (human T-lymphocyte virus type-1) mice has shown that red wine solids significantly delayed tumour onset (Ebeler et al., 1997). Although the actual wine components are unknown, work is underway to identify them. It has also been reported that rats fed a diet containing 5% quercetin had a 48% lower incidence of mammary cancer induced by 7,12-dimethylbenz[a]anthracene (Verma et al., 1988).

Polyphenols are potent antioxidants and scavenge free radicals, which can damage DNA and cause mutations. Phenolic compounds can also quench and inactivate carcinogens. Quercetin is able to suppress hydroperoxide induced cytotoxicity (Nakayama, 1994). Caffeic and ferulic acids are also reported to inhibit the formation of the carcinogenic nitrosamines by reacting with nitrate (Kuenzig et al., 1984).

1.5.2 Anti-inflammatory

The root of *Polygonum cuspidatum* Sieb. et Zucc. has traditionally been used for the treatment of many diseases including arteriosclerosis, hyperlipidemia, allergic and inflammatory conditions (Kimura et al., 1985). The active ingredient has been identified as *trans*-resveratrol and its glucoside. Resveratrol was noted to inhibit cyclo-oxygenase and lipoxygenase enzymes. These enzymes produce a range of compounds including the prostaglandins, thromboxanes and leukotrienes, which modulate inflammatory responses. Other phenolic compounds including quercetin and myricetin are also known to inhibit the cyclo-oxygenase and lipoxygenase enzymes (Koshuhara et al., 1983).

1.5.3 Anti-viral

Quercetin, morin and rutin have been shown to possess antiviral activity against eleven types of virus (Selway et al., 1986). In studies on Herpes Simplex Virus – Type 1 (HSV-1), a relationship was observed between the inhibition of the virus and the ability of the flavonoid to stimulate cAMP synthesis in cells (Mucsi and Pragai, 1985). In general the flavonoid antiviral activity is believed to due to their ability to bind to viral proteins and thus interfere with viral nucleic acid synthesis. In addition to this direct response phenolic compounds can enhance the anti-viral activity of other agents such as interferon and tumor necrosis factor (Formica and Regelson, 1995).

1.5.4 Estrogenic

The stilbene resveratrol has been reported to have estrogenic activity due to its structural similarity to the estrogenic agent diethylstilbestrol. Using estrogen-positive MCF-7 human breast cancer cells, resveratrol was found to competitively inhibit binding of ^{3}H-estradiol to type 1 estrogen receptors as well as activate the receptors. This ability to antagonise estrogen binding may have a role to play in the prevention or treatment of breast cancer (Williams et al., 1996). In countries with high intakes of soya-derived phytoestrogen isoflavones the development of osteoporosis, menopausal symptoms and breast and prostate cancer are relatively rare (Cassidy et al., 2000).

1.6 Antioxidant properties of phenolics

The production of free radicals is a natural response to the metabolism of oxygen. Free radicals can react with and damage DNA, proteins, and lipids and they have been implicated in a number of diseases including artherosclerosis, tumour production and Parkinson's disease. A free radical is a molecule or an ion that contains an unpaired electron. They can be highly reactive and undergo a range of reactions, which can either generate more radicals or dimerise and pair the free electron. Three of the most common radical reactions are shown in Figure 1.13.

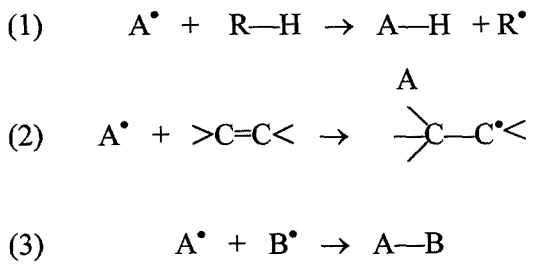


Figure 1.13. Common reactions of free radicals

Free radicals can be classified into oxygen, nitrogen, carbon and sulphur based molecules (Rice-Evans and Diplock, 1991).

- Oxygen based radicals include O_2 (dioxygen, two unpaired electrons), $O_2^{\bullet-}$ (superoxide), $HO_2^{\bullet}$ (hydroperoxyl), $RO_2^{\bullet-}$ (peroxyl) and $OH^{\bullet}$ (hydroxyl). The hydroxyl radicals are the most reactive and will react with the first available molecule. They can be produced from hydrogen peroxide (H_2O_2) through the action of transition metal ions.

- NO, previously known as endothelium derived relaxation factor (ERDF), has only recently been identified. It reacts rapidly with oxygen so will only be found in high levels when oxygen is absent.

- The most biologically important carbon centred radicals are the polyunsaturated fatty acid (PUFA) allyl radicals. This is the chemical basis of the oxidation of low density lipoprotein (LDL).

- The sulphur based radicals $RS^{\bullet}$ and $RS—SR^{\bullet-}$ are involved in cellular function.

While the body has an array of endogenous defences, dietary antioxidants have a vital role to play in quenching the radicals and in preventing damage. As well as the common dietary antioxidants, vitamins C and E, polyphenolic compounds are now believed to be involved in these processes.

1.6.1 Antioxidant actions of phenolic compounds

Polyphenolic compounds are purported to act as antioxidants through a number of roles.

1.6.1.1 Free radical scavengers

Phenolic compounds can scavenge or "mop-up" free radicals such as hydroxyl and peroxyl compounds. In addition to preventing the oxidation of proteins and other biological matrices, this action can prevent the oxidation of low-density lipoproteins (LDL). The uptake of oxidised LDL is implicated in the pathogenesis of artherosclerosis.

1.6.1.2 Metal ion chelation

In addition to the action of free radicals, LDL oxidation can also be initiated by the action of metals such as copper and iron. Both these metals can initiate chain reactions in the fatty acids present in LDL. Flavonoids are known to chelate metal ions and inhibit the metal induced oxidation of LDL.

1.6.1.3 Regeneration of vitamins E and C

Vitamins E and C are the traditional dietary antioxidants described. Phenolic compounds, particularly flavonoids, have been hypothesised to act as "secondary" antioxidants. Rather than scavenging free radicals themselves they act to regenerate vitamins E or C when they accept/donate an unpaired electron or hydrogen ion from a free radical.

1.6.2 Methods to determine antioxidant activity

A number of important factors must be taken into account when investigating the action of phenolic compounds as antioxidants in *in vitro* and *in vivo* conditions. It is necessary to ensure that the phenolic compounds do not react with other antioxidants present and affect their activity. In addition, once the phenolic compound has donated a hydrogen ion it becomes a free radical itself. It must be ensured that the phenolic free radical is not more active than the free radical that it has scavenged.

Free radicals are abundant in both hydrophilic and lipophilic phases. Vitamin E is a fat-soluble dietary antioxidant while vitamin C is water-soluble. Likewise some phenolics are likely to be active in only one phase.

Some antioxidant assays do not take account of the rate of reaction. They quantify the antioxidant activity of a compound at a particular time rather than observe it over the time period of the reaction until completion.

A number of techniques have been developed that can quantify the activity of an antioxidant. Not all methods are suitable for every antioxidant. While some techniques are based on a chemical reaction, others make use of a biological response. It must be noted that examining an antioxidant with a number of different assays can yield contradictory results. In a study comparing the deoxyribose hydroxyl radical assay and the peroxyl based AAPH method, 80% of wine deoxyribose antioxidant activity was lost with the removal of alcohol, compared with only 50% with the AAPH assay (Ghiselli et al., 1998).

1.6.2.1 Electron spin resonance spectroscopy

ESR spectroscopy has been applied to the assessment of the antioxidant activity of wines against a number of free radicals (Sato et al., 1995, Yamiguchi et al., 1999, Gardner et al., 1999).

This technique is only applicable to transactions involving unpaired electrons. The decay of radical resonance of either a water-soluble or organic-soluble radical is examined over time. This enable the kinetics and the stochiometry of the donation of the H^+ from the antioxidant to the radical to be determined (Duthie, 1999).

1.6.2.2 TEAC – trolox equivalent antioxidant activity

The TEAC assay was developed commercially by the Rice-Evans group based in Guy's Hospital, London (Miller et al., 1993). This approach has been used to determine the antioxidant activity of wines (Simonetti et al., 1997; Pelligrini et al., 2000). It determines the ability of hydrophilic H donating antioxidants to scavenge the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) radical compared with Trolox (a water soluble analogue of vitamin E). The $ABTS^{\bullet+}$ radical is not found naturally in the body. 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. In addition the $ABTS^{\bullet+}$ radical is strong compared with other oxidising species.

In the presence of such species most compounds will act as antioxidants (Duthie, 1999).

1.6.2.3 Deoxyribose method

The deoxyribose method assesses the ability of antioxidants to scavenge hydroxyl radicals. Hydroxyl radicals (generated via iron-EDTA/H_2O_2 reaction) react with deoxyribose, which can be treated with thiobarbituric acid (TBA) to yield a coloured product. The addition of a hydroxyl scavenging antioxidant will inhibit the colour formation enabling the rate of the reaction to be determined (Halliwell et al., 1987). The ability of wine fractions to scavenge hydroxyl radicals has been determined using this method (Ghiselli et al., 1998).

1.6.2.4 AAPH method

The ability of compounds to trap peroxyl radicals is assessed using the 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) assay. A steady stream of peroxyl 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; Natella et al., 1999).

1.6.2.5 DPPH method

The radical scavenging effect of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) has been used to determine the antioxidant activity of wines and phenolic compounds (Chen et al., 1997; Fauconneau et al., 1997; Larrauri et al., 1999).

As with many of the other antioxidant assays this method 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.

1.6.2.6 Hypoxanthine-xanthine oxidase system

The superoxide radical scavenging ability of a wine and other compounds can be determined using the hypoxanthine-xanthine oxidase superoxide generating system. A steady stream of superoxide ions is produced through the action of xanthine oxidase on hypoxanthine. The quenching of the radicals by antioxidants can be examined using a number of approaches including ESR (Sato et al., 1996), chemiluminescence (Hodgson and Fridovich, 1976) and spectroscopy via the reduction of nitro blue tetrazolium (Robak and Gryglewski, 1988).

1.6.2.7 Chemiluminescence

When a chemiluminescent substrate, luminol, is oxidised by hydrogen peroxide in a reaction catalysed by horseradish peroxidase, light is emitted. Light emission is dependent on the steady production of free radicals. The presence of an antioxidant will diminish the light emission. The length of time of light quenching is related to the amount of the antioxidant present (Whitehead et al., 1992). This approach has been used to determine the antioxidant capacity of plasma (Whitehead et al., 1992) and phenolic compounds (Heilman et al., 1995).

1.6.2.8 FRAP assay

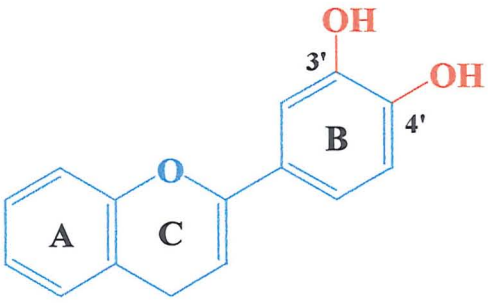
The ferric reducing antioxidant potential (FRAP) assay is a simple and inexpensive assay which can provide an index of antioxidant activity (Benzie and Strain, 1996). It describes the ability of a compound to reduce Fe^{3+} to Fe^{2+}. This reduction is accompanied by the formation of a blue colour in the presence of a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex.

1.6.3 Antioxidant activity of phenolic compounds

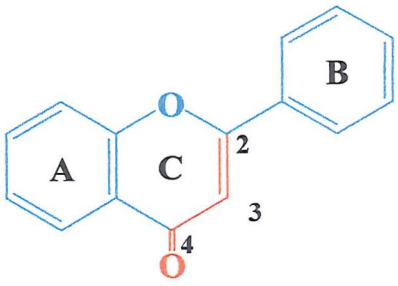
Polyphenols act as antioxidants as a result of their structure, particularly the number and orientation of their hydroxyl groups. The hydroxyl groups enable

the compound to donate H^+ and delocalise the resulting free electron, scavenge free radicals and provide a metal chelating capacity.

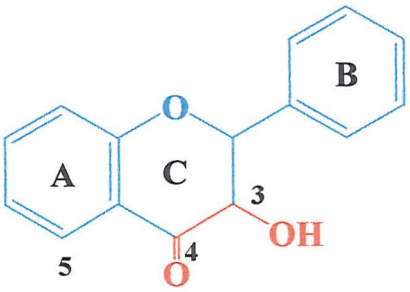
Studies have shown that quercetin is a potent antioxidant due to the number and distribution of its hydroxyl groups (Rice-Evans et al., 1995). (+)-Catechin has been reported to be the most active antioxidant of the polyphenolic compounds tested (Teissedre et al., 1996). The common flavonoids, myricetin, quercetin, rutin and gallic acid all showed a greater antioxidant activity than the conventional antioxidant vitamin α-tocopherol.



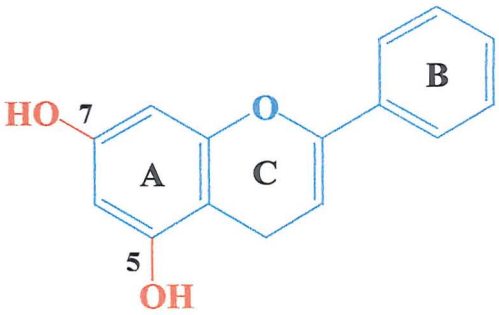
Scheme I
(3',4' dihydroxy)



Scheme II
(2,3 double bond + 4-one)



Scheme III
(3-OH and 4-one)



Scheme IV
(5,7-dihydroxy)

Figure 1.14. Structure-activity relationship of flavonoids.

The antioxidant activities of polyphenolic compounds, particularly flavonoids, are closely related to their structure. The important structural characteristics

responsible for the activities have been identified. They are presented in Figure 1.14 and include, in order of importance:

(I) 3′,4′ dihydroxy pattern in ring B (Scheme 1)

In general the greater the number of hydroxy groups on a structure, the greater the antioxidant activity (Salah et al., 1995). However particular combinations are found to offer greater activity. Quercetin has a 3′,4′ dihydroxy pattern and an antioxidant activity of 4.7 ± 0.1 Trolox equivalents (Rice-Evans et al., 1995). The addition of a hydroxy group on the 5′ position to produce myricetin decreases the activity to 3.1 ± 0.3 and the removal of the 3′ hydroxyl group to form kaempferol decreases the activity to 1.34 ± 0.08.

(II) 2,3 double bond and 4-one (Scheme 2)

The ability of polyphenols to accept or donate an electron has been attributed to the extensive conjugation possible between the B and C rings. Electrons can delocalise from the B ring through the 2,3 double bond of the C ring and down to the ketone group on the 4 position.

(III) 3-OH and 4-one (Scheme 3)

With many flavonoid compounds conjugation with sugar groups occurs preferentially at the hydroxyl group at the 3 position of ring C. Such conjugation has been noted to decrease the antioxidant activity of a compound compared with the aglycone. Quercetin was reported to have a TEAC value of 4.7 ± 0.1 compared with a value if 2.4 ± 0.1 for quercetin-3-rutinoside (rutin) (Rice-Evans et al., 1995).

(IV) 5,7 hydroxyl pattern in ring A (Scheme 4)

The *meta* arrangement of hydroxyl groups at the 5 and 7 positions of ring A are reported to increase the antioxidant activity of a compound. This is likely to be due to increased dissociation possible in the A ring (Rice-Evans et al.,

1997). The flavone apigenin has three free hydroxyl substituents at the 5,7,4′ positions compared with the isoflavone genistin, which only has hydroxyl groups at the 5 and 4′ positions. In the absence of a 5,7 dihydroxyl arrangement genistin has a TEAC value of 0.79 ± 0.04 compared wuth 1.45 ± 0.08 for apigenin.

1.6.4 Antioxidant activity of wines

Plant derived products, including wines contain a range of phenolic compounds and exhibit antioxidant activity. Many of the techniques described above have been used to assess the activity of wines and investigate the relationship between the phenolic content of a wine and its antioxidant activity.

The antioxidant activity of a wine is known to be closely related to its total phenolic content (Sato et al., 1996; Paquay et al., 1997; Simonetti et al., 1997; Gardner et al., 1999). The superoxide scavenging activity of wines correlated strongly with Folin-Ciocalteu total phenolics (r = 0.991) but only slightly with the anthocyanin content, estimated by the optical density (OD) at 520 nm (r = 0.752). Further studies investigated the contribution of flavonols and flavan-3-ols to the total phenol derived antioxidant activity (Simonetti et al., 1997). Flavonols contributed less than 3% to the total antioxidant activity of wine, whereas the total flavan-3-ols were strongly correlated (r = 0.927). Gardner et al. (1999) has also reported the minimal contribution of flavonols to the antioxidant activity of wine using an ESR system to quantify the H^+ donating capacity of phenolics.

Fractionation of a wine enabled the antioxidant activity of anthocyanins, proanthocyanidins and phenolic acids to be investigated using two free radical generating systems, hydroxyl and peroxyl (Ghiselli et al., 1998). The anthocyanin fraction was effective against both the radicals while the proanthocyanidins inhibited only hydroxyl production. The phenolic acids had only a slight inhibitory effect on both fractions. The antioxidant activity of the anthocyanin fraction was not only due to their abundance (70% of total

phenolics), but the anthocyanins had a higher antioxidant efficiency than the other fractions. Further work is necessary to determine what are the major contributors to the antioxidant activity of wine.

1.6.5 Antioxidant activity of plasma after wine consumption

Plasma contains endogenous antioxidant defences such as superoxide dismutase and glutathione transferase. Dietary antioxidants such as vitamins C and E and the carotenoids also contribute to the antioxidant activity of plasma. Vitamins E and C, urate and thiol groups are reported to contribute 67% of the total antioxidant activity of plasma, with the carotenoids, retinol, bilirubin and polyphenols providing the balance of 23% (Ghiselli et al., 1997).

The antioxidant activity of plasma can be assessed and quantified using the methods detailed above and the effect of the consumption of foods and beverages, including wine, investigated. Using the chemiluminescence assay the antioxidant activity of serum was shown to increase rapidly to a maximum at 90 min after the consumption of Bordeaux red wine (5.7 mL/kg). Levels remained high even 4 h after consumption (Maxwell et al., 1994).

In a further study by the same group nine subjects consumed 300 mL of red wine. The antioxidant activity increased from average basal levels of 486 to 572 μmol/L after 1 h. The increase in antioxidant activity was noted to be independent of body weight (Whitehead et al., 1995).

The effect of the ingestion of water, alcohol free red and white wine on the antioxidant activity of plasma has been compared (Serafini et al., 1998). Consumption of alcohol free red wine increased the plasma antioxidant activity, reaching a peak after 50 min. No effect was observed with alcohol free white wine and water. Plasma levels of polyphenols were noted to reach a maximum at 50 min after alcohol free red wine consumption. Polyphenol levels were not affected by white wine or water consumption.

The increase in plasma antioxidant activity after wine consumption is related to the intake of polyphenolic compounds rather than the effect of alcohol. This was reaffirmed by investigations into the effect of the consumption of concentrated grape juice.

Grape juice (125 mL) was consumed daily for seven days by seven subjects (Day et al., 1997). Antioxidant activity was determined by chemiluminescence at 0, 30, 60, 240, 480 min and 7 days after the start of consumption. Maximum levels of antioxidant activity were recorded at 60 min (478 μmol/L) compared with 441 μmol/L at time 0. After seven days basal levels of plasma antioxidant activity had increased by 50 μmol/L.

Regular consumption of grape juice and alcohol free red wine will increase plasma levels of polyphenolic compounds that concomitantly increase plasma antioxidant activity, without the deleterious effects of alcohol.

1.7 Wine phenolics and coronary heart disease

The death rate from coronary heart disease (CHD) in England and Wales is 448 and 167 per 100,000 for men and women respectively, aged 35-74. This is compared with 101 and 32 for men and women in the South of France. This huge difference between the two populations may be due to cultural variations in genetics, lifestyle factors and diet.

Atherosclerosis is due to a combination of hyperlipidemia and the oxidation of lipoproteins. These conditions may arise due to genetic predisposition. Lipid metabolism may be abnormal and/or the processes controlling and preventing excessive oxidation may be at fault. Altering the dietary fat intake can act to lower plasma lipid levels and decreases the occurrence of CHD. The dietary antioxidant vitamin E is known to prevent the oxidation of lipoproteins. Recent studies have shown that phenolic compounds are potent antioxidants both *in vitro* and *in vivo*.

1.7.1 Development of coronary heart disease.

The exact chemical nature of the pathogenesis of atherosclerosis remains unknown. However the major mechanisms have been described (Fig. 1.15). Low density lipoproteins (LDL) present in blood bind to glycoproteins on the arterial walls. They are internalised, trapped and begin to accumulate in the vessel cell wall. At this stage LDL is oxidised through lipid peroxidation. Oxidised LDL binds to monocytes and is accumulated internally. This leads to the production of cytokines. These 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.

1.7.2 Alcohol and CHD

The ever-increasing human and economic cost of CHD has prompted extensive investigations into the associated risk factors such as diet, exercise, lifestyle and alcohol. Large-scale mixed population studies have consistently demonstrated a negative correlation between alcohol consumption and the risk of CHD. A U-shaped correlation was observed, with consumers of up to 2 drinks daily having a lower overall mortality rate compared with abstainers or heavy drinkers (Klatsky et al., 1997). This inverse association has been reported to be independent of sex, age, smoking habits and exercise (see Goldberg, 1995).

It is now well established that the intake of alcohol can protect against the development of CHD. The effect of the type of alcoholic beverage consumed on the incidence of CHD has been investigated. A number of thorough reviews have been published in this field (Gaziano et al., 1996; Kannel and Ellison, 1996; Rimm et al., 1996; Gustafsson and German, 1997; Cao and Prior, 2000).

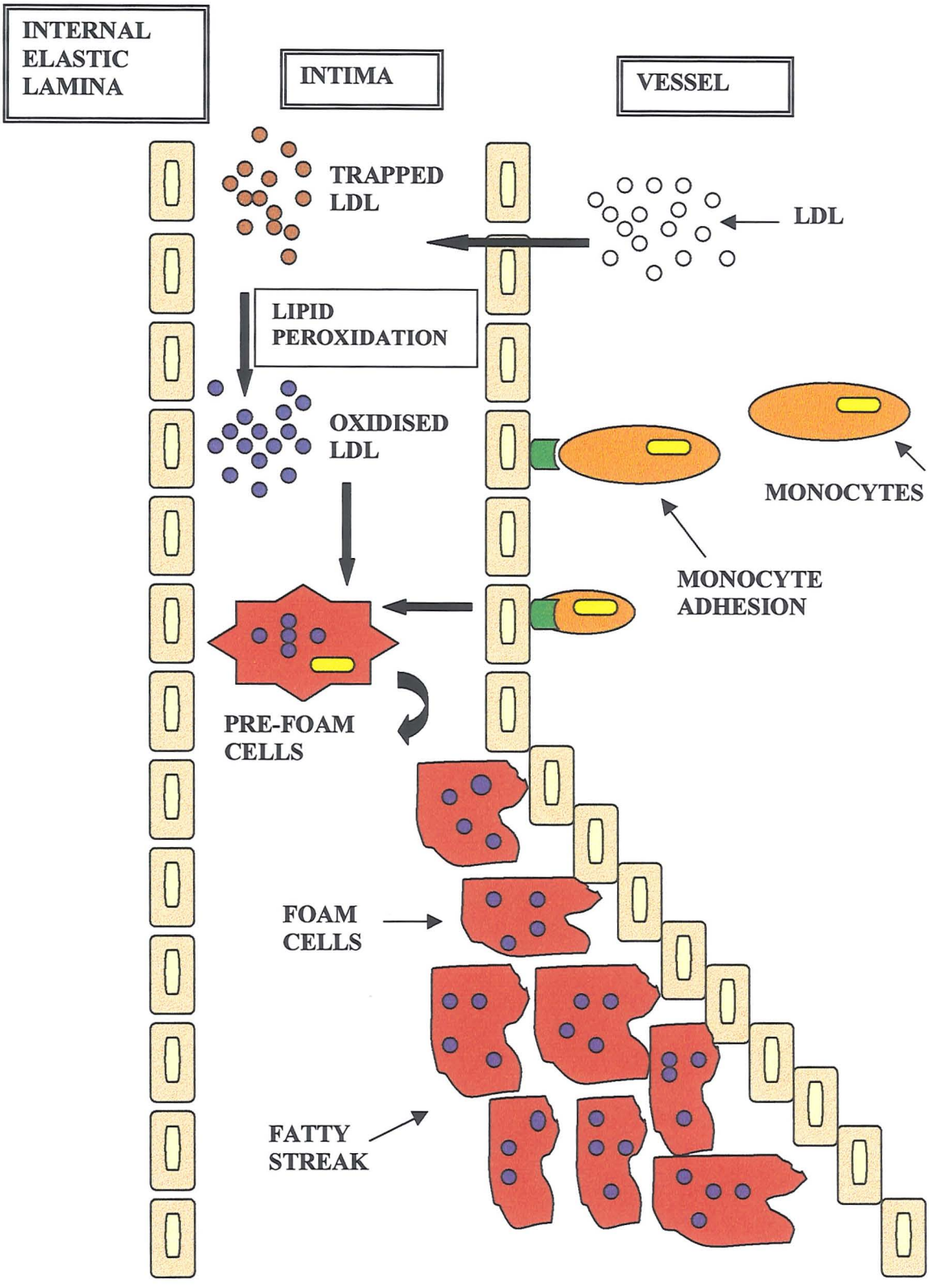


Figure 1.15. Pathogenesis of atherosclerosis

Alcohol can be consumed in the form of wine, beer or spirits. Investigations into the effect of alcohol type and CHD do not present a clear picture. In some studies the greatest beneficial effect of an alcohol type are found with beer (Stampfer et al., 1988) or spirits (Rimm et al., 1991). However one of the earliest studies in the field compared the effect of red wine, white wine, alcoholic water, whisky and beer on the development of atherosclerosis in rabbits (Klurfeld and Kritchevsky, 1981). The consumption of red wine significantly reduced the development of atherosclerosis while alcoholic water, white wine and whisky caused only a small reduction. Intake of beer had no significant effect.

Similar results were observed in a large-scale Danish prospective study (Grønbæk et al., 1995). The intake of beer or spirits was not associated with a reduction in the risk of mortality from CHD. However the consumption of a moderate amount of red wine (3-5 drinks/day) reduced the relative risk of mortality from 1.00 for abstainers to 0.51.

Alcohol consumption is thought to offer protection against CHD through a number of complementary mechanisms. It is known to increase levels of high density lipoproteins (HDL), which is involved in the transport and excretion of exogenous cholesterol via the liver (Gaziano et al., 1993). By removing excess cholesterol from circulation, its accumulation on the arterial wall is reduced. Aggregation of platelets and the resulting thrombotic tendency is also inhibited by alcohol intake (Gryglewski et al., 1987; Pace-Asciak et al., 1995).

1.7.3 Epidemiological investigations

Red wine has been reported to offer protection above and beyond that due to alcohol alone. Investigating death from CHD in 18 countries it was observed that while there was a positive association with saturated fat, a strong negative association was observed when wine consumption was taken into account (St Leger et al., 1979). Renaud and de Logeril (1992) went on to publicise this popular association by highlighting the 'French Paradox'. In general the

mortality rate from CHD is positively correlated with the consumption of dietary fat. However in certain cities in France, notably Toulouse, although the saturated fat intake remains high, there is a low mortality from CHD. In addition it was noted that blood pressure and serum cholesterol levels were no different to other countries. One explanation for this paradox was that the Mediterranean diet traditionally found in France, along with the regular wine intake offered a form of protection. The active components of the wine, and also the diet, have been hypothesised to be the antioxidant phenolics.

Phenolic compounds are also widely dispersed throughout many commonly consumed fruits and vegetables. The dietary flavonoid intake of 805 Dutch men (aged 65-84) was assessed over a five-year period and correlated with their medical history. The consumption of quercetin, and four other flavonoids, was found to be inversely correlated to the death from, and the development of, CHD (Hertog et al., 1993a). Similar observations were reported in an extended study that examined the intake of flavonoids and the risk of CHD in 16 cohorts in seven countries (Hertog et al., 1995). Mortality from CHD was assessed over a 25 year follow up period. Differences in CHD mortality between the populations could be partially explained by variations in flavonoid intake. The incidence of smoking and intake of saturated fat were also important factors.

Contradictory observations were reported in a 6 year prospective study of nearly 40 000 male health professionals (Rimm et al., 1996). A significant inverse association was not observed between the dietary intake of flavonoids and subsequent rates of mortality from CHD.

1.7.4 In vitro studies on the inhibition of LDL oxidation

Intake of phenolic compounds is widely reported to protect against the development of CHD. The mechanism of action of phenolic compounds is under investigation. However there is considerable evidence that phenolic compounds can prevent the oxidation of LDL. Generally the oxidation of LDL is assayed by static headspace gas chromatography (GC) of the hexanal

produced (Meyer et al., 1997) or by changes in the absorbance of conjugated dienes (Frankel et al., 1993a), formed by the copper catalysed oxidation of human LDL.

Red and white wines have been reported to inhibit LDL oxidation *in vitro* (Frankel et al., 1993a, 1995; Kanner et al., 1994; Teissedre et al., 1996; Satùe-Gracia et al., 1999). The wines are tested at a standardised concentration of 5, 10 or 20 μM GAE. An investigation of 20 Californian wines, standardised to 10 μM 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). Spanish sparkling white wines were also reported to inhibit LDL oxidation to a similar extent as red wines (Satùe-Gracia et al., 1999).

The ability of grapes and grape juices to inhibit LDL oxidation has also been investigated (Meyer et al., 1997; Frankel et al., 1998). Twelve *Vitis vinifera* grapes were extracted and at concentrations of 10 μM GAE were found to inhibit LDL oxidation by between 22 and 60%. At 20 μM the extent of inhibition increased to between 62 and 91% (Meyer et al., 1997). Commercial grape juices (10 μM) inhibited oxidation by between 62 and 75% (Frankel et al., 1998). Highest inhibition was observed with white grape juices (70-75%). The inhibition of LDL oxidation by wines, grape juices and grapes has been attributed to their total phenolic content. The inhibition of LDL 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 ($r = 0.56$, $p < 0.05$) (Meyer et al., 1997).

The correlation between the antioxidant activity of Californian wines and LDL oxidation was 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 strongly correlated with the prevention of LDL oxidation. The ability of pure phenolic compounds to inhibit LDL oxidation has also been investigated. One of the original studies in this field demonstrated that the presence of quercetin prolonged the oxidation lag phase of LDL by around 4 h (de Whalley et al., 1990). Other studies with *trans-*

resveratrol, quercetin and (-)-epicatechin have reported on their ability to inhibit LDL oxidation. Quercetin and (-)-epicatechin were observed to have twice the inhibition potential of *trans*-resveratrol (Frankel et al., 1993b). A recent investigation of structurally similar phenolics noted that (+)-catechin, quercetin, cyanidin and caffeic acid all had high antioxidant activities against LDL oxidation and functioned in a dose-dependent manner (Meyer et al., 1998).

The mechanism of action of the phenolics against LDL oxidation remains uncertain. The phenolics may act to re-generate vitamin E, which acts as an endogenous antioxidant in LDL, by donating H^+ to the oxidised vitamin E. It may also be preferentially oxidised to spare the endogenous vitamin E. Additionally the metal ion chelating activity of the phenolics may prevent copper and iron catalysed lipid peroxidation.

1.7.5 In vivo studies on the inhibition of LDL oxidation

Few studies have reported on the effect of red wine consumption on the oxidation of LDL *in vivo*. Those that have been published have presented conflicting results.

One of the original studies in this field compared the effect of red and white wine consumption on the susceptibility of LDL to oxidation (Fuhrman et al., 1995). LDL oxidation was determined by a number of methods including the formation of conjugated dienes and lipid peroxidation levels.

Consumption of 400 mL/day red wine for 2 weeks was found to decrease the ability of LDL to undergo copper-induced lipid peroxidation. There was a 72% decrease in the content of lipid peroxides and a 54% decrease in the formation of conjugated dienes. White wine consumption resulted in an increase in the propensity of LDL to undergo oxidation. After two weeks of red wine consumption the concentration of total phenols in LDL increased by 4-fold. No changes were noted in levels of the plasma antioxidant vitamins E and C. The protective effects of red wine consumption are attributed to the

content of polyphenolic compounds, which are considerably lower in white wine. The pro-oxidant effect of white wine may be as a result of the combination of the alcohol content of the wine and the absence of the protective polyphenols.

The effect of alcohol was eliminated in a later study that reduced the alcohol content of red and white wine to 3.5% (de Rijke et al., 1996). Subjects followed a low flavonol diet to avoid interference from food-derived phenolics. Subjects drank 550 mL of white wine for 2 weeks prior to the study, which served as a washout period. They then consumed either red or white wine for another 4 weeks. LDL oxidation was determined by the formation of conjugated dienes. No effect on the oxidizability of LDL was observed after consumption of either wine. It was speculated that the removal of alcohol might have impeded the absorption of the phenolic compounds.

A recent study set out to investigate the contradictory results produced by the above groups (Nigdikar et al., 1998). Subjects consumed either red wine, a capsule of powdered red wine polyphenols, the same extract dissolved in either white wine or an alcoholic drink, for two weeks. After this period the levels of polyphenols in plasma increased by 38% for red wine, and 27 and 28% for the 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 extract. The results of this group confirm the protective effects of red wine observed by the Israeli group (Fuhrman et al., 1995).

Nigdikar et al. (1998) proposed that the lack of protection observed in the Dutch study (de Rijke et al., 1996) was due to technical differences in the analysis of LDL oxidation. After the collection of plasma or LDL EDTA can be added to prevent any oxidation by removing copper or iron. The EDTA is removed prior to analysis of the LDL by either dialysis or an absorption column. The Dutch group however added excess copper to overcome the effect of EDTA. This action may have allowed the EDTA present to inhibit the copper induced oxidation of LDL.

Further studies are necessary to completely clarify the situation and to determine the mechanism through which the red wine polyphenols function.

1.8 Phenolics and vascular function

The inability of blood vessels to contract and relax efficiently is implicated in the development and progression of vascular diseases such as atherosclerosis, hypertension and diabetes. The vascular endothelium forms a barrier between the vascular smooth muscle and the flow of blood. It controls the contraction and relaxation of the vascular muscle by responding to stresses and stimuli.

Nitric oxide (NO) is produced by the vascular endothelium and induces an endothelium-dependent relaxation of blood vessels. Compounds that can generate increased levels of nitric oxide may have a therapeutic role in the prevention and treatment of medical conditions involving vascular dysfunction.

Certain plant extracts, including wine, have been shown to stimulate the generation of NO, and its secretion by the endothelial cells of the blood vessel wall *in vitro* resulting in vasodilation.

1.8.1 Mechanism of action of phenolics and vasodilation

The mechanism by which phenolic compounds cause vasodilation has yet to be fully elucidated. Two pathways have been reported, endothelium dependent and endothelium independent. Evidence to date suggests that while the endothelium dependent pathway requires the synthesis of NO to catalyse smooth muscle contraction, the endothelium independent mechanism involves the direct action of the stimulus on the smooth muscle (Huang et al., 1999).

Phenolic compounds appear to activate vasodilation of endothelium-denuded aortic rings only at high concentrations (Chen and Pace-Asciak, 1996). It has been speculated that the endothelium independent activity of phenolic compounds may be as a result of their inhibition of cyclic nucleotide

phosphodiesterases which break down cAMP and cGMP (Andriambeloson et al., 1997). By inhibiting their breakdown cAMP (cyclic adenosine monophosphate) and cGMP (cyclic guanosine monophosphate) these compounds accumulate and result in smooth muscle relaxation. However the endothelium dependent vasodilation takes place in the presence of phenolic compounds at concentrations comparable to those found *in vivo*.

Phenolic compounds induce endothelium dependent relaxation by NO. NO is constitutively produced at basal levels in the endothelium from L-arginine by NO synthase. NO subsequently activates guanylate cyclase in the vascular smooth muscle to produces cyclic guanosine monophosphate and protein kinase G. Muscular contraction is inhibited by protein kinase G through its action on the muscle myosin-actin complex (Fig. 1.16).

Ca^{2+} has been reported to be essential for NO mediated endothelium dependent vasodilation. Removing Ca^{2+} from the reaction media abolishes the effect of phenolic compounds. They are believed to act to encourage the entry of Ca^{2+} from the reaction mixture into the intracellular environment (Huang et al., 1999; Andriambeloson et al., 1999).

Nitric oxide has a very short half-life and is known to react rapidly with superoxide to form peroxynitrite. This reactive compound can subsequently nitrate and damage other biological comounds (Mateo et al., 2000).

Phenolic comounds are reported to prevent the inactivation of the vasoactive NO, and the damaging nitration through two separate mechanisms. Superoxide ions are quenched by phenolic compounds and are prevented from removing NO from the site of vasorelaxation (Hodgson and Fridovich, 1976; Robak and Gryglewski, 1988; Sato et al., 1996). Hydroxycinnamates are reported to inhibit the peroxynitrite dependent nitration of tyrosine, by either preferential nitration or electron donation (Pannala et al., 1998).

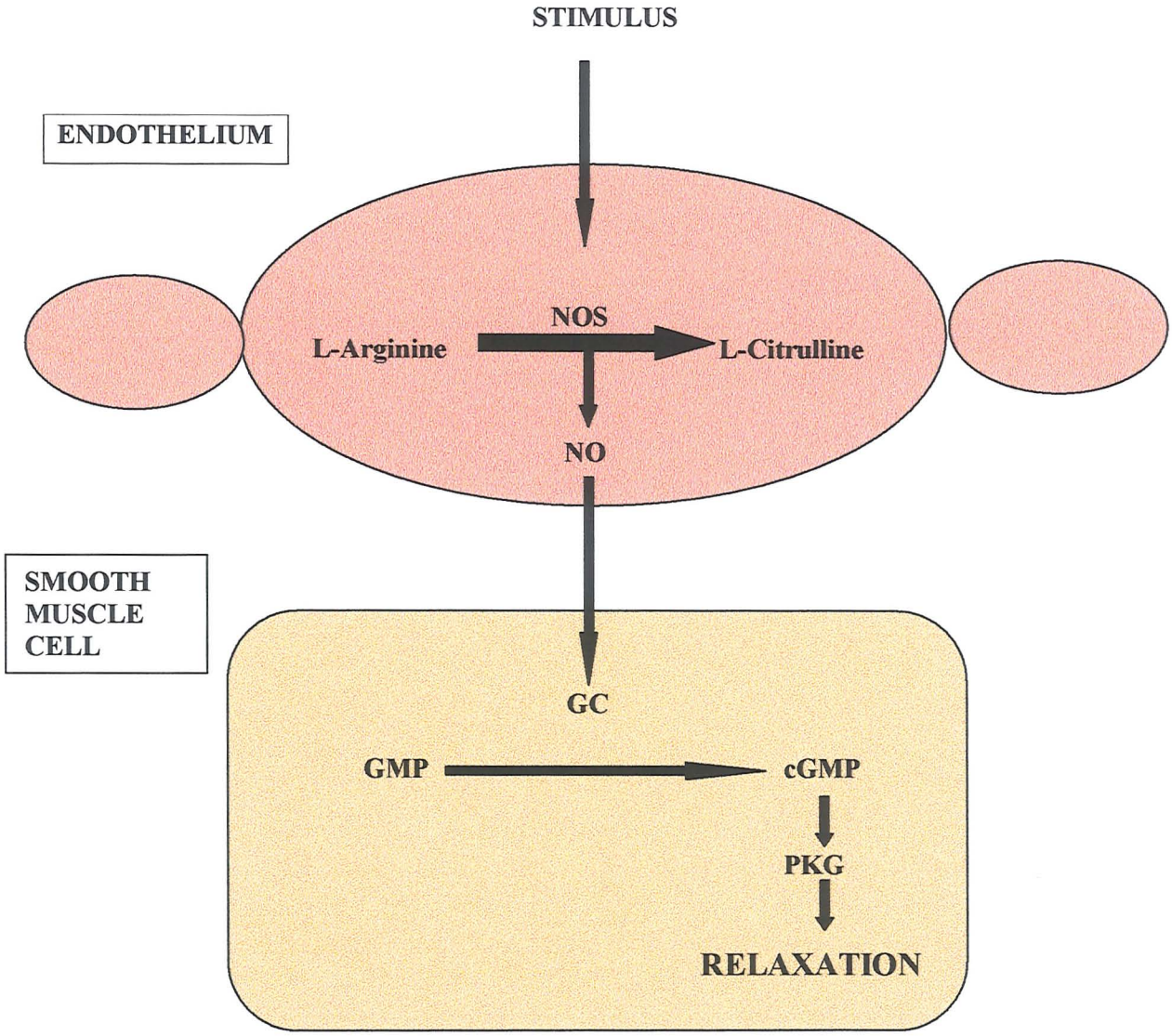


Figure 1.16. Pathway mediating vascular smooth muscle relaxation

NOS, nitric oxide synthase; NO, nitric oxide; GC, guanylate cyclase; GMP, guanosine monophosphate; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G.

1.8.2 Vasodilation capacity of grape and wine phenolics

It has been speculated that the beneficial effects of wine on the risk of coronary heart disease could be due to their vasodilation capacity in addition to their antioxidant activity.

A number of studies have investigated the vasodilation activity of a range of plant extracts, including red wine, grape juice and grape skin extract (Fitzpatrick et al., 1993, 1995; Andriambeloson 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).

Pulp and skin extracts of red and white grapes were tested in the same system. Neither pulp extract produced a vasodilatory effect, however the skin extracts resulted in relaxations of 100 and 96% for red and white grapes respectively. This suggests that although both red and white grapes have the capacity to produce vasodilation, only red wine will show a similar effect. The active components are skin derived, and white wine contains little skin-derived phenolic as opposed to red wine, due to the minimal grape skin extraction during white wine vinification. Ethanol at concentrations comparable to a typical wine showed no relaxation capacity.

The identity of the active ingredients in grape skins and red wines remains under investigation. A powdered extract of red wine polyphenols was separated into ten fractions using a Sephadex LH-20 column. The phenolics were separated on the basis of their molecular weight. Each fraction was investigated for its ability to relax pre-contracted aorta (Andriambeloson et al., 1998). The fraction containing the large tannins showed no vasodilation activity. However those fractions containing the oligomeric tannins (di-, tri- and tetramers of the catechins) and the anthocyanins showed similar vasodilation activity as the original wine extract. The anthocyanin-containing fraction was more potent than the oligomeric tannin fraction.

1.8.3 Vasodilation capacity of pure dietary phenolics

The ability of individual phenolic compounds to relax pre-contracted aortic rings has been investigated in an attempt to identify the active ingredient responsible for the vasodilation capacity of grapes and wines.

Contradictory results have been obtained for several of the common wine phenolics. In one of the original studies in this field *trans*-resveratrol was found to show no vasodilation capacity, while quercetin produced maximal dilation through a non-NO dependent mechanism (Fitzpatrick et al., 1993). In a later study both quercetin and *trans*-resveratrol were reported to cause endothelium dependent and independent dilation (Chen and Pace-Asciak, 1996). The endothelium independent dilation was observed with only very high concentrations of phenolics. The contradiction was attributed to the low Ca^{2+} concentration in the earlier study and their use of anaesthetics on the rats.

Individual anthocyanins have also been investigated (Fitzpatrick et al., 1993; Andriambeloson et al., 1998). In both studies only the aglycones malvidin, cyanidin or delphinidin were used. Only delphinidin elicited a vasorelaxation response (Andriambeloson et al., 1998). However, in wines anthocyanins are primarily found as sugar conjugates with malvidin conjugates present in significantly higher levels than the other compounds. Delphinidin was reported to produce a response comparable to the wine extract, however delphinidin and its conjugates are minor components of wine and on this basis is unlikely to be the active component.

To date no studies investigating the condensed tannins, the compounds found in the active fractions of the wine extract, have been reported. The monomer (+)-catechin is reported to only produce a relaxation, endothelial dependent or independent at high concentrations (Andriambeloson et al., 1997). However green tea derived (-)-epicatechin induces a NO mediated endothelium dependent relaxation (Huang et al., 1999). Tannin acid, also known as the hydrolysable tannin pentagalloglucose is reported to produce maximum vasodilation (Fitzpatrick et al., 1993).

It is apparent that much work is still required in order to identify the nature of the compound(s) responsible for the relaxation activity of wines. Investigations using the anthocyanins and purified condensed tannin (proanthocyanidins) will serve to clarify the picture.

1.8.4 Vasodilation capacity of other plant extracts

In addition to grapes and wines, other foods and plant-derived products have been found to induce a relaxation of pre-contracted aortic tissue. The foods investigated included extracts of fruits, vegetables, teas, herbs and spices (Fitzpatrick et al., 1995).

Table 1.4. Vasodilation activity of fruit and vegetable extracts

Extract	Percentage relaxation
Vegetables	
Corn	72.2 ± 20.3
Lima beans	56.5 ± 10.5
Aubergine (skin)	77.7 ± 6.2
Fruits	
Cranberries	87.4 ± 6.7
Red Apples (skin)	92.0 ± 0.8
Red Apples (pulp)	81.2 ± 15.4
Plum (skin)	88.1 ± 4.3
Plum (pulp)	92.6 ± 2.0
Tomato (skin)	10.1 ± 2.5
Tomato (pulp)	5.0 ± 2.6
Strawberry	0.0 ± 0.0
Nuts, herbs and spices	
Peanut (skin)	97.0 ± 3.0
Peanut (meat)	60.1 ± 10.3
Garlic (fresh extract)	64.8 ± 8.5
Garlic (Kwai extract)	58.0 ± 8.6
Cinnamon	98.1 ± 1.9
Bilberry	79.9 ± 4.5
Pepper (red)	18.2 ± 4.8
Pepper (white)	17.0 ± 2.2
Pepper (black)	0.0 ± 0.0

Greater than 50% relaxation was observed with extracts of corn, lima beans, cranberries and aubergine skins. Of the nuts investigated the greatest vasodilation capacity was obtained with peanut skins (97%), compared with

60% for the meat. In general nut skins proved to be very active. Likewise the herbs and spices were particularly active. Garlic extracts induced both a 58% and 65% relaxation, with cinnamon and bilberry producing relaxations of 78 and 98% respectively. Pepper had a very poor vasodilation capacity, inducing only 18, 17 and 0% relaxation for red, white and black pepper respectively.

Many of the fruits had high vasodilation capacity with a number of unexpected exceptions. Strawberries produced no relaxation even though they contain anthocyanins that are reported to be vasoactive components in wine. Likewise tomatoes showed very little activity in either their skins or pulp. However apples and plums, particularly their skins, were very active, producing 92 and 88% relaxation respectively.

1.9 Absorption and metabolism of phenolics

Although epidemiological and *in vitro* evidence strongly indicates an important pharmacological role for polyphenols, there is considerable conflicting data on both the ability and the extent of their absorption and subsequent metabolism. Originally much of the interest in the absorption of phenolic compounds arose from studies into their toxicity. To this end animals were fed compounds at doses far exceeding that which could be obtained from the diet. The value of such studies is questionable as the mechanism for absorption could be limiting, and the enzyme systems used to metabolise the phenolic compounds may become saturated. The information on levels of circulating and eliminated phenolic compounds and their metabolites does not represent what would happen under normal dietary conditions. However recent years have seen intervention studies with phenolic compounds from food sources or pure compounds at a concentration comparable to that found in the diet.

1.9.1 Flavonoids

The majority of work on polyphenol absorption has focused on flavonoids, particularly the flavonol quercetin and its conjugates. Recently interest has focused on the flavan-3-ols due in part to their large consumption from tea.

1.9.1.1 Flavonols

Initial studies on the absorption of flavonoid compounds were contradictory. It was originally suggested that flavonoid glycosides, in contrast to the aglycone were precluded from intestinal absorption due to the presence of sugar residues (Kühnau, 1976). Brown (1980) advanced on this idea and suggested that the hydrophilic nature of the glycosides and their relatively high molecular weights were responsible for their poor absorption in the small intestine. Although Kühnau believed the flavonoid aglycones to be absorbed, it was subsequently reported that after oral administration of quercetin no measurable plasma concentration could be detected (Gugler et al., 1975). However the limit of detection in this study was 100 ng/mL compared with 5 ng/mL achievable now.

There is currently a significant body of evidence supporting the theory that the flavonol conjugates are preferentially absorbed, and that the nature of the conjugation may be important (Hollman et al., 1995). Ileostomy subjects consumed either an onion test meal or capsules containing quercetin or quercetin rutinoside. It was found that 52 ± 15% of the quercetin glucosides from onions, 17 ± 15% of quercetin rutinoside and 24 ± 9% for quercetin were absorbed. Absorption of quercetin was defined as (oral intake – ileostomy excretion), and was corrected for 14% degradation within the ileostomy bag. In studies on the absorption and excretion of flavonols after onion consumption, it has been shown that quercetin-4′-glucoside and isorhamnetin-4′-glucoside accumulate in human plasma and are excreted in urine to a much greater extent than their aglycones (Aziz et al., 1998; Aziz, 2000). Conjugated quercetin can be similarly detected in human plasma after the consumption of red wine (Crozier et al., 2000).

A recent report determined that although conjugation with different glycosides can affect the absorption of quercetin, quercetin glucosides are efficiently absorbed independently of the glucose group (Olthof et al., 2000). The site of absorption of flavonol conjugates is dependent upon the nature of the conjugation. A time course of the appearance of quercetin in plasma after the consumption of quercetin-3-glucoside or quercetin-3-rutinoside suggests that while quercetin-3-glucoside is absorbed from the small intestine, quercetin-3-rutinoside is absorbed later from the colon after deglycosylation. It has been proposed that quercetin glucosides are absorbed across the small intestine by interacting with the sodium-dependent glucose transport receptors (Gee et al., 1998).

1.9.1.2 Flavan-3-ols

In general flavan-3-ols are found in plasma methylated and/or conjugated to sulphate or glucuronic acid (Lee et al., 1995; Piskula et al., 1998). Glucuronidation is the first step in the metabolism of the tea-derived polyphenol (-)-epicatechin. Within 30 min of ingestion 90% of the total (-)-epicatechin metabolites were glucuronidated. The site of action is the intestinal mucosa (Piskula et al., 1998). Sulphate conjugation takes place in the liver along with methylation, although the latter also occurs in the kidney. While methylation is reported to take place on the 3′ carbon of the flavan-3-ol, no information is available on the nature of sulphate and/or glucuronide conjugation.

Significant circulatory plasma levels of flavan-3-ols have been reported after the consumption of decaffeinated tea. EGCG, EGC and (-)-epicatechin were detected in plasma after four volunteers consumed 1.2 g of green tea in 200 mL of water. The tea contained 88 mg of EGCG, 82 mg of EGC, 33 mg of ECG and 32 mg of (-)-epicatechin. Maximum polyphenol plasma concentrations were found after 1 h. Levels of EGC varied from 82-206 ng/mL compared with 48-80 ng/mL for (-)-epicatechin and 46-268 ng/mL for EGCG. The polyphenols were found conjugated to sulphate and glucuronic acid. The nature and extent of conjugation was characteristic of the individual

polyphenols. Two-thirds of (-)-epicatechin was found conjugated to sulphate and one third conjugated to glucuronic acid. In comparison between 57 and 71% of EGC was glucuronidated, 23-36% sulphated and 3-13% free. Sulphate conjugates accounted for between 58 and 72% of EGCG metabolites, 12-28% were free and 8-19% were conjugated to glucuronic acid. However this study did not describe the conjugation of polyphenols with both sulphate and glucuronide, or the appearance of methylated metabolites.

A recent study has demonstrated the presence of (+)-catechin metabolites in human plasma after the consumption of red wine or de-alcoholised red wine. Maximum levels of 50-170 nmol/L of total (+)-catechin metabolites were recorded after 1 h. Although free (+)-catechin and its metabolite, 3′-*O*-methylcatechin were detected, their levels were low compared to those of (+)-catechin sulphate and/or glucuronide conjugates (Donovan et al., 1999). After 1 h free (+)-catechin was <2% of the total unmethylated metabolites, while 3′-*O*-methylcatechin was only ca. 7% of the methylated metabolites. Differences were also observed in the elimination of the metabolites. The sulphated conjugates were rapidly eliminated, followed by the methylated metabolites. The unmethylated compounds had the longest half-life. No significant differences were observed in the absorption and metabolism of flavan-3-ols between the red wine and the de-alcoholised red wine. However the half-life of total catechins and unmethylated metabolites were longer in the absence of alcohol. Similar findings were reported in a later study by the same group (Bell et al., 2000). The presence of alcohol increased the half-life of (+)-catechin by 22%.

Very little is known about the absorption and metabolism of proanthocyanidins in animals and man. The limited availability of pure standards has made feeding studies extremely difficult.

1.9.1.3 Anthocyanins

Reliable studies into the absorption and metabolism of anthocyanins have been limited. Some of the original investigations in this field used impure extracts

from anthocyanin containing fruits and provided no information on the identity of the anthocyanins present. Absorption was confirmed by the presence of anthocyanin-like absorbing compounds in acidified urine and plasma (Clifford, 2000a). Even with the advances in detection methods (i.e. mass spectroscopy) recent publications still make use of such vague approaches to the identification of compounds (Paganga et al., 1997; Lapidot et al., 1998). In non-supplemented volunteers anthocyanins were detected, but not characterised, in plasma. Identification was on the basis of an absorbance maxima in the region of 520 nm (Paganga et al., 1997). Anthocyanin-like compounds have also been detected in human urine after consumption of 300 mL red wine (Lapidot et al., 1998). After the acidification of post red wine urine a pink colour developed which absorbed in the region of 500 nm. Two compounds present in red wine were observed unchanged in urine along with other compounds that may be structurally modified anthocyanins.

Two recent, and very similar, reports describe the absorption and metabolism of cyanidin glucosides in rats and humans (Tsuda et al., 1999; Miyazawa et al., 1999). Rats were fed pure cyanidin-3-glucoside (0.9 mmol/kg body weight) and killed at 15, 30, 60, 120 and 240 minutes (Tsuda et al., 1999). The distribution of anthocyanin metabolites was examined in plasma, liver, kidney, stomach and the small intestine. Cyanidin-3-glucoside was identified unchanged in plasma, reaching maximum levels at 30 min (0.31 $\pm$ 0.03 μmol/L). No aglycone, or sulphate and glucuronide metabolites were detected in the course of the study. Methylated cyanidin-3-glucoside was detected in liver and kidney with the kidneys containing 2-fold higher levels of methylcyanidin-3-glucoside. The position of methylation is uncertain but speculated to be either the 3′ or 4′ hydroxyl group.

Similar observations were reported after the administration of cyanidin-3-glucoside- and cyanidin-3, 5-diglucoside-containing red fruit extract to rats and humans (Miyazawa et al., 1999). Only intact anthocyanins were found in plasma. Two anthocyanin metabolites were observed in the liver. One was identified as peonidin-3-glucoside while the other remained unidentified. No

methylated anthocyanin metabolites were detected in plasma suggesting that the metabolites may be rapidly metabolised and eliminated.

1.9.2 Non-flavonoids

In addition to flavonoids, the bioavailability of other wine phenolics is now coming under investigation.

1.9.2.1 Hydroxybenzoates

The absorption and metabolism of gallic acid has been investigated after the consumption of tea and red wine. With both beverages the predominant metabolites were methyl ether derivatives of gallic acid. After the administration of 50 mg gallic acid, 4-*O*-methylgallic acid and gallic acid were detected in urine and plasma (Shahrzad et al., 1998). Levels of these compounds increased after hydrolysis of both urine and plasma. The nature of the conjugation of the metabolites was not investigated or speculated.

Further metabolites were detected after the prolonged consumption of black tea (Hodgson et al., 2000). 4-*O*-methylgallic acid, 3-*O*-methylgallic acid and 3,4-*O*-dimethylgallic acid may be used as markers of black tea consumption. It remains uncertain whether all gallic acid metabolites are derived from gallic acid alone. Gallate esters may contribute, along with breakdown products of the flavonoids.

After the prolonged ingestion of red wine levels of 4-*O*-methylgallic acid in urine increased (Adu-Amsha Caccetta et al., 2000). It is proposed that this increase may be due to gallic acid derived from tannic acid aswell as the free gallic acid (Zhu et al., 1992).

1.9.2.2 Hydroxycinnamates

Animal and human studies have investigated the absorption and metabolism of hydroxycinnamates. After ingestion of a diet rich in fruits and vegetables,

caffeic, *p*-coumaric and ferulic acids were detected in urine (Bourne and Rice-Evans, 1998a). Identification was on the basis of retention time and spectral characteristics. The same group reported on the absorption of ferulic acid from tomatoes. They found that after 7 h maximum concentrations of 7 μM ferulic acid were detected in urine (Bourne and Rice-Evans, 1998b).

Administration of a 1g dose of caffeic acid resulted in the detection of methylated metabolites in the urine (Jacobson et al., 1983). Ferulic acid and isoferulic acid were detected, along with intact caffeic acid and vanillic acid. Methylation takes place in the liver. The mechanism of absorption of hydroxycinnamates remains uncertain. It is speculated that the sodium dicarboxylate co-transporter (SDCT1) may be involved (Clifford et al., 2000b).

1.9.2.3 Stilbenes

Despite the increasing interest surrounding *trans*-resveratrol and the other stilbenes few studies have been carried out in animals or humans to investigate their bioavailability. *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. Considerably higher levels of *trans*-resveratrol were detected in kidney and liver tissue compared with plasma. It is likely that the kidneys are involved in the elimination of the stilbene (Bertelli et al., 1996).

A recent paper has investigated the absorption and metabolism of *trans*-resveratrol using isolated rat small intestine model (Kuhnle et al., 2000). *Trans*-resveratrol was metabolised to its glucuronide conjugate, which crosses the small intestine. Conjugation was proposed to take place at the 4′ hydroxyl position. The previous feeding studies have not reported the presence of *trans*-resveratrol glucuronide in plasma. It is speculated that soon after absorption across the small intestine the glucuronide is cleaved to the aglycone, which enters circulation.

1.10 Analysis of phenolics

A number of different techniques have been employed over the years for the isolation and quantification of phenolic compounds. Many of the original methods have been consigned to the history books as they have been overtaken by increasingly advanced techniques.

The majority of approaches have been based on chromatography and several excellent reviews have been published on the subject (Markham, 1975; Hostettmann and Hostettmann, 1982; Markham, 1989; Robard and Antolovich, 1997; Merken and Beecher, 2000.)

1.10.1 Paper chromatography

Although paper chromatography (PC) is the oldest of the chromatographic methods it is still used in some labs because of its ease and low cost. Indeed two-dimensional paper chromatography (2D-PC) is still often used for the preliminary analysis of crude phenolic extracts or as a preparative tool. Information on the structures of flavonoids can be elucidated on the basis of their R_f values (mobility) or their response to UV exposure and various spray reagents (Markham, 1989).

1.10.2 Thin layer chromatography

The approach of this method is very similar to that of paper chromatography, however separation occurs on thin layer chromatography plates coated with polyamide, silica, C_8 or C_{18} depending on the classes of phenolics to be investigated. While the anthocyanins can be detected by direct examination, other flavonoids are viewed either under UV or with spray reagents (see Markham, 1975).

1.10.3 Column chromatography

The major applications of column chromatography are in the purification of crude plant extracts or the preparative isolation of large quantities of compounds where it has the advantages of convenience and low cost. The column packing used include cellulose (microcrystalline), silica (0.06-0.3 mm partical size), polyamide, Sephadex LH-20, Amberlite XAD-7 and reversed phase C_8 and C_{18} supports.

1.10.4 Gas chromatography

Because very few flavonoids are volatile without derivatisation, this widespread technique is not often employed in their analysis. Only the polymethoxyl flavones are sufficiently volatile and gas chromatography (GC) has been used for their separation in orange peel (Robards and Antolovich, 1997).

Less volatile compounds can be derivatised with silyl groups to facilitate their analysis by GC. Soleas et al., (1997a) developed a multi-residue derivatisation GC assay which allowed for the simultaneous analysis of fifteen phenolic compounds including caffeic and *p*-coumaric acids, the flavan-3-ols (+)-catechin and (-)-epicatechin, the stilbenes *cis* and *trans*-resveratrol and the flavonol quercetin. One of the potential problems with this approach is that derivatization efficiency can vary from compound to compound so that even with an internal standard accuracy of quantitative analysis is poor.

1.10.5 High performance liquid chromatography

"Liquid chromatography is a method for the separation of mixtures in which the sample is introduced into a system of two phases. Differences in distribution shown by the solutes cause them to travel at different speeds in the system" (Lindsay, 1992).

1.10.3.1 Reversed phase

Reversed-phase HPLC makes use of a non-polar stationary phase (a column) and a polar mobile phase. Separation occurs as non-polar solutes will be soluble in the stationary phase and will travel through the system more slowly than polar solutes, which favour the mobile phase. Solutes are eluted in order of polarity, with the most polar eluting first. Changing the polarity of the stationary phase, or ideally the mobile phase can alter the elution times of solutes.

1.10.3.2 Normal phase

In contrast to reversed-phase HPLC, with normal phase HPLC the stationary phase is relatively polar and the mobile phase is non-polar.

1.10.3.3 Description of a typical HPLC system

The layout of a typical HPLC system is shown in Figure 1.17. It comprises of a system controller, two pumps, an injector, a column oven with column and finally the required detectors.

In brief, the system controller is used to monitor the flow rate and composition of the mobile phase (solvent). It can also be used to control an auto-injector allowing multiple samples to be analysed without the intervention of the operator.

The pumps are needed to vary the composition of the mobile phases. The system can be used in isocratic or gradient mode. Isocratic mode means that a steady composition of the mobile phase is used throughout the run, whereas gradient implies that the composition of the mobile phase varies.

The auto-injector has replaced manual injection of samples over the past few years. This allows samples to be prepared and left within a controlled environment until automatic injection. The column oven houses the column,

which after the solvents used in the mobile phase, is the most influential component of the system. The column oven can be used to aid or delay elution of the solutes by varying the temperature. In addition it may just be used to maintain a constant temperature if the ambient environment is variable.

1.10.3.4 Column

As mentioned before, in reverse phase chromatography the column used is non-polar, usually C_{18} or C_8. The column is made up using packing of micoparticulate silicas. These are small spherical or irregular porous silica particles, and can have a diameter of 3, 5 or 10 μm. The silica is chemically modified, in the case of C_{18} columns bonded with octadecylsilane groups.

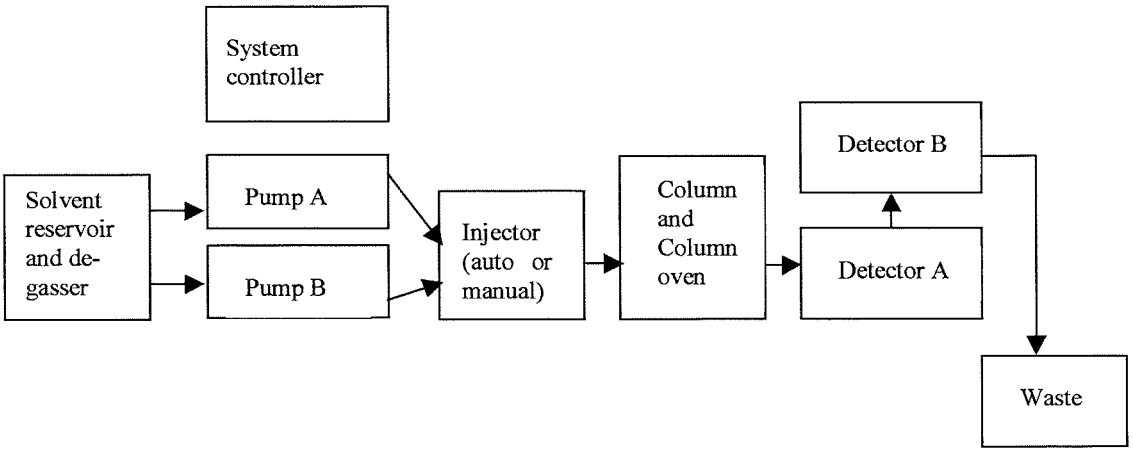


Figure 1.17. Basic layout of a HPLC system showing solvent flow

1.10.3.5 Detector

The objective of a detector is to utilise a particular property of the solute emerging from the column and create a proportional electrical output. Ideally a detector must be sensitive, selective and linear in its response. Commonly used detectors are UV absorbance monitors, photodiode array detectors, fluorescence detectors, electrochemical detectors and mass spectrometers.

The most commonly used detector is the UV absorbance monitor, however this will only detect those compounds that absorb UV or visible radiation such as aromatic compounds. The detector can generally monitor the eluting solute peak at either one or two wavelengths. The absorption of the solutes is proportional to their concentration which can be determined using either the Beer-Lambert Law; A = ,cl, or the use of an internal or external standard.

An extension to the UV monitor is the photodiode array detector. In this case the eluting solutes are examined at a wide range of wavelengths, typically 200-600 nm. This enables examination of the absorption spectra of each eluting peak, allowing determination of the purity of the peak as well as quantification of the solute present.

The related fluorescence detectors make use of the property of a compound to absorb UV radiation and subsequently emit radiation of a longer wavelength. By varying the excitation and emission wavelengths the detector can be highly selective. Not many compounds have the ability to fluoresce, mainly those that have conjugated cyclic structures.

Electrochemical detectors work in a very different way to those detectors already discussed. They determine the current associated with the oxidation or reduction of a solute. To use this detection method the solutes in question must be easily oxidisable or reducible.

The mass spectrometer is able to separate and analyse ions in the gas phase, and can be operated in such a way as to be either universal in its response, or highly selective.

Chapter 2 . Materials and Methods

2.1 Chemicals

Quercetin, myricetin, kaempferol, (+)-catechin, (-)-epicatechin, caffeic acid, *p*-coumaric acid, *trans*-resveratrol, ellagic acid and gallic acid were obtained from Sigma (Poole, Dorset, UK). Isorhamnetin and *trans*-resveratrol-*O*-β-glucoside were supplied by Apin (Abingdon, Oxford, UK). *Trans*-resveratrol-*O*-β-glucoside was also isolated and crystallised from the root of *Polygonum cuspidatum* by Professor Takao Yokota, Teikyo University, Utsunomiya, Japan. Malvidin-3-glucoside was purchased from Extrasynthase (Lyon, France). *Cis*-resveratrol was obtained by isomerisation of *trans*-resveratrol in methanol during 12 h exposure to high white light. Dr Creina Stockley of the Australian Research Institute generously provided a sample of caftaric acid.

Methanol (HPLC Grade), ethanol and acetonitrile (HPLC Grade) were from Rathburn Chemicals (Walkerburn, UK). Trifluoroacetic acid (TFA), formic acid, aluminium nitrate, citric acid, Folin and Ciocalteu's phenol reagent (2.0 normal), acetylcholine chloride, phenylephrine hydrochloride were supplied by Sigma. Concentrated hydrochloric acid, acetic acid (glacial), disodium hydrogen phosphate (Na_2HPO_4), and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Loughborough, Leicestershire, UK). Ethanol (Analar grade) and disodium carbonate (Na_2CO_3) were from BDH Laboratory Supplies (Poole, UK). All other chemicals and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK).

2.2 Wines, musts and grapes

2.2.1 Bottled wines

All bottled wines were supplied by Safeway Stores plc. Details of origin, principal grape variety and price are shown in Tables 2.1 and 2.2. Wines were supplied in two batches, batch 1 in summer 1997 and batch 2 in summer 1998.

Table 2.1. Details of red wines analysed for their phenolic content (batch 1)

	Wine	Year	Origin	Principal Grapes	Price
1	Chilean Cabernet Sauvignon	1997	Lontué, Chile	Cabernet Sauvignon	£3.99
2	Californian Oak Aged Cabernet Sauvignon	1995	California, USA	Cabernet Sauvignon	£4.99
3	Young Vatted Cabernet Sauvignon	1996	Sliven, Bulgaria	Cabernet Sauvignon	£3.29
4	Bulgarian Matured Cabernet Sauvignon	1992	Svischtov, Bulgaria	Cabernet Sauvignon	£3.29
5	Cono Sur Pinot Noir 20 Barrels	1995	Rapel Valley, Chile	Pinot Noir	£8.99
6	Fetzer Santa Barbara Pinot Noir	1994	California, USA	Pinot Noir	£7.29
7	Domaine Rossignol Trapet, Gevrey Chambertin	1995	Burgundy, France	Pinot Noir	£13.99
8	Villa Montes Oak Aged Merlot	1994	Curicó, Chile	Merlot	£4.99
9	Merlot	1996	Languedoc, France	Merlot	£3.49
10	Cosme Palacio y Hermanos Rioja	1995	Rioja, Spain	Tempranillo	£5.99
11	Viña Albali Tempranillo	1996	Valdepeñas, Spain	Tempranillo	£2.99
12	Fetzer Vineyards Zinfandel	1995	California, USA	Zinfandel	£5.99
13	Beaujolais	1996	Beaujolais, France	Gamy	£3.99
14	Domaine Roche Vue, Minervois	1995	Aude, France	Carignon	£3.99
15	Valpolicella	1996	Veneto, Italy	Corvina, Molinara	£3.49
16	Chianti Classico	1995	Tuscany, Italy	Sangiovese, Trebbiano	£5.75

Table 2.2. Details of red wines analysed for their phenolic content (batch II)

	Wine	Year	Origin	Principal Grapes	Price
17	Connectable Talbot St Julien	1994	Bordeaux, France	Cabernet Sauvignon, Merlot	£11.49
18	Chateau Soudars	1994	Bordeaux, France	Cabernet Sauvignon, Cabernet Franc, Merlot	£9.99
19	Safeway Claret	NS	Bordeaux, France	Cabernet Sauvignon, Merlot	£3.69
20	Pommard Premier Cru	1996	Burgundy, France	Pinot Noir	£16.99
21	Safeway Cotes du Rhone	1997	Rhone, France	Carignon, Cinsault	£3.25
22	Peter Lehman Cabernet Sauvignon	1995/96	Australia	Cabernet Sauvignon	£7.99
23	Chateau Reynella Shiraz	1995	Australia	Syrah	£11.99
24	De Bortoli Yarra Valley	1995/96	Australia	Pinot Noir	£10.99
25	Safeway Oaked Shiraz	1997	Australia	Syrah	£4.99
26	Tim Knapstein Cabernet Franc	1995	Australia	Cabernet Franc	£7.99
27	Peter Lehman Seven Surveys	1996	Australia	Grenache, Syrah, Mouvedre	£6.99
28	Church Road Cabernet/Merlot	1996	New Zealand	Cabernet Sauvignon, Merlot	£8.99
29	Rafael Estate Tempranillo	1997	Argentina	Tempranillo	£3.49
30	Balbi Vineyards Malbec	1997	Argentina	Malbec	£4.49
31	Safeway Chilean Cabernet Sauvignon	1997	Chile	Cabernet Sauvignon	£3.99
32	Errazuriz Syrah Reserva	1996	Chile	Syrah	£9.99
33	Safeway South African Cinsault	1997	South Africa	Cinsault	£3.99
34	Safeway South African Pinotage	1997	South Africa	Pinotage	£4.49
35	Stellenbosch Cabernet Sauvignon	1997	South Africa	Cabernet Sauvignon	£4.49
36	Safeway Ribbatejo	1997	Portugal	Castelão Frances	£2.99
37	Safeway Barrique Aged Cabernet Sauvignon	1995	Italy	Cabernet Sauvignon	£6.99
38	Tenuta San Vito (Organic)	1996	Italy	Sangiovese, Canaiolo	£5.99

NS, not specified.

Wines were chosen to represent a cross-section of grape varieties, geographical locations and price. While batch 1 includes mainly old world wines, batch 2 includes predominately new world wines. In each instance aliquots of wine were removed from the bottle for analysis and the remaining wine was stored under nitrogen and re-corked. Unless stated otherwise wine was untreated prior to analysis.

2.2.2 Musts

Samples of musts were collected during a field trip to Viña San Pedro, Curicó, Chile. Four wines were followed during the first 7 to 9 days of vinification. Samples were collected at the same time each day and processed in the same manner.

Must, 100 mL, was filtered (Wattman 0.7μm) to remove particulate matter and 50 mL ethanol added to halt fermentation. The liquid was then decanted into 375 mL bottles, purged with carbon dioxide and corked. Prior to analysis the alcohol was removed by rotary evaporation. The samples were subsequently stored in amber bottles under nitrogen between analyses. Unless stated otherwise samples were untreated prior to analysis.

2.2.2.1 Sample concentration

Samples were concentrated for the analysis of those phenolics found in low levels. Five mL sample was dried down using a rotary evaporator with a water bath operating at 35 °C. The sample was re-dissolved in distilled water to a known volume.

2.2.3 Grapes

Grape samples were collected by randomly selecting fruit from different aspects, clusters and vines. Samples were weighed and stored at –20 °C prior to transportation to laboratory facilities within the Universidad Catolica, Chile. There they were frozen with liquid nitrogen and packed in dry-ice for transport

to the University of Glasgow. On arrival samples were immediately stored at –80 °C prior to analysis.

2.2.3.1 Preparation of methanolic grape extract

A weighed aliquot of grapes was de-frosted at room temperature prior to homogenisation with 30 mL methanol containing 2% formic acid. Samples were centrifuged at 10000 g for 10 min and the supernatent stored at -80 °C until analysis.

2.3 Analysis of phenolics by high performance liquid chromatography

Red wine contains a large number of phenolic compounds and their derivatives, many of which are found in very low levels. A range of methodologies was developed for the detection and quantification of many of the major phenolic compounds found in wine. These include the flavonols, myricetin, quercetin, kaempferol and isorhamnetin; the flavan-3-ols, (+)-catechin and (-)-epicatechin; the hydroxycinnamates; caftaric, caffeic and *p*-coumaric acids; the stilbenes, *trans*-resveratrol, *cis*-resveratrol and *trans*-resveratrol-*O*-β-glucoside; the hydroxybenzoates, gallic and ellagic acids and the anthocyanins; malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-acetyl)glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside.

Red wines were analysed using a Shimadzu (Kyoto, Japan) LC-10Avp series automated liquid chromatograph comprising a SCL-10Avp system controller, two LC-10ATvp pumps, a SIL-10ADvp auto-injector with sample cooler, a CTO-10Avp column oven operating at 40 °C and linked to either a Waters 996 photodiode array (PDA) detector (Waters, Milford, MA, USA) or a Shimadzu SPD-10A UV-vis detector. Data from the detectors were collected and processed via either a Millennium Chromatography Manager (Waters) or a Reeve Analytical (Glasgow, UK) 2700 data handling system respectively. Fluorescence was monitored on a RF-10A fluorimeter (Shimadzu). Sample

treatment, HPLC column, solvent conditions and the detector systems used for the different phenolics are summarised below. In order to optimise resolution, mobile phase conditions for isocratic analyses were designed to provide k' values of ca. 4-5 for the compounds of interest. In addition, for each group of compounds, different reverse-phase columns with varying selectivities and polarities were evaluated with red wines to ensure that impurities did not impinge on the homogeneity of quantified peaks.

2.3.1 Flavonols

Free and conjugated myricetin, quercetin, kaempferol and isorhamnetin were analysed in samples, before and after acid hydrolysis (McDonald et al., 1998). Samples were hydrolysed at 90 °C for 2 h in 1.2 M HCl in 50% aqueous methanol. 450 μL of wine was added to a solution of 1200 μL of 80% methanol containing 20mM diethyldithiocarbamic acid and 300 μL 6M HCl. A micro-scale hydrolysis procedure was used with a 3 mL glass V-vial. A Teflon coated magnetic stirrer was placed in the vial that was sealed tightly with a PTFE-faced septum prior to heating in a Reacti-Therm heating/stirring module (Pierce, Rockford, IL). Extract aliquots of 50 μL were taken both before and after hydrolysis and made up to 250 μL with distilled water adjusted to pH 2.5 with trifluroacetic acid (TFA).

Samples were eluted using a 150 x 3.0 mm i.d., 4 μm C_{18} Genesis column (Jones Chromatography, Mid-Glamorgan, UK) eluted at a flow rate of 0.5 mL/min with a 20 min gradient of 20-40% acetonitrile in water adjusted to pH 2.5 with TFA. Sample volumes analysed were equivalent to 4.6 μL of wine. After passing through the UV detector operating at 365 nm, column eluate was mixed with 0.1 M methanolic aluminium nitrate in 7.5% acetic acid pumped at a flow rate of 0.5 mL/min and fluorescent flavonol complexes detected with a fluorimeter (excitation 425 nm, emission 480 nm) as described by Aziz et al. (1998). Postcolumn derivatization was optimised from the method published by Hollman et al., (1996). They used a viscous solution of 1.0 M aluminium nitrate, however we decreased this to 0.1 M which enabled the use of a low pressure pump. Lowering the concentration decreased the sensitivity of the

fluorescence detection only 2-3 fold, and had the advantages of minimising cost, lowering the back-pressure and also the risk of corrosion from aluminium nitrate.

2.3.2 Flavan-3-ol

A 150 x 4.6 mm i.d. 5 μm C_{18} Luna column (Phenomenex, Macclesfield, UK) eluted at a flow rate of 1 mL/min with 10% acetonitrile in water adjusted to pH 1.5 with TFA was used to analyse the (+)-catechin and (-)-epicatechin content of 5 μL volumes of red wines. (+)-Catechin and (-)-epicatechin were detected with a fluorimeter operating at excitation 280 nm and emission 310 nm (Arts and Hollman, 1998) and by absorbance at 280 nm. The method also allowed the separation of epigallocatechin, epigallocatechin gallate and epicatechin gallate. These compounds could only be detected using absorbance at 280 nm and were not found in detectable levels in the wines analysed.

2.3.3 Anthocyanins

Malvidin-3-*O*-glucoside and two derivatives, malvidin-3-*O*-(6-*O*-acetyl)glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside were separated using a 250 x 4.6 mm i.d. 4 μm C_{18} Nova-Pak column (Waters, Milford, MA, USA) eluted with a gradient over 40 min of 5-30% ACN in 5% formic acid at a flow rate of 1mL/min. Anthocyanins were detected by absorbance at 520 nm. When quantifying anthocyanins present in musts the samples were concentrated by rotary extraction as detailed previously in Part 2.2.2.1.

2.3.4 Hydroxybenzoates

The gallic acid content of 5 μL volumes of red wines was analysed on a 150 x 3.0 mm i.d., 4 μm C_{18} Genesis column (Jones Chromatography, Mid-Glamorgan, UK) eluted at a flow rate of 1.0 mL/min with 2% methanol in water adjusted to pH 1.5 with TFA using a PDA detector at 280 nm. Ellagic

acid in 5 µL volumes of red wines was analysed on a 150 x 3.0 mm i.d., 4 µm C_{18} Genesis column (Jones Chromatography, Mid-Glamorgan, UK) eluted at a flow rate of 1.0 mL/min with 30% acetonitrile in water with 1% formic acid using an absorbance detector operating at 255 nm.

2.3.5 Hydroxycinnamates

Five µL volumes of samples were analysed before and after alkaline hydrolysis, which was used to cleave conjugated caffeic acid and *p*-coumaric acid. This was achieved by mixing 1 mL of red wine and 1 mL 4 N NaOH in a 3 mL glass V-vial which was incubated in darkness at room temperature for 2 h before being acidified with 1 mL 6 M HCl. Five µL volumes of hydrolysate are equivalent to 1.67 µL of wine. The method was adapted and optimised from that of Rapisarda et al. (1998).

Samples were analysed on a 150 x 3.0 mm i.d., 5 µm C_{18} Nemesis column (Phenomenex, Macclesfield, UK) eluted at a flow rate of 1 mL/min with either 7, 11 or 12% ACN in water adjusted to pH 1.5 with TFA and a PDA detector operating at 313 nm. Caffeic acid and caftaric acid were analysed with 7% ACN while an 11% ACN mobile phase was used for free *p*-coumaric acid. Caffeic acid and *p*-coumaric acid released by alkaline hydrolysis were analysed with 12% ACN. Using an 11% ACN mobile phase ferulic and sinapic acids could also be separated, but were not found in detectable levels in the wines under study.

2.3.6 Stilbenes

Trans- and *cis*-resveratrol in 10 µL volumes of red wine were analysed on a 250 x 4.6 mm i.d., 5 µm ODS Hypersil (Shandon, Astmoor, UK) column, packed in-house and eluted at a flow rate of 1 mL/min with 25% ACN in water adjusted to pH 1.5 with TFA using a PDA detector at 307 nm. *Trans*-resveratrol-*O*-β-glucoside was analysed under similar conditions except that the mobile phase was 17% ACN in water adjusted to pH 1.5 with TFA.

2.3.7 Identification of phenolics

Phenolics were identified on the basis of their retention time, co-chromatography with pure standards and, where a PDA was used, their absorbance spectra. The exception to this was in the case of the anthocyanins. Due to the lack of standards for many of the anthocyanins they were identified by comparison with the established literature and confirmed by LC-MS.

2.3.7.1 LC-MS identification of anthocyanins

The anthocyanins in 10 μL volumes of sample were analysed on a 250 x 4.6 mm i.d. 4 μm C_{18} Novapak column (Waters, MA, USA) eluted with a 40 min gradient of 5-30% ACN in 5% aqueous formic acid with a flow rate of 1 mL/min (Fig. 2.1).

Anthocyanins were directed to an absorbance monitor operating at 520 nm, and then to a Shimadzu LC Q8000 quadropole mass spectrometer. The mass spectrometer was operated with an atmospheric pressure chemical ionisation interface (APCI) in positive ion full scan mode, scanning from 250 to 800 atomic mass units (amu). Examination of the mass spectra of the main anthocyanins (Fig. 2.1) enabled their identification as (A) malvidin-3-*O*-glucoside, (B) malvidin-3-*O*-(6-*O*-acetyl)glucoside, (C) malvidin-3-*O*-(6-*O*-*p*-coumaroyl) glucoside.

2.4 Fractionation of wine

Chilean Cabernet Sauvignon 1999 from Lontúe was fractionated with the aim of isolating the zone of antioxidant activity and determining the phenolics present.

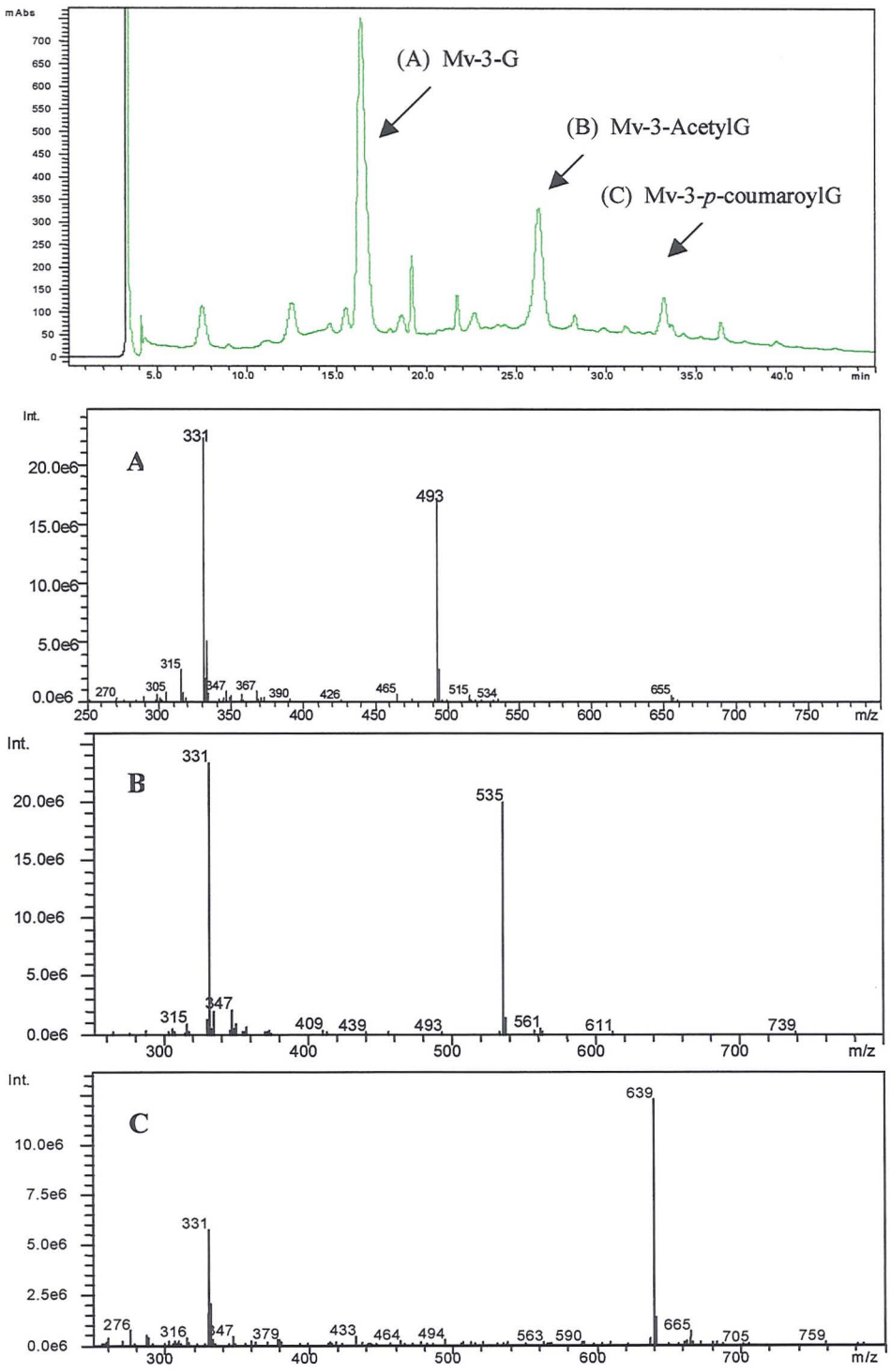


Figure 2.1. HPLC-MS analysis of the major anthocyanins in wine

Analysis of the major anthocyanins in 10 μL volumes Chilean Cabernet Sauvignon, 1999. Column, 250 x 4.6 mm i.d. 4 μm C_{18} Novapak column (Waters). Mobile phase, 40 min gradient of 5-30% ACN in 5% aqueous formic acid. Flow rate, 0.8 mL/min. Detection, absorbance at 520 nm and positive ion MS analysis with APCI interface. (A) malvidin-3-*O*-glucoside, (B) malvidin-3-*O*-(6-*O*-acetyl)glucoside, (C) malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside.

2.4.1. Pre-fractionation sample preparation and concentration

Wine, 500 mL, was cleaned and concentrated prior to fractionation. The first step was to remove the alcohol from the 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 phenolics were eluted with four column volumes of methanol. The methanol was removed by rotary evaporation in vacuo, prior to loading the sample onto a column, 44 x 44 mm i.d., packed with 5 μm, C_{18} silica gel (Aldrich, UK). The column was initially washed with 10% MeOH in 0.1 % aqueous TFA, and then eluted with four column volumes of 100% MeOH. Methanol fraction taken to dryness *in vacuo* and re-dissolved with 5% ACN in 0.1% aqueous TFA, to a final volume of 30 mL.

2.4.2 Fractionation of wine

Wine extract was analysed using a preparative HPLC system comprising a Hewlett Packard 85B gradient controller (CA, USA), two ConstaMetric® 3000 solvent delivery systems (LDC/Milton Roy, FL, USA), Rheodyne preparative injector with 20 mL sample 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 absorbance detector (Rainin Instrument Co. Inc., MA, USA). Fractions were collected using a Gilson FC 203 (WI, USA). Data was collected and processed via a Reeve Analytical (Glasgow, UK) 2700 data handling system.

Twenty mL volumes of wine extract were analysed on the above system operating at 10 mL/min with a 40 min gradient of 5 to 40% ACN in 0.1 % aqueous TFA and then held at 40% for 5 min. Absorbance was monitored at 280 nm. Fractions were collected every 45 seconds after discarding the first 3.5 minutes of eluent (accounting for the dead volume of the column). Each

fraction contained 7.5 mL of eluent. A total of sixty fractions were collected. In addition the acetonitrile wash was collected as 2 x 50 mL fractions.

2.4.3 HPLC analysis of fractions

The phenolic profile of each fraction was determined using HPLC-PDA. A 250 x 4.6 mm i.d. Max RP 80A column (Phenomenex, Macclesfield, UK) was run at 1mL/min using a 40 min gradient of 5-40% ACN in 0.1% trifluoracetic acid then held at 40% for 5 min. Five μL volumes of each fraction were monitored using a PDA operating at 280, 313, 365 and 520 nm.

2.4.4 LS-MS analysis fractions

The phenolic compounds in 20 μL volumes of fractions were analysed on a 250 x 4.6 mm i.d. C_{18} Max-RP column (Phenomenex, Macclesfield, UK) eluted with 3% ACN in 1% aqueous formic acid with a flow rate of 1 mL/min.

Eluent was directed to a PDA absorbance monitor scanning between 150 and 600 nm, and then to a LCQ DUO mass spectrometer (Thermoquest, CA, USA). The mass spectrometer was operated with an Electrospray interface in positive ion full scan mode, scanning from 100 to 1500 atomic mass units (amu).

2.5 Colorimetric methods

2.5.1 Determination of total phenolics

The total phenol contents of the wines were determined using the Folin-Ciocalteu method of Singleton and Rossi (1965). In brief 0.2 mL of a 1:5 diluted wine, must or extract was added to 10 mL of 1:10 diluted Folin and Ciocalteu reagent and 1.8 mL water. After 5 min 7.0 mL of 115g/L Na_2CO_3 was added and the reaction mixture was left for 2 h at room temperature. The absorbance of the solution was then read at 765 nm against a water blank on a Cecil 3000 series UV/visible spectrophotometer (Cecil Instruments Ltd,

Cambridge, UK). The optical density (O.D.) compared to a standard curve prepared with 50 to 500 mg/L gallic acid, and data expressed as gallic acid equivalents.

2.5.2 Colorimetric analysis of anthocyanins

The anthocyanin content of red wines was estimated using a pH shift method adapted from Ribéreau-Gayon and Stonestreet (1965). Two test tubes were set up each containing 1 mL wine and 1 mL 0.1% concentrated HCl in 95% ethanol. Ten mL of 2% concentrated HCl (pH 0.6) was added to one tube and 10 mL of pH 3.5 buffer (300 mL 0.2 M Na_2HPO_4 and 700 mL 0.1 M 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 correction of the 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 coefficient $\varepsilon = 28000$. At < pH 1 anthocyanins are found entirely in their red flavylium form allowing the determination of the total anthocyanins. However at pH 3.5 the flavylium 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.

2.6 Electron-spin resonance spectroscopic determination of antioxidant activity

This was the method used throughout the study to determine the antioxidant activity of wines and musts. The greater majority of other antioxidant assays rely on a colour change to give an indication of the capacity of compounds to act as antioxidants. Because of the wide range of hues found when dealing with red wines it was essential to use an assay that did not depend on colour change as it may have compromised measurements.

The ability of red wines to reduce the Fremy's salt (potassium nitrosodisulphonate) was measured as described by Gardner et al. (1998). The wines were diluted to 5% (v/v) with ethanol/water (12:88, v/v). Three mL aliquots were reacted with an equal volume of 1mM Fremy radical in ethanol/water (12:88, v/v). The ESR spectra of the low field resonance of the Fremy's radical were obtained after 20 min by which time the reaction was complete. Signal intensity was obtained by double integration and concentration calculated by comparison with a control reaction using ethanol/water (12:88, v/v) without red wine. Spectra were obtained at 21 °C on a Bruker ECS 106 spectrometer equipped with a cylindrical (TM110 mode) cavity and operating at ca. 9.5 GHz (X-band frequency). The microwave power and modulation amplitude were set at 2 mW and 0.01 mT, respectively.

2.7 Methods used by collaborators

2.7.1 Ferric reducing potential as an index of anti-oxidant activity

The ferric reducing potential of the wines was assessed by a method based on that of Benzie and Strain (1996). This method measures the ability of a compound or extract to reduce Fe^{3+}, in the reagent mix, to Fe^{2+}. The reagent amounts were scaled up by a factor of 10, 0.1 mL of sample was added to a tube containing 3 mL FRAP reagent and 0.3 mL water. The absorbance at 593 nm was measured 4 min after addition of the reactants, and the difference between this and the absorbance of the blank was related to the same absorbance change in the Fe^{2+} standard solution (0 to 1 mM $FeSO_4$).

2.7.2 Assay of vasodilation capacity

New Zealand white adult rabbits (ca. 2.5 kg) were studied. They were killed by sodium pentobarbitone (200 mg/kg), the thoracic aorta removed and cleaned of adhering fat and connective tissue. The aorta was cut into rings (4-5 mm long), suspended from force displacement transducers in 10 mL organ

baths and bathed in Krebs-buffer solution (pH 7.4) (composition (mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$ 2.5, glucose 11.0, and EDTA 23.0) at 37 °C. The buffer was bubbled continuously with 16% O_2/5% CO_2 balanced with N_2 to give values similar to those found *in vivo*. Tension (2 g) was then applied to all rings. Following a 1h equilibration period the response to 50 mM KCl was determined, followed by wash-out and further equilibration. All tissues were contracted submaximally with phenylephrine (PE, 0.1 μM) and once a stable plateau had been reached, cumulative concentration-dependent response curves (CCRCs) to the wine extracts (1-5000 μg/mL) using various dilutions of these extracts were constructed. Only one wine extract was used for each ring. All drugs and solutions were prepared in distilled water. Extracts were prepared by removing water and alcohol from the wine first by vacuum and then under nitrogen to ensure they were dry. The samples were then diluted to give an initial solution of 500 mg/mL, from which further dilutions were made. Fresh wine extract dilutions were made up daily and used within 24 h. As an index of potency, the pIC_{50} (the concentration in μg/mL at which each extract caused 50% of maximum vasodilation) for each wine extract was determined using graphical interpolation for each CCRC constructed and expressed as mean ± standard error (SE). Graphically the potency is displayed as $^1/pIC_{50}$ x 10^3.

2.7.3 Spectral assay for total catechins

The total catechin content of wine samples was determined using a method adapted from that of Kivits et al. (1997). Wine samples were diluted 10-fold with 12.5% ethanol prior to the addition of 3 mL of 6 mM dimethylaminocinnamaldehyde (DMACA), dissolved in methanol/perchloric acid/water (8:1:1, v/v). After 6 min a scan of absorption between 500 and 750 nm was obtained. Peak area was related a standard curve obtained with catechin standard. The shape of the absorption scans varied due to anthocyanin absorption around 500 nm, as such only the 604-684 nm region of the scan was used in catechin quantification.

2.8 Statistics

Data are presented as mean values ± standard error (SEM) (n=3). Each sample was analysed in triplicate and the standard error describes the analytical mean. A matrix plot was used to graphically represent the data obtained in Chapter 4. Some relationships between results were apparently non-linear therefore non-parametric Spearman Rank (Chapter 4) or Pearson (Chapter 5) correlations were used to assess the strength of the association between them using Minitab software version 12 (Minitab Inc., Addison-Wesley Publishing Co., Reading, MA, USA).

Chapter 3 HPLC methods for the separation and quantification of phenolic compounds

3.1 Introduction

Wine is a complex fluid containing water, alcohols and sugars, in addition to a wide range of phenolic compounds. As interest in the pharmacological properties of red wine phenolic compounds increases, so does the value of accurate and reliable information about their content and distribution. Many of the phenolic compounds in wine are structurally very similar. This can cause their retention times to be close and their absorbance spectra to be almost super-imposable in many instances. Consequently, their accurate identification can be problematical. Those compounds that are present in low concentrations can be particularly difficult to identify and quantify. If they do not have a selective feature, such as an absorbance at a higher wavelength, they can be lost in an erratic baseline. Throughout the decades many techniques have been used for the separation, detection and identification of phenolic compounds. Indeed when quercetin was first isolated from grape leaves it was identified on the basis of its melting point. Many of the other techniques employed in phenolic analysis are described in section 1.10. However in the past two decades the method of choice has increasingly been reversed-phase HPLC utilising a C_{18} support.

3.2 Approaches to HPLC

In brief, reversed-phase HPLC separates phenolic compounds on the basis of their polarity. It makes use of a polar mobile phase and a non-polar C_{18} stationary phase chemically bonded onto a silica gel support.

3.2.1 Separation of phenolics

Two main approaches are evident in the literature, either one long general separation, or small selective analyses. The approach taken will be dependent

on the requirements of the study, whether the investigators are interested in only one particular family, or the complete phenolic profile of a sample.

3.2.1.1 The 'long gradient' method

This approach involves separating out a wide range of classes, in one long run. A gradient is run from a low to a high organic solvent concentration, with the most polar compounds eluting first. This approach has been employed by some of the major groups investigating wine phenolics.

The Goldberg group based in the University of Toronto collaborators at the Liquor Control Board of Ontario developed a tertiary HPLC gradient method. They separated out the phenolics in 20 μL volumes of wine by eluting a column with 15-40% gradient of methanol in 0.1% aqueous acetic acid over 40 min (Fig. 3.1). The eluent was monitored at wavelengths of (A) 280 and (B) 306 nm (Goldberg et al., 1996b).

Another approach that has been widely utilised is the direct HPLC separation of phenolics method developed by Lamuela-Raventós and Waterhouse (1994). It suffers from the same problems as the Goldberg method when the eluent is monitored at 280 nm. The baseline is erratic and congested making the peak identification and quantification less than straightforward.

The major advantage of this approach is rapid speed of analysis. A large number of phenolics can be separated, generally in less than one hour. This is opposed to several shorter runs, with solvent conditions optimised for specific groups of phenolics. This approach will provide enhanced resolution but does so at the expense of speed of analysis. These considerations would be of particular importance to groups working commercially, or with a large number of samples.

The difficulties of using a long gradient become apparent when examining the traces obtained (Fig. 3.1 [A] and [B]). Baseline separation of many of the compounds is not achieved (e.g. Fig. 3.1 [B] peak 1 and its neighbour). This

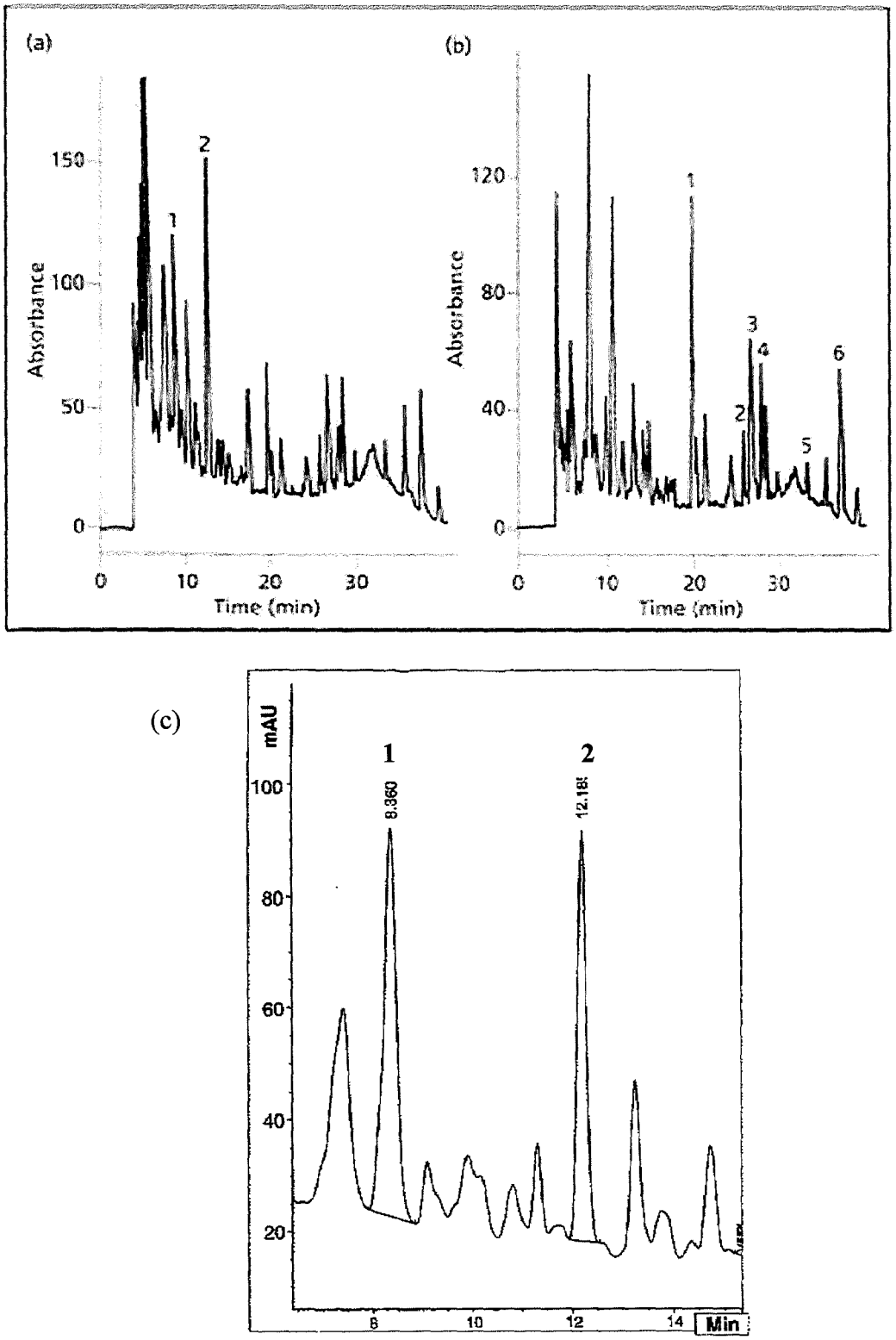


Figure 3.1. Long gradient RP-HPLC of wine phenolics

Gradient of 15-40% methanol in 0.1% aqueous acetic acid. (A) Absorbance monitored at 280 nm. Peak 1, (+)-catechin; peak 2, (-)-epicatechin. (B) Absorbance monitored at 306 nm. Peak 1, *trans*-resveratrol glucoside; peak 2, rutin; peak 3, *trans*-resveratrol; peak 4, *cis*-resveratrol; peak 6, quercetin. Data from Goldberg et al., 1996b replotted by Cserháti et al., 2000. (C) As for (A) but chromatogram expanded (Goldberg et al., 1998a).

makes accurate quantification difficult, and similarly it can be almost impossible to detect the presence of impurities that co-elute with the peak of interest. Another major problem with the use of long gradients is the identification and quantification of peaks that elute early, e.g. (+)-catechin, peak 1, in Fig. 3.1 (A). Modern detectors have software packages with spectral match functions. This assigns a numerical value to the relationship between a peak and its corresponding standard. While such facilities undoubtedly improve peak identification, they are no substitute for good chromatography and if not used with care, can lead to inaccurate conclusions being drawn.

3.2.1.2 The 'selective run' method

This method tends to be employed when investigating only one particular compound or family of compounds. This is demonstrated with the analysis of the stilbenes. Their proposed health benefits have elicited significant interest and a number of methods have been described for their analysis in wine and grape tissue. Recent methods involve the direct injection of wine and monitoring absorbance at 307 nm. Compared to many other phenolics the stilbenes are found in trace amounts. By separating the compounds of interest either isocratically or with a shallow gradient optimum resolution is achieved and it is possible to separate the low-concentration compound from other peaks that would undoubtedly interfere with the analysis on a long gradient designed to separate diverse phenolic compounds (Fig. 3.2).

3.2.2 Detection methods

Once the compounds of interest have been separated appropriate means of detection are required. Ideally, the method should be sensitive and linear in its response. The most common detectors make use of the ability of a compound to absorb radiation.

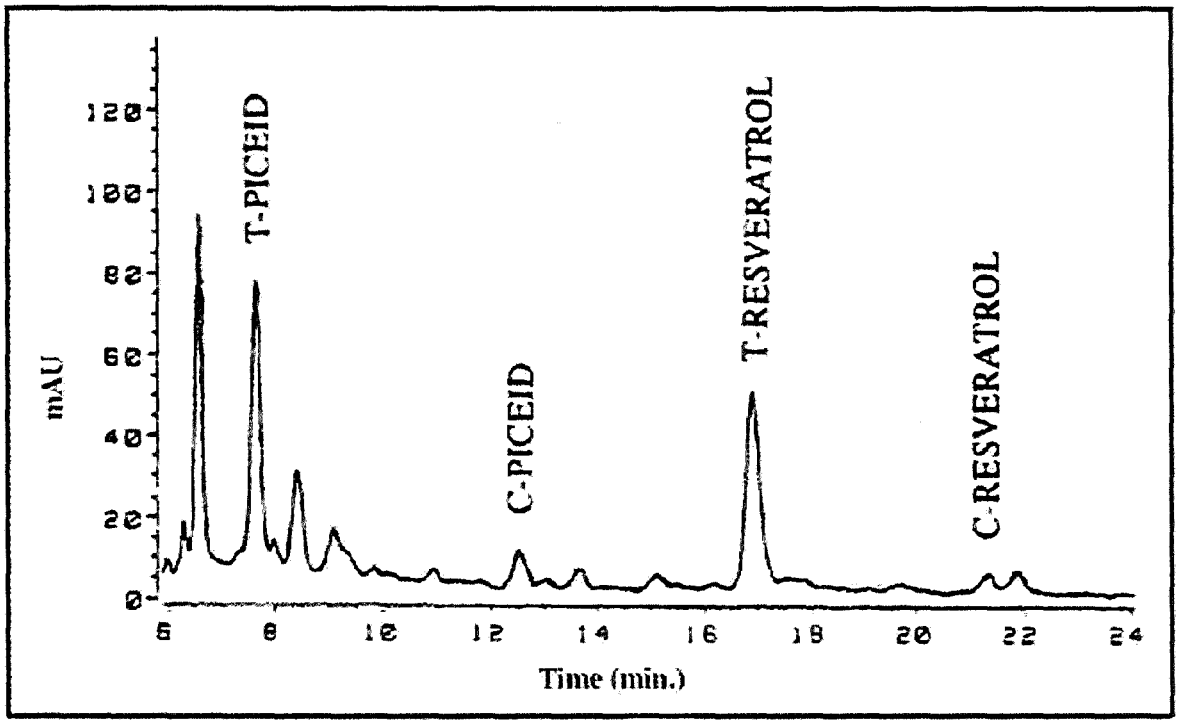


Figure 3.2. Shallow gradient HPLC separation of stilbenes

Analysis of *cis*- and *trans*-resveratrol and their glucosides in 100 μL wine. Mobile phases, (A) acetic acid in water, pH 2.4, (B) 20% A with 80% acetonitrile. Flow rate, 1.5 mL/min. Gradient 18% B for 10 min, up to 23% B by 17 min and then 23.5% B at 21 min (Lamuela-Raventós et al., 1995).

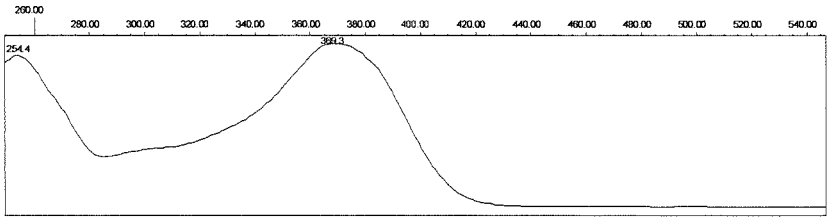
3.2.2.1 Absorbance detection

All phenolics absorb light in the UV region due to their aromatic ring (Fig. 3.3). The absorbance spectra of a compound is characteristic of its chemical class. Flavonoids generally have two absorbance bands. Band II in the region of 280 nm is common to all phenols, but flavonols, flavones (not shown) and anthocyanins show absorbance in higher wavelength regions (Table 3.1). Monitoring a sample at different wavelengths can yield a lot of information about its composition (Fig. 3.4). For instance monitoring at 520 nm allows the selective detection of anthocyanins. Likewise flavonols characteristically show absorbtion in the region of 365 nm. Highest sensitivity is obtained by monitoring the eluent at the maximum wavelength, λ max, of the compound of interest. Compounds with a λ max of a higher wavelength, such as anthocyanins, are in general easier to analyse as far fewer impurities are detected and thus interfere with the analysis.

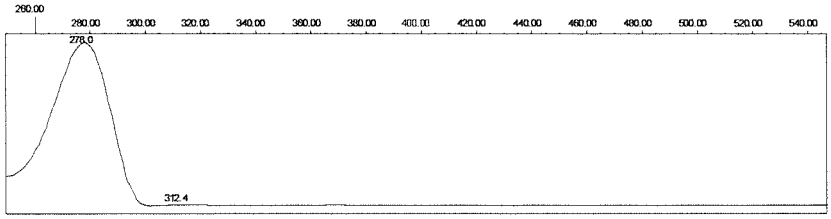
3.2.2.2 Fluorescence detection

While all phenolic compounds have UV absorbance not all show the ability to fluoresce with or without chemical derivatization. This provides a higher level of selectivity and an accompanying increase in sensitivity (Crozier et al., 1997a) (Fig. 3.5). The chromatograms A and B represent an injection of the same standard mix monitored with both absorbance and fluorescence detection after post-column derivatization. By looking at the respective scales it can be seen that fluorescence is 20-fold more sensitive. The selectivity of this approach is also apparent. While standards nine flavonol/flavone compounds are shown with UV absorbance at 365 nm, only five can be detected by fluorescence. A comparison of the pre and post-hydrolysed wine samples (Fig. 3.5 [C] and [D]) reveals the presence of impurities in the absorbance trace that are all but absent when the post-column fluorescence procedure is utilised.

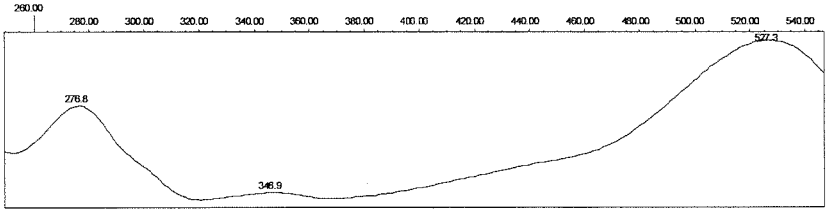
Flavonol [quercetin]



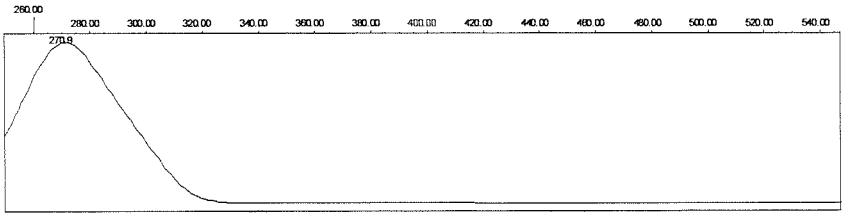
Flavan-3-ol [(+)-catechin]



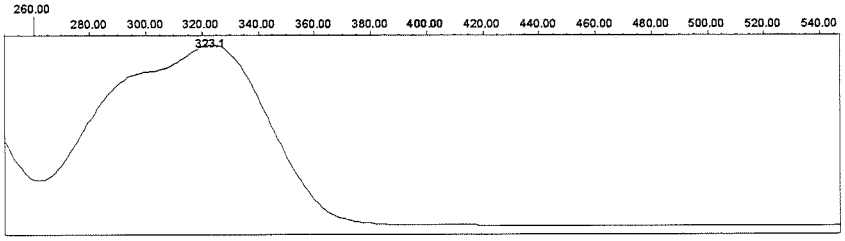
Anthocyanidin [malvidin]



Hydroxybenzoate [gallic acid]



Hydroxycinnamate [*p*-coumaric acid]



Stilbene [*trans*-resveratrol]

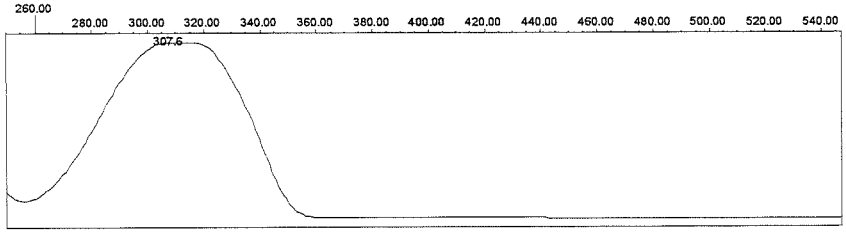


Figure 3.3. Comparison of the UV absorbance spectra of the major classes of phenolics

Table 3.1. UV absorption spectra for the major phenolic classes

Class	Band II	Band I
flavonol	250-280	350-385
flavan-3-ol	250-300	
anthocyanin	265-275	465-560
hydroxybenzoate	235-305	
hydroxycinnamate	227-245, 310-332	
stilbene	250-340	

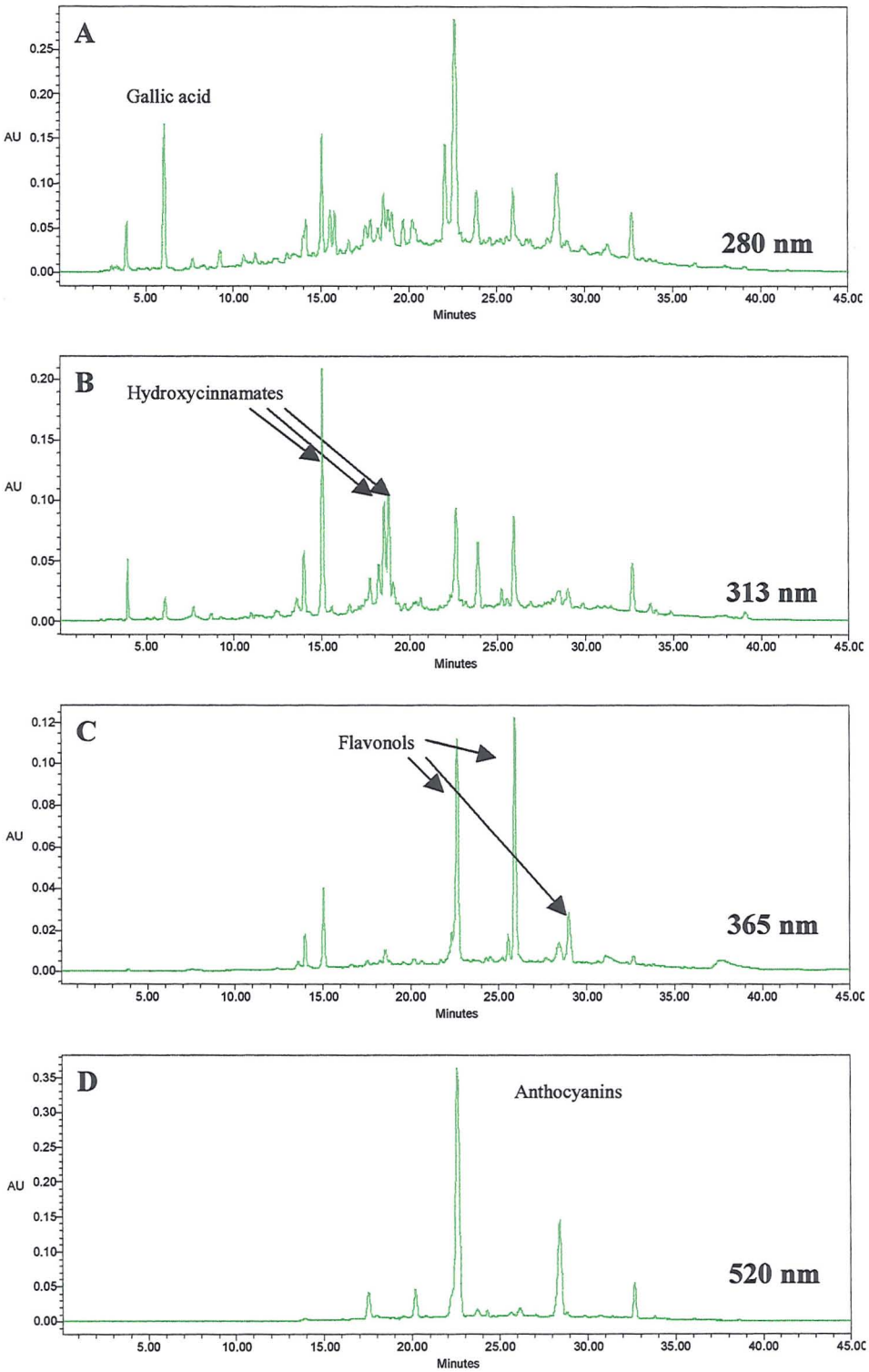


Figure 3.4. Wine monitored at four different wavelengths

Gradient RP-HPLC separation of phenolics in a 1 μL sample of concentrated Chilean Cabernet Sauvignon; Column, 250 x 4.6 mm i.d., Max RP 80 A at 40 °C, Mobile phase, 40 min gradient of 5-40% of ACN in 0.1% TFA; Flow rate, 1 mL/min. Eluent monitored at (A) 280 nm, (B) 313 nm, (C) 365 nm and (D) 520 nm.

3.3 Methods developed for the analysis of phenolics

There were two main requirements for the methods developed in this study. As much of the work was to be quantitative, the chromatography had to be of a sufficient standard to allow for the accurate determination of peak area for the relevant analysis. Ideally the baseline should be flat and the peaks of interest must be homogenous with baseline separation from impurities being achieved.

In addition a large number of samples were to be analysed. These included grape, must and wine samples. With time constraints in mind, minimal sample preparation would be beneficial. Many of the long-gradient approaches make use of extensive sample purification or fractionation. This would remove many impurities providing a cleaner chromatogram. However such treatments would require both more sample and time, and recoveries of some of the compounds of interest may be poor.

With both these considerations in mind it was decided to develop short selective HPLC methods for the separation of each family of compounds of interest. Although this would mean that analyses would take longer than they would with the long gradient approach, the resolution gained would make identification and quantification more accurate.

3.3.1 Flavonols

Initial investigations into the identification of flavonols focused only on levels of the aglycones after acid hydrolysis (Hertog et al., 1992a, b, 1993c). Myricetin and quercetin were separated isocratically from wine, however the chromatography was poor. Myricetin was eluted within 5 minutes and the peaks showed tailing. Extensive investigations into the analysis of flavonols have shown that only certain reversed-phase columns are suitable for their separation. On some supports the flavonol aglycone peaks are broad and asymmetrical, perhaps due to incomplete end-capping leaving residual groups

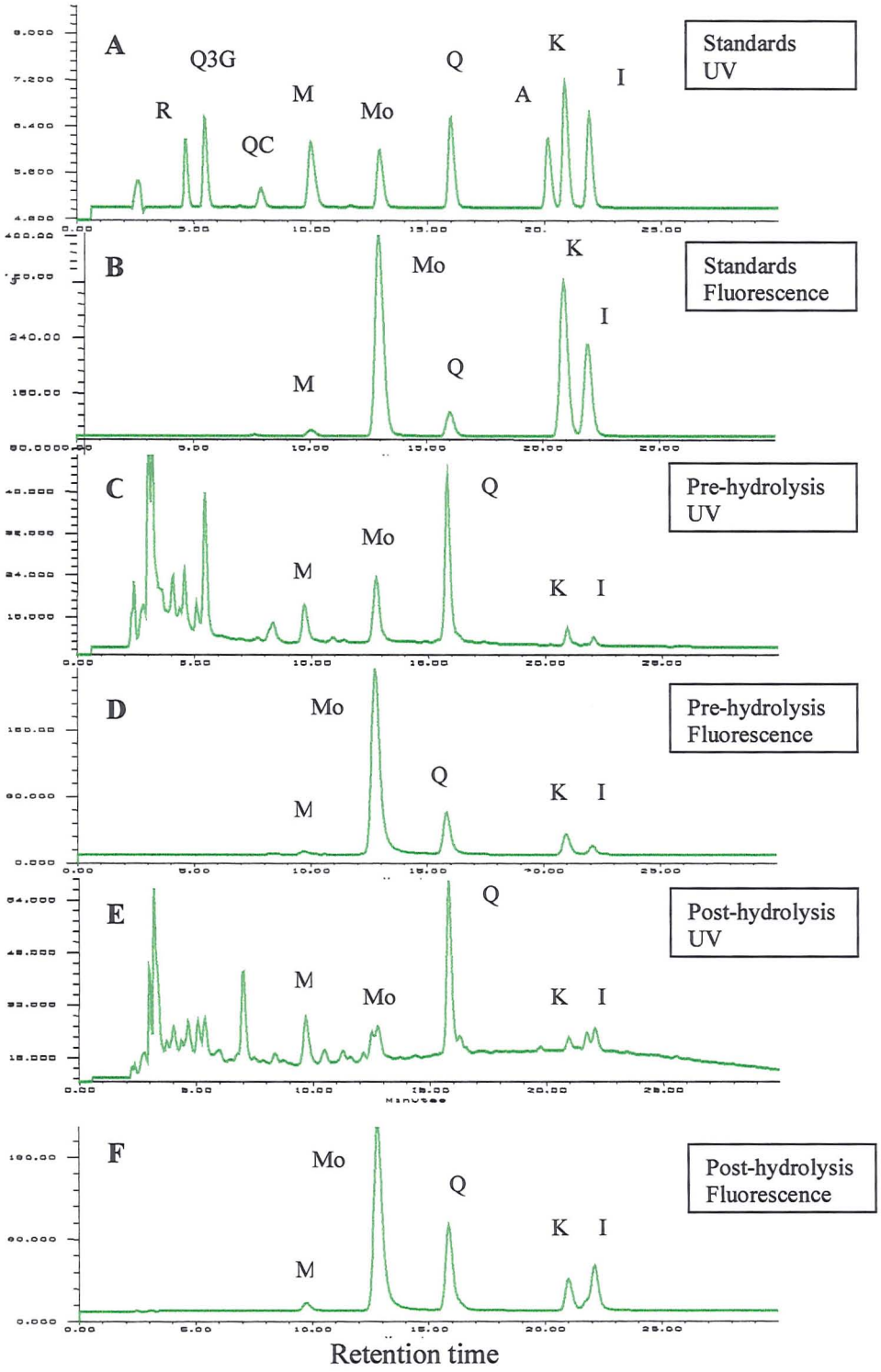


Figure 3.5. HPLC trace of the separation of flavonoids

Analysis of free and conjugated flavonols in wine 14, a 1995 French Minervois; Column, 150 x 3.0 mm i.d. 4μm Genesis C_{18} at 40 °C; Mobile phase, 20 min gradient of 20–40% ACN in water adjusted to pH 2.5 with TFA; Flow rate, 0.5 mL/min; samples extract aliquot equivalent to 4.6 μL wine before [(C) + (D)] and after [(E) + (F)] hydrolysis; Detection, (A), (C), and (E) absorbance at 365 nm (B), (D) and (F), fluorescence (excitation = 425 nm, emission = 480 nm) after post column derivatisation with methanolic aluminium nitrate. Peaks R-rutin; Q3G-quercetin-3-glucoside; QC-quercitrin; M-myricetin; Mo-morin; Q-quercetin; A-apigenin; K-kaempferol; I-isorhamnetin. Morin included as an internal standard in wine extract.

on the silica gel support. Further work optimised the conditions for the gradient elution of a total of nine flavone, flavonols and flavonol glucosides (Crozier et al., 1997a). More recent studies in our laboratory have shown that relatively few supports provide good chromatography of free flavonols.

While initial investigations used only UV absorbance to detect the flavonols myricetin and quercetin in wines (McDonald et al., 1998), recent studies have made use of post-column derivatization to produce fluorescent flavonol complexes (Hollman et al., 1996; Aziz et al., 1998). This technique has allowed four flavonol aglycones to be quantified in wines (Fig. 3.5). As with compounds which fluoresce naturally, this provided both enhanced selectivity and sensitivity which is evident when comparing the A_{365} trace and fluorescence traces obtained with wine before and after acid hydrolysis. A small impurity was observed forming a shoulder on isorhamnetin (Fig. 3.5 (E). Attempts to separate this from isorhamnetin proved unsuccessful. However it was excluded as much as possible from quantification with careful and consistent peak integration.

Flavonols were quantified before and after acid hydrolysis thereby allowing the content of free and conjugated flavonols to be determined. Although this method supplies invaluable information on the concentration of flavonol conjugates, it provides none on the nature of conjugation. However direct HPLC analysis of wine flavonol glucosides proved problematic. They do not fluoresce, they elute earlier than the aglycones, co-elute with many other compounds and in most instances, unlike their aglycones, reference compounds were not available.

3.3.2 Flavan-3-ols

Much of the literature on the analysis of flavan-3-ols focuses on tea, where they are found in high levels (Finger et al., 1992). Wine contains the flavan-3-ols (+)-catechin and (-)-epicatechin in addition to gallocatechins and gallate esters, although the latter two groups are present in only very low levels.

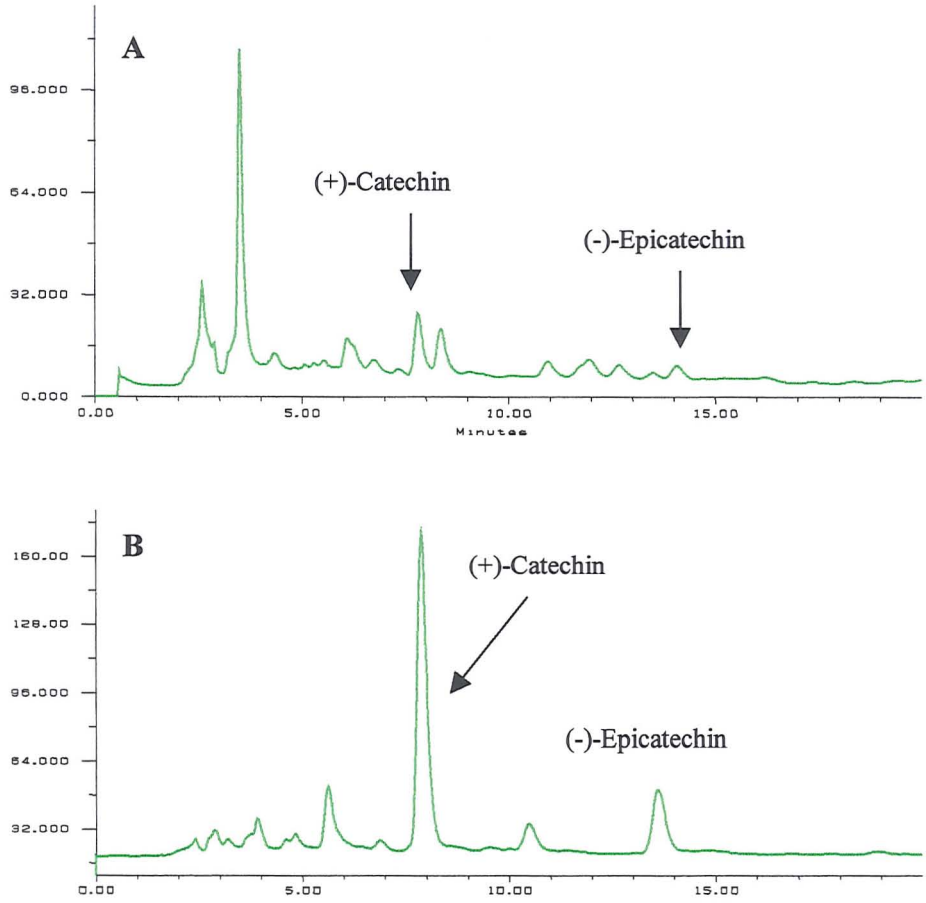


Figure 3.6. HPLC traces of the separation of flavan-3-ols

Analysis of (+)-catechin and (-)-epicatechin in 10 µL volumes of wine 14, a 1995 French Minervois. Column, 150 x 4.6 mm i.d. 5µm C_{18} Luna column; Mobile phase, 10% ACN in water adjusted to pH 1.5 with TFA; Flow rate, 1mL/min (A) 10 µL wine monitored at 280 nm; (B) as (A) but with fluorescence (excitation = 280 nm and emission = 310 nm).

A comprehensive analysis of the levels of (+)-catechin and (-)-epicatechin in red wines (Goldberg et al., 1998a) separated wine phenolics using a long gradient (Fig. 3.1). (+)-Catechin and (-)-epicatechin eluted at 8.36 and 12.19 minutes respectively. Examining the chromatogram in Figure 3.1[A] we can see that while these peaks are sharp, the compounds are eluting in the early stages of the run where the baseline is sloping steeply. This will make accurate integration of the flavan-3-ol peaks difficult, if not impossible, to achieve. While the original description of the method presents the full, complex chromatogram, the following publication discussing levels of (+)-catechin and (-)-epicatechin expands the chromatogram between 6 and 16 min and shows only this section (Fig. 3.1 [C]). This gives a rather misleading impression of the resolution of the phenolic compounds using this method.

A feature of the flavan-3-ols is that they absorb maximally at 280 nm. This is a region where all phenolic compounds absorb and consequently it can be difficult to detect and quantify flavan-3-ols in a complex sample such as wine. Fortunately both (+)-catechin and its isomer (-)-epicatechin are naturally fluorescent. This property of flavan-3-ols was initially exploited for the analysis of a variety of fruits, including grapes (Arts and Hollman, 1998) but has been extended to wine (Arts et al., 2000). The increase in sensitivity can be seen by comparing chromatograms [A] and [B] in Figure 3.6. They correspond to the same injection of sample but [A] represents absorbance at 280 nm and [B] fluorescence with excitation at 280 nm and emission at 310 nm. Fluorescence detection shows approximately 5-fold greater sensitivity than absorbance. In addition it can be seen that fluorescence detection offers greater selectivity. A number of other compounds elute in the same region as (-)-epicatechin [A] but are not visible in the fluorescent trace (B).

3.3.3 Anthocyanins

Anthocyanins, and their aglycone anthocyanidins, are the major contributors to the colouring of many fruits, including grapes. The anthocyanin profile differs between grapes, a young wine and an aged wine (Fig. 3.7). In grapes mono- or di-glucosides of the main anthocyanidins predominate and can be identified

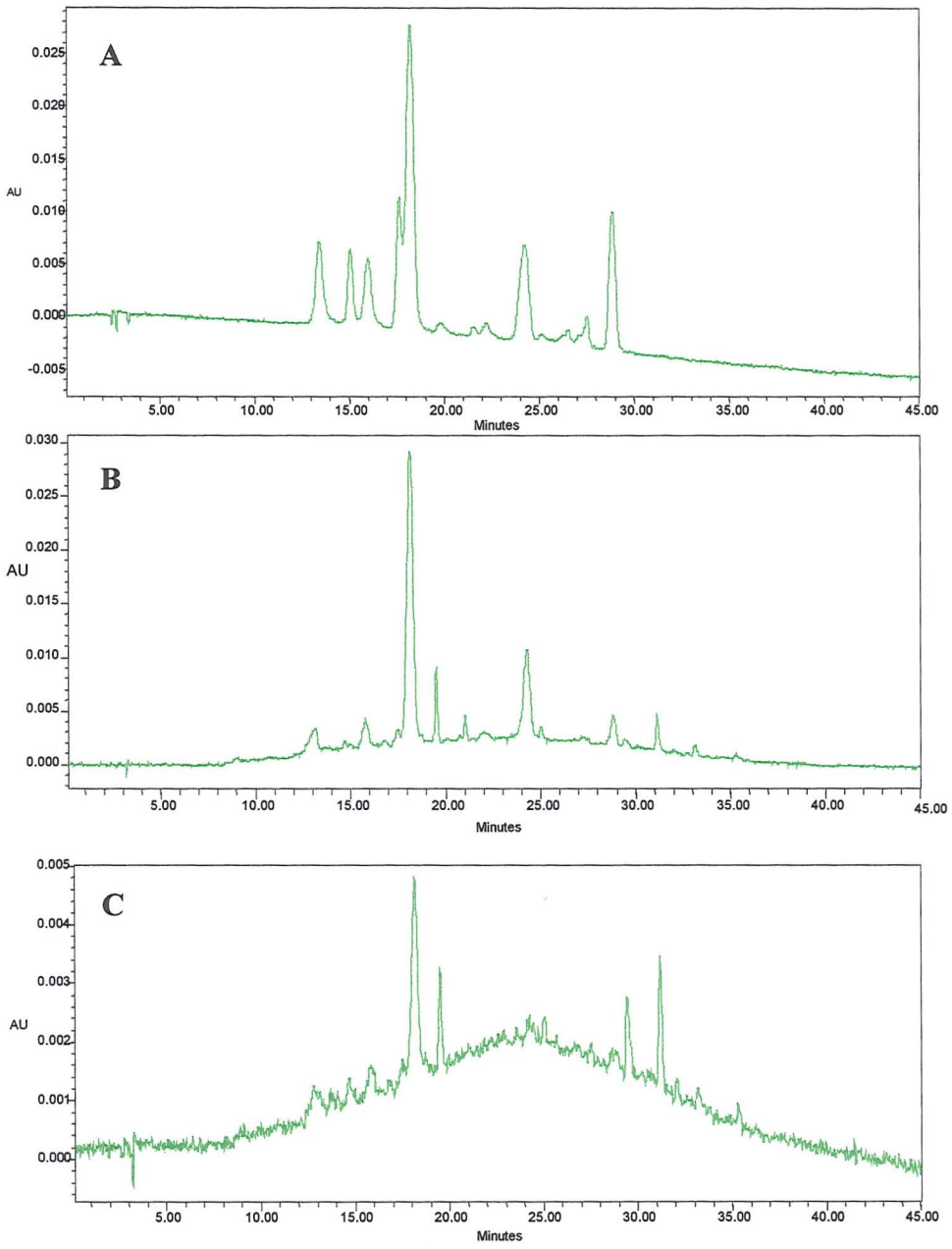


Figure 3.7. Comparison of the anthocyanin content of grapes, young wine and old wine.

Anthocyanins in 10 μL aliquots of (A) grape extract, (B) Chilean Cabernet Sauvignon, 1999 and (C) Australian Cabernet Sauvignon, 1997. Column, 250 x 4.6 mm i.d. 4μm C_{18} Nova-Pak. Mobile phase, 40 min gradient of 5-30 % ACN in 5% aqueous formic acid. Flow rate, 1 mL/min Detection, absorbance at 520 n.

and quantified with relative ease. As a wine ages larger polymeric pigments are formed (Haslam, 1998). These are condensation products of anthocyanins and other phenolics and do not chromatograph as defined peaks so their presence results in a broad underlying peak spread over the centre of the chromatogram. Due to their poor diffuse elution profile it has proved difficult to identify many of the complex pigments, or quantify the low levels of free anthocyanins still present.

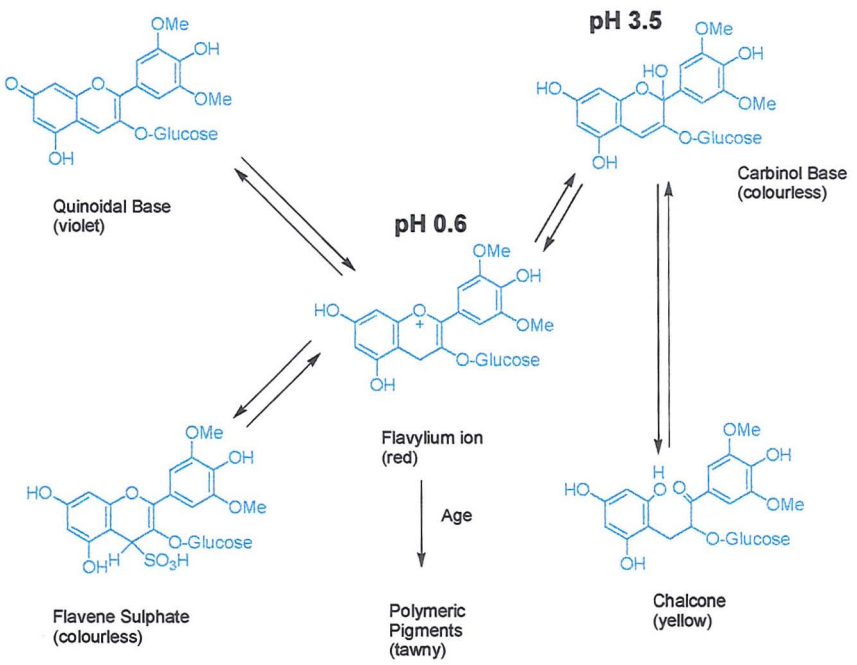


Figure 3.8. The chemical nature of the colours of anthocyanins

The major problem occurring with the chromatography of anthocyanins is due to their chemical structure. It is only at a low pH that they are ionised and in their red coloured flavylium form (Fig. 3.8 adapted from Bohm, 1998). Reversed phase HPLC methods to analyse anthocyanins have used, almost exclusively, a high acid content in both the aqueous and organic solvents. In addition to stabilising the anthocyanin structure this appears also to have been required for column efficiency. Many of the older C_{18} supports, which are probably not efficiently end-capped, required up to 10-15% acetic acid in the mobile phase to retain even medium peak efficiency and prevent excessive tailing. The effect of increasing acid concentration can be seen in Figure 3.9. It causes the peaks to be sharper as ionisation of the anthocyanins is suppressed.

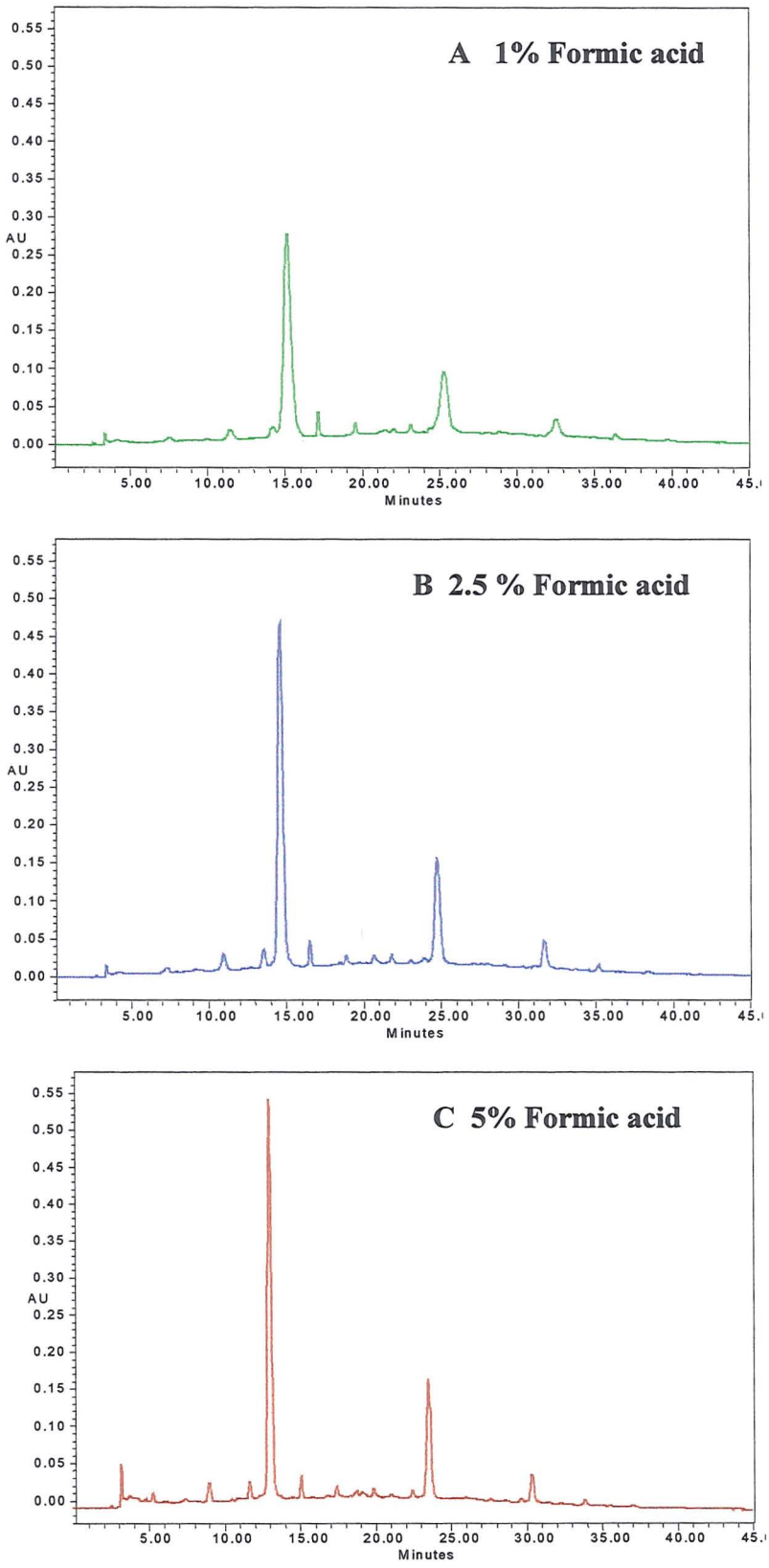


Figure 3.9. Effect of increasing acid concentration on the elution of anthocyanins

Analysis of anthocyanins in 10 μL volumes of Chilean Cabernet Sauvignon, 1999, Column, 250 x 4.6 mm i.d. 4μm C_{18} Nova-Pak; Flow rate, 1 mL/min; Mobile phase, 40 min gradient of 5-30% ACN in aqueous formic acid. (A) 1%, (B) 2.5%, (C) 5% formic acid. Detection was by absorbance at 520 nm.

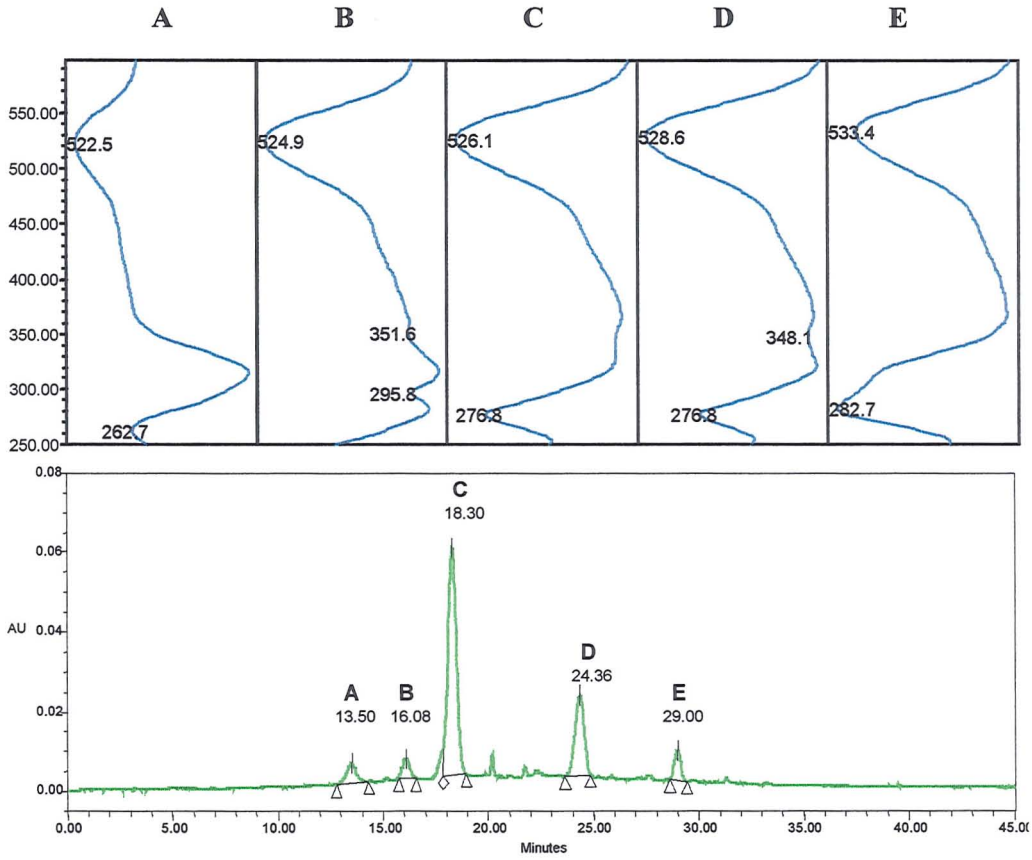


Figure 3.10. HPLC trace and absorbance spectra of the gradient RP-HPLC separation of anthocyanins

Analysis of anthocyanins in 10 µL volume of Chilean Cabernet Sauvignon, 1999, Column, 250 x 4.6 mm i.d. 4µm C_{18} Nova-Pak; Flow rate, 1 mL/min; Mobile phase, 40 min gradient of 5-30% ACN in 5% formic acid. Detection was by absorbance at 520 nm. (A) delphinidin-3-*O*-glucoside; (B), petunidin-3-*O*-glucoside; (C), malvidin-3-*O*-glucoside; (D), malvidin-3-*O*-(6-*O*-acetyl)glucoside; (E), malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside.

The retention times of the compounds are also noted to decrease at higher acid levels. Both isocratic and gradient elution have been used to separate anthocyanidins and their conjugates (Hong and Wrolstadt, 1990; Baldi et al., 1995; Revilla et al., 1999). Although it is necessary to ensure that the anthocyanins are efficiently chromatographed, because they have an absorbance maxima above 520 nm there is little, if any, interference from other phenolics. The method employed here is the gradient elution of the anthocyanins. It is able to separate the major peaks detected in grapes, musts and young wines (Fig. 3.10). Peaks are identified on the basis of retention times and absorbance spectra.

3.3.4 Hydroxybenzoates

The major hydroxybenzoate found in red wines is gallic acid. Its dimer, ellagic acid, is present in much lower concentrations.

3.3.4.1 Gallic acid

Gallic acid is one of the most simple of all phenolic compounds. It has only a single aromatic ring and consequently a simple absorbance spectrum. It shows maximum absorbance in the region of 270 nm (Fig. 3.3). Much of the information on its analysis is based on tea where it is quantified along with the flavan-3-ols (Finger et al., 1992). Most published methods used show gallic acid eluting with a low k′ value, very shortly after the void volume peak. Rather than combine gallic acid analysis with that of the flavan-3-ols it was decided to develop a method where gallic acid was eluted with a k′ of 4-5 in order resolve it from any early eluting impurities. It was vital to operate the elution of the compound with a low pH to ensure that ionisation was fully suppressed otherwise gallic acid produced a double peak. However the higher the acid content of the mobile phase, the shorter the retention time. After analysing gallic acid on a number of columns, acceptable chromatography was eventually achieved with a C_{18} Genesis support eluted with a mobile phase of 2% methanol in water adjusted to pH 1.5 with trifluoracetic acid (Fig. 3.11 [A]).

3.3.4.2 Ellagic acid

Few methods exist in the literature for the HPLC analysis of ellagic acid. Traditionally identification has been by paper chromatography after Sephadex LH-20 column chromatography (Quinn and Singleton, 1985). HPLC was first used to quantify the gallic acid dimer in a study on fruits in which a C_{18} column was eluted with a gradient of methanol in ammonium dihydrogen buffer (Daniel et al., 1989). Ellagic acid eluted as a very broad (5-7 min) peak with a retention time of 40 min, casting doubt on the accuracy of the analysis.

An improved HPLC method was developed for isocratic elution of ellagic acid in wines. The dimer was well retained on a 150 x 4.6 mm i.d. Genesis C_{18} column eluted with a mobile phase of 30% methanol in 0.1% aqueous TFA. The ellagic acid standard from Sigma was supposedly 95% pure but it clearly contained higher levels of impurities (Fig. 3.11 [B]).

3.3.5 Hydroxycinnamates

The major hydroxycinnamates found in wine are the tartrate esters of caffeic and *p*-coumaric acids. Although standards of the aglycones are widely supplied, those of the conjugates are not available commercially. The Australian Wine Research Institute kindly supplied a sample of caftaric acid (the tartrate ester of caffeic acid). However it was not possible to obtain a standard of coutaric acid (the tartrate ester of *p*-coumaric acid). Consequently coutaric acid could not be identified and could only be estimated after alkaline hydrolysis, which cleaves the tartrate bond and enables the aglycone to be identified and quantified (Rapisarda et al., 1998).

Caffeic acid has a λ max around 323 nm, while that of *p*-coumaric acid is in the region of 308 nm, as a compromise is was decided to monitor the eluent at 313 nm as no diode-array was available at this time. At this wavelength both of the compounds exhibit significant absorbance.

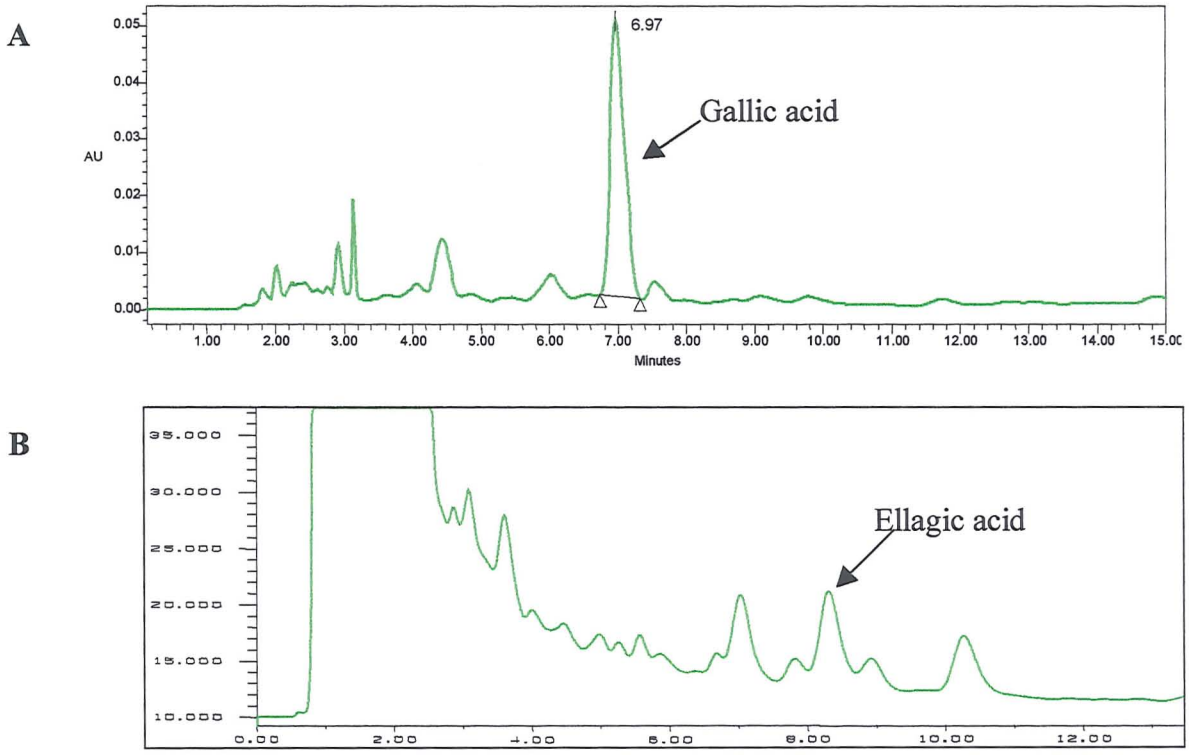


Figure 3.11. HPLC trace for the separation of gallic acid and ellagic acid in wine

(A) Gallic acid was analysed in 5 µL volumes of Chilean Cabernet Sauvignon 1999. Column, 150 x 3.0 mm i.d. 4µm Genesis C_{18}. Mobile phase, 2% MeOH in water adjusted to pH 1.5 with TFA. Flow rate, 1.0 mL/min. Detection, absorbance at 280 nm. Ellagic acid was analysed in 5 µL volumes of French Minervois, 1995. Column, 150 x 3.0 mm i.d. 4µm Genesis C_{18}. Mobile phase, 30% ACN in 0.1% aqueous formic acid. Flow rate, 1.0 mL/min. Detection, absorbance at 255 nm.

Hydroxycinnamates have previously been separated by both isocratic and gradient elution (Somers et al., 1987; Betés-Saura et al., 1996; Rapisarda et al., 1998). On the basis of the traces in these publications, in the current study it was decided to use isocratic elution. While caftaric acid and free caffeic acid could be quantified on the same isocratic run, free *p*-coumaric acid had to be analysed separately. Although this proved time consuming, enhanced peak resolution and an absence of interfering impurities were obtained by the use of the two isocratic methods (Fig. 3.12).

3.3.6 Stilbenes

Although much interest has surrounded the presence of stilbenes, particularly *trans*-resveratrol, in wines, they are found in only low concentrations. The combination of their low levels in wines and a lack of commercial standards has made their detection and quantification problematical. The *cis* isomers of resveratrol and its glucoside can be produced by UV exposure, although, quantification must be on the basis of the *trans* isomer. While recent studies have primarily used HPLC to quantify stilbenes (Waterhouse and Lamuela-Raventós, 1994; Goldberg et al., 1995c) GC has also been utilised (Jeandet et al., 1993; Goldberg et al., 1995b). However this requires the stilbenes to be derivitised to increase their volatility, a time-consuming step which may not always go to completion and in the absence of a suitable internal standard this will result in low recoveries.

The majority of HPLC methods for the analysis of stilbenes use isocratic elution (Sieman and Creasy, 1992; Goldberg et al., 1995c; Waterhouse and Lamuela-Raventós, 1994). Their low levels make them unsuitable for many of the long gradient HPLC methods where they would be in danger of co-eluting with one of the many other compounds present.

Two isocratic methods were developed for the analysis of *trans*-resveratrol (Fig. 3.13 [A]) and *trans*-resveratrol glucoside (Fig. 3.13 [B]). In both instances detection was by absorbance at 307 nm. Absorbance detection has been the method of choice in stilbene analysis, however recently it has been

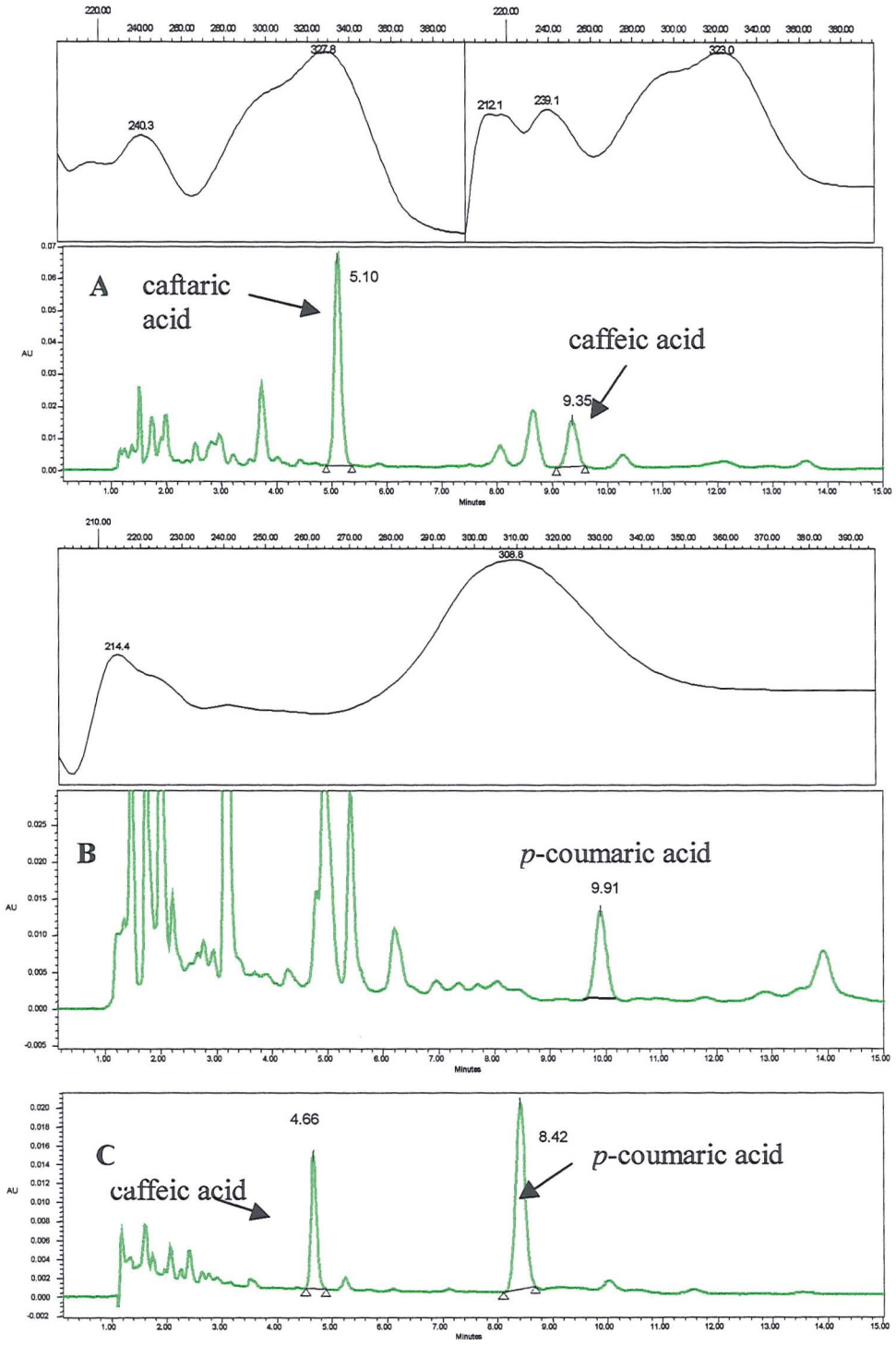


Figure 3.12. Absorption spectra and RP-HPLC separation of hydroxycinnamates

5µL volumes were analysed before [A and B] and after [C] alkaline hydrolysis. Column, 150 x 3.0 mm i.d. 5µm C_{18} Nemesis. Mobile phase, (A) 7% ACN in water adjusted to pH 1.5 with TFA, (B) 11% ACN in water adjusted to pH 1.5 with TFA and (C) 12% ACN in water adjusted to pH 1.5 with TFA. Flow rate, 1 mL/min. Detection, absorbance at 313 nm.

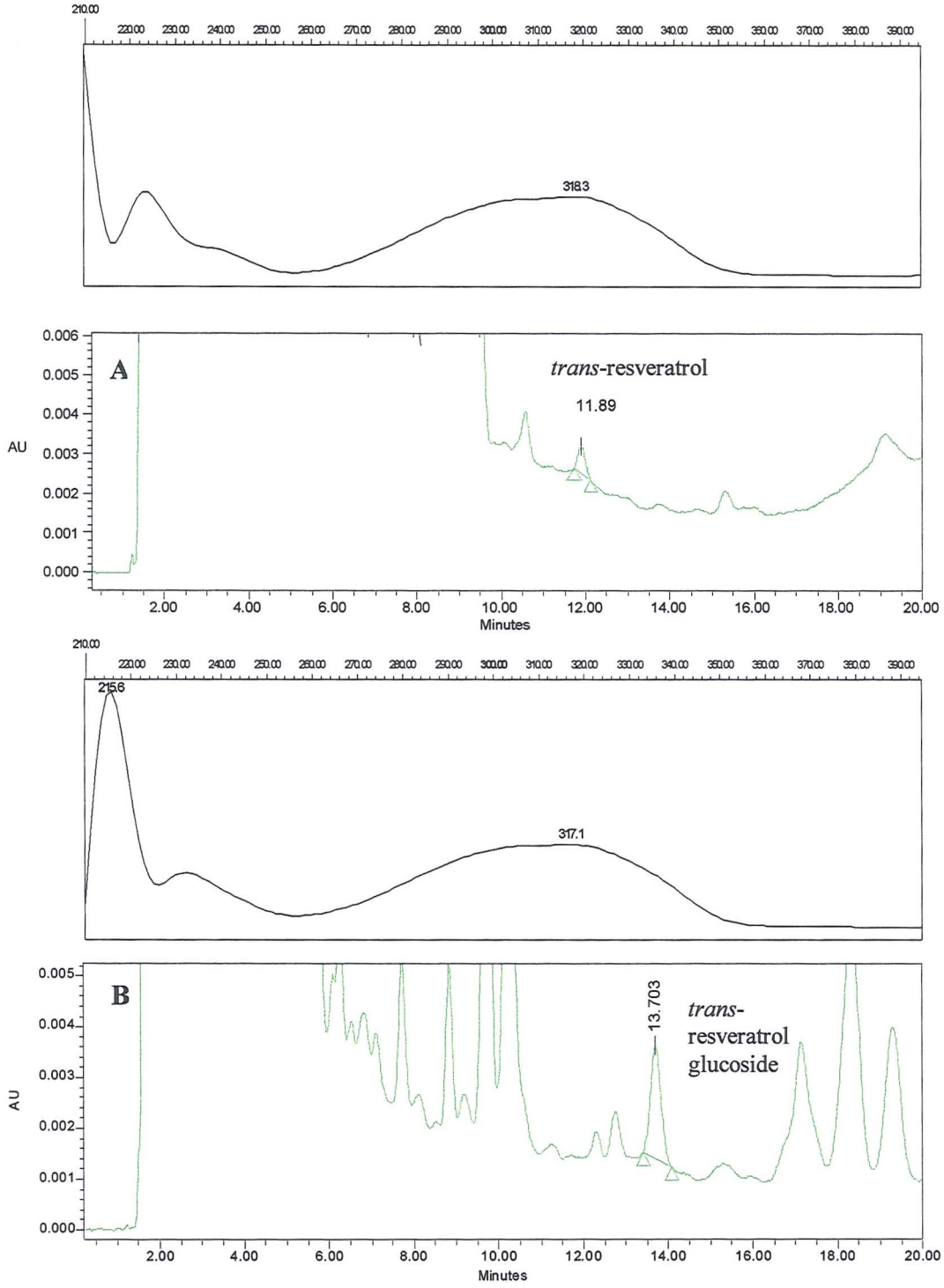


Figure 3.13. RP-HPLC separation and absorbance spectra of *trans*-resveratrol and *trans*-resveratrol glucoside

10 µL volumes of Chilean Cabernet Sauvignon, 1999 were analysed. Column, 250 x 4.6 mm i.d. 5µm ODS-Hypersil column. Mobile phase, (A) 25% ACN in water adjusted to pH 1.5 and (B) 17% ACN in water adjusted to pH 1.5. Flow rate, 1mL/min. Detection, absorbance at 307 nm.

shown that *trans*-resveratrol exhibits natural fluoresce. Using excitation at 298 nm and emission at 385 nm *trans*-resveratrol was detected in six white and six red wines (Carando et al., 1999a). Although chromatograms of fluorescence detection are illustrated in this publication, there is none of the corresponding UV detection to allow comparison of the sensitivity and selectivity.

3.4 Conclusion

A selection of HPLC methods was developed for the analysis of phenolics in red wines. The exception was the method for the analysis of flavonols which was adapted from that of Crozier et al. (1997b). Method development should be viewed as a continuing process. Improved supports, offering differences in selectivity continue to be marketed and were investigated. Detector availability is also important and has been shown in several instances in this section to markedly affect analyses. At the beginning of this study in October 1997 the sole detectors available were a dual wavelength absorbance monitor and a fluorescence detector. Subsequently routine access to a photo-diode array detector was possible and this was followed by occasional use of a single quadropole LC-MS that has recently been replaced with an ion-trap LC-MS-MS.

Chapter 4 Relationship between anti-oxidant activity and phenolic content of red wines

4.1 Introduction

Red wine contains wood- and yeast-derived phenolics, in addition to large amounts of phenolic compounds that originate from grapes, particularly the skins (Singleton, 1982). Although structurally diverse, phenolics can be classified into two groups – the flavonoids and the non-flavonoids. The flavonoid family includes the flavonols, myricetin, quercetin, kaempferol and isorhamnetin, which exist as both aglycones and as sugar conjugates; the flavan-3-ols (+)-catechin and (-)-epicatechin; and the anthocyanins such as malvidin-3-glucoside. The non-flavonoids encompass gallic acid; hydroxycinnamates, including *p*-coumaric, caffeic and caftaric acids; and the stilbenes, *trans*-resveratrol, *cis*-resveratrol and *trans*-resveratrol-*O*-β-glucoside. A significant proportion of the phenolic content of wine originates from the tannins which are sub-divided into condensed and hydrolysable tannins. The condensed tannins, known as procyanidins, are oligomers and polymers of (+)-catechin and (-)-epicatechin sub-units, while the hydrolysable tannins are based on gallic acid and its derivatives. Red wines contain, in total 1500-2500 mg/L of phenolics (Frankel et al., 1993a), although their presence and structures are affected by a number of factors including grape variety, sun exposure, vinification techniques and ageing (Price et al., 1995; McDonald et al., 1998).

Red wines do not contain significant amounts of vitamins or selenium, and their protective effects have been ascribed to phenolic components. It has been proposed that they act as antioxidants. The antioxidant capacity of phenolic compounds is essentially due to the ease with which an hydrogen atom from an aromatic hydroxyl group can be donated to a free radical, and the ability of the phenolic moiety to support an unpaired electron due to

delocalisation around the π-electron system (Kanner et al., 1994). Such activity could reduce free radical-mediated oxidation of low-density lipoprotein (LDL) and so decrease artherogenicity (Frankel et al., 1993a, 1995; Fuhrman et al., 1995; Nigdikar et al., 1998). Other mechanisms have been proposed to explain the beneficial effects of red wine in the prevention of CHD including inhibition of platelet aggregation (Gryglewski et al., 1987; Pace-Asciak et al., 1995) and endothelium-dependent relaxation of blood vessels, mediated by the NO-cGMP pathway (Fitzpatrick et al., 1993).

This chapter reports on a study to investigate the relationship between the phenolic content of a wine and its antioxidant activity. Two batches of wine were analysed. Wines were chosen to include a cross-section of those commonly consumed. While batch I is composed mainly of wines from the old world, batch II represents predominately the new world. The antioxidant activity of the wines was measured by electron-spin resonance (ESR) spectroscopy and with batch I wines parallel measurements of vasodilation capacity were also made. In addition, a variety of high-performance liquid chromatography (HPLC) procedures were used to identify and quantify the phenolic components present in the individual wines, and anthocyanins were determined using a spectrophotomeric method.

4.2 Relationship among phenolic content, antioxidant activity and vasodilation capacity of wines (batch I)

4.2.1 Antioxidant activity

The ability of the 16 wines to reduce the Fremy's salt free radical in the ESR-based antioxidant assay was assessed and values ranging from 4.13×10^{21} to 9.29×10^{21} radicals reduced/L were obtained (Table 4.1). This compares with a range of 6.59×10^{21} to 8.55×10^{21} radicals/L for seven red wines studied previously (Gardner et al., 1999). In this chemical model system, Beaujolais (wine 13) and Valpolicella (wine 15) showed the lowest activities, while

Table 4.1. Antioxidant activity, vasodilation activity and phenolic content of red wines (batch I).

Wine	ESR-based antioxidant activity[a]	Vasodilation activity[b]	Folin-Ciocalteu total phenolics[c]	HPLC total phenolics[d]
1	7.0 ± 0.2	39 ± 8	10.5 ± 0.2	1.0 ± 0.0
2	6.0 ± 0.2	188 ± 64	10.2 ± 0.1	1.1 ± 0.0
3	9.3 ± 0.3	9 ± 3	18.6 ± 0.1	1.7 ± 0.0
4	6.2 ± 0.2	127 ± 27	11.1 ± 0.1	1.0 ± 0.0
5	7.3 ± 0.3	46 ± 6	13.3 ± 0.1	1.6 ± 0.0
6	6.6 ± 0.1	239 ± 51	11.9 ± 0.0	1.1 ± 0.0
7	8.0 ± 0.1	28 ± 7	15.7 ± 0.1	1.3 ± 0.1
8	6.0 ± 0.1	46 ± 6	10.4 ± 0.1	0.9 ± 0.0
9	8.0 ± 0.2	28 ± 6	14.6 ± 0.1	1.4 ± 0.0
10	7.5 ± 0.3	37 ± 5	14.2 ± 0.0	1.2 ± 0.0
11	6.6 ± 0.2	50 ± 10	12.1 ± 0.0	1.2 ± 0.0
12	6.4 ± 0.1	54 ± 10	11.6 ± 0.0	1.2 ± 0.0
13	4.1 ± 0.1	256 ± 123	6.5 ± 0.0	0.9 ± 0.0
14	8.3 ± 0.1	21 ± 4	17.4 ± 0.1	2.0 ± 0.0
15	4.5 ± 0.0	380 ± 142	7.7 ± 0.1	0.8 ± 0.0
16	7.1 ± 0.1	27 ± 6	13.5 ± 0.1	1.2 ± 0.0

[a]Antioxidant capacity of red wines, measured by ESR spectroscopy, presented as the number of Fremy's radicals reduced by one litre of wine x 10^{21}. [b]Vasodilation capacity expressed as concentration of wine extract required to give 50% maximal contraction of aortic rings, pIC_{50}. [c,d]Total phenol content of red wine determined by the Folin-Ciocalteu method (mM gallic acid equivalents GAE) and from HPLC analysis of individual phenolics (mM). All data expressed as mean values ± SEM

Bulgarian Young Vatted Cabernet Sauvignon (wine 3) and Minervois (wine 14) were ranked first and second, respectively.

4.2.2 Vasodilation activity

The vasodilation activity of the wines was also assessed and the pIC_{50} values determined (Table 4.1). Although all showed activity, a varying response was observed across the range of wines. The Young Vatted Bulgarian Cabernet Sauvignon (wine 3) and the Minervois (wine 14) were once again found to be the most active, while Beaujolais (wine 13) and Valpolicella (wine 15) exhibited the lowest activity.

4.2.3 Analysis of individual phenolic compounds

In order to investigate the phenolic content of the red wines in detail, samples were analysed using a number of HPLC systems custom designed for the different categories of phenolic components. Anthocyanins were measured using a spectrophotometric method. The data obtained were as follows:

4.2.3.1 Flavonols

The flavonols were analysed by gradient RP-HPLC with detection at $A_{365\ nm}$, as used in previous studies with fruits, vegetables (Crozier et al., 1997b) and wines (McDonald et al., 1998). However, in this instance an additional, on-line post-column derivatization step was used to provide sensitive and selective detection of flavonols (Aziz et al., 1998).

The flavonol contents of the 16 red wines are presented in Table 4.2. All the wines contained free and conjugated myricetin, quercetin, kaempferol and isorhamnetin in varying concentrations and with different aglycone:conjugate ratios. As in our previous study with red wines (McDonald et al., 1998), there are more than 10-fold differences in total flavonol content with values ranging from 17.6 μM in the 1992 Bulgarian Cabernet Sauvignon (wine 4) to over 187 μM in Chilean Cabernet Sauvignon (wine 1) and Pinot Noir (wine 5) and

Table 4.2. Free and conjugated flavonol content of red wine (batch 1).

wine	free M	conj. M	total M	free Q	conj. Q	total Q	free K	conj. K	total K	free I	conj. I	total I	total flavonols	% free
1	6.0 ± 0.2	51.0 ± 3.3	55.1 ± 4.3	11.9 ± 0.1	87.8 ± 11.2	95.8 ± 14.3	2.3 ± 0.0	7.3 ± 0.3	8.8 ± 0.7	2.3 ± 0.1	25.8 ± 2.8	27.4 ± 3.5	187.1 ± 19.1	12.0
2	12.5 ± 0.5	16.6 ± 1.6	29.1 ± 1.0	26.8 ± 0.8	41.1 ± 3.3	67.9 ± 2.6	3.8 ± 0.1	1.5 ± 0.5	5.4 ± 0.4	6.1 ± 0.2	7.0 ± 1.8	13.1 ± 1.6	115.5 ± 4.6	42.6
3	8.5 ± 0.1	12.4 ± 0.4	20.9 ± 0.3	3.5 ± 0.1	13.2 ± 0.7	16.7 ± 0.8	1.7 ± 0.0	0.7 ± 0.2	2.3 ± 0.2	0.3 ± 0.0	13.4 ± 0.9	13.6 ± 0.9	53.5 ± 1.7	26.2
4	2.3 ± 0.1	3.5 ± 0.2	5.8 ± 0.3	1.8 ± 0.1	3.2 ± 0.1	5.0 ± 0.1	n.d.	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	6.3 ± 0.1	6.6 ± 0.1	17.6 ± 0.5	25.0
5	18.1 ± 0.1	13.8 ± 1.5	32.5 ± 0.6	36.6 ± 1.6	51.2 ± 0.2	88.6 ± 2.1	2.7 ± 0.1	2.7 ± 0.2	5.4 ± 0.3	6.8 ± 0.1	54.2 ± 3.6	61.1 ± 3.5	187.6 ± 7.9	34.2
6	8.4 ± 0.4	8.8 ± 1.0	17.2 ± 0.6	17.1 ± 1.4	29.9 ± 2.5	47.1 ± 1.6	1.8 ± 0.0	1.6 ± 0.1	3.4 ± 0.2	2.4 ± 0.1	30.5 ± 0.9	33.0 ± 0.9	100.7 ± 2.4	29.5
7	3.2 ± 0.2	9.1 ± 0.6	12.4 ± 0.4	21.2 ± 0.5	20.0 ± 0.2	41.7 ± 0.1	2.1 ± 0.1	0.3 ± 0.0	2.5 ± 0.0	1.8 ± 0.0	16.8 ± 0.1	18.6 ± 0.1	75.2 ± 0.5	37.6
8	10.7 ± 0.7	22.7 ± 4.8	27.6 ± 0.8	11.0 ± 0.6	28.8 ± 5.5	34.6 ± 0.7	1.7 ± 0.1	1.5 ± 1.0	2.4 ± 0.2	2.8 ± 0.1	12.1 ± 2.8	13.6 ± 1.6	78.2 ± 3.9	35.5
9	6.4 ± 1.4	12.8 ± 2.9	19.2 ± 3.1	32.8 ± 3.8	29.2 ± 4.9	62.0 ± 5.1	4.0 ± 0.3	0.9 ± 0.3	4.8 ± 0.1	4.4 ± 0.5	16.9 ± 0.9	21.3 ± 1.1	107.3 ± 9.2	44.4
10	20.7 ± 1.1	18.4 ± 5.0	39.1 ± 5.5	3.5 ± 0.1	20.3 ± 3.7	23.9 ± 3.8	1.8 ± 0.0	1.8 ± 0.6	3.6 ± 0.6	0.3 ± 0.0	17.6 ± 3.1	17.9 ± 3.1	84.5 ± 11.9	31.1
11	17.1 ± 0.4	9.0 ± 1.8	26.1 ± 1.4	15.0 ± 0.1	15.3 ± 1.4	30.2 ± 1.5	0.9 ± 0.0	0.7 ± 0.1	1.6 ± 0.1	3.2 ± 0.0	11.4 ± 0.8	14.6 ± 0.8	72.5 ± 3.7	49.9
12	7.6 ± 0.1	4.5 ± 0.7	12.1 ± 0.7	7.4 ± 0.2	13.3 ± 1.1	20.8 ± 1.0	1.6 ± 0.0	0.6 ± 0.1	2.1 ± 0.1	0.7 ± 0.0	5.0 ± 0.4	5.7 ± 0.4	40.7 ± 1.7	42.5
13	3.4 ± 0.1	4.9 ± 0.4	8.3 ± 0.5	6.8 ± 0.2	9.2 ± 1.2	16.0 ± 1.3	0.2 ± 0.0	0.6 ± 0.1	0.7 ± 0.1	1.2 ± 0.0	8.7 ± 0.9	9.9 ± 1.0	34.9 ± 2.7	33.2
14	20.7 ± 4.4	30.9 ± 8.6	51.7 ± 4.3	41.9 ± 4.6	62.8 ± 14.7	104.7 ± 10.5	4.5 ± 0.2	3.2 ± 1.1	7.6 ± 0.9	7.1 ± 0.6	24.3 ± 6.1	31.4 ± 5.6	195.4 ± 21.2	38.0
15	3.1 ± 0.6	8.8 ± 1.2	12.0 ± 0.6	2.3 ± 0.2	16.6 ± 1.7	18.9 ± 1.5	1.1 ± 0.0	1.0 ± 0.1	2.0 ± 0.2	0.2 ± 0.0	16.0 ± 1.6	16.2 ± 1.6	49.1 ± 3.8	13.6
16	4.9 ± 1.3	17.0 ± 1.4	21.9 ± 0.5	20.9 ± 2.5	36.1 ± 2.4	57.0 ± 1.5	1.6 ± 0.1	0.8 ± 0.1	2.4 ± 0.0	0.5 ± 0.0	7.6 ± 0.2	8.1 ± 0.2	89.4 ± 1.9	31.2

Data are expressed as μM ± SEM, (n=3). M, Myricetin; Q, Quercetin; K, Kaempferol; I, Isorhamnetin. n.d., not detected. % free, free flavonols as % of total. Conj., conjugated.

Minervois (wine 14). The slightly higher flavonol levels observed in the present study are attributable to the additional detection of isorhamnetin and kaempferol with post-column derivatization and fluorescence detection.

4.2.3.2 Stilbenes

Information on the levels of *cis*- and *trans*-resveratrol and *trans*-resveratrol-*O*-β-glucoside in the wines under study are presented in Table 4.3. *Cis*-resveratrol-*O*-β-glucoside was not detected in any of the wines analysed. Most wines contained much higher levels of the aglycones than the conjugate although high concentrations of the glucoside were present in the French Gevrey Chambertin (wine 7) and Minervois (wine 14). Total resveratrol content ranged more than 20-fold from 4.3 μM in wine 2, a Californian Cabernet Sauvignon, to 87.9 μM in the conjugate-rich, Pinot Noir-based Gevrey Chambertin (wine 7). Wines 5 and 6, Pinot Noirs from California and Chile also contained high total levels of resveratrol.

4.2.3.3 Gallic acid

The levels of gallic acid varied almost 10-fold from 416.6 μM in wine 3, a 1996 Bulgarian Cabernet Sauvignon, to 45.9 μM in wine 8, a Chilean Merlot (Table 4.3).

4.2.3.4 Hydroxycinnamates

Quantitative estimates of caftaric acid, caffeic acid, *p*-coumaric acid and conjugated *p*-coumaric acid are presented in Table 4.4. Wine 13, a Beaujolais produced by light extraction of Gamay grapes contained by far the highest concentration of caftaric acid, 331.8 μM. Wine 14, the Minervois contained the highest levels of both free and conjugated *p*-coumaric acid, 210.6 μM and 462.2 μM, respectively. As a consequence, the overall concentration of hydroxycinnamates in the Minervois, 903.4 μM, was ca. 2-fold higher than the levels detected in any of the other wines. With the exception of the Minervois, the total hydroxycinnamate content did not vary greatly in the wines that were

Table 4.3. Stilbene and gallic acid content of red wine (batch 1).

wine	*trans*-resveratrol	*cis*-resveratrol	*trans*-resveratrol glucoside	total resveratrol	gallic acid
1	2.1 ± 0.3	2.5 ± 0.5	2.8 ± 0.3	7.4 ± 0.6	130.8 ± 4.9
2	2.3 ± 0.1	2.0 ± 0.1	n.d.	4.3 ± 0.1	167.1 ± 0.6
3	29.4 ± 0.6	22.8 ± 0.7	8.3 ± 0.2	60.5 ± 1.1	416.6 ± 4.0
4	27.9 ± 0.3	8.2 ± 0.2	3.1 ± 0.0	39.2 ± 0.5	344.7 ± 0.7
5	39.0 ± 2.2	28.1 ± 0.4	7.8 ± 1.6	74.9 ± 2.9	205.2 ± 2.6
6	46.3 ± 2.6	32.7 ± 0.7	n.d.	79.0 ± 3.1	282.9 ± 1.7
7	30.4 ± 0.8	27.1 ± 0.4	30.4 ± 0.8	87.9 ± 2.1	300.7 ± 4.1
8	2.1 ± 0.1	6.6 ± 0.2	n.d.	8.7 ± 0.1	45.9 ± 1.7
9	22.8 ± 2.2	22.1 ± 1.1	7.0 ± 0.4	51.9 ± 2.1	300.0 ± 1.8
10	15.1 ± 0.2	11.3 ± 0.4	2.9 ± 0.2	29.3 ± 0.4	145.1 ± 0.5
11	9.1 ± 0.3	6.0 ± 0.1	7.1 ± 0.2	22.2 ± 0.5	225.8 ± 1.4
12	5.7 ± 0.0	4.6 ± 0.3	2.6 ± 0.1	12.9 ± 0.4	245.8 ± 1.7
13	11.0 ± 0.5	9.9 ± 0.1	1.3 ± 0.2	22.2 ± 0.5	89.1 ± 0.4
14	18.6 ± 0.8	7.1 ± 0.8	18.6 ± 0.8	44.3 ± 0.9	274.4 ± 4.1
15	9.2 ± 0.9	4.4 ± 0.1	9.2 ± 0.9	22.8 ± 1.9	147.4 ± 2.6
16	9.1 ± 0.8	4.7 ± 0.7	9.1 ± 0.8	22.9 ± 1.0	335.5 ± 2.4

Data are expressed as μM *trans*-resveratrol or gallic acid ± SEM (n=3). n.d., not detected.

Table 4.4. Hydroxycinnamate content of red wines (batch I).

wine	caftaric acid*	free caffeic acid	free *p*-coumaric acid	conj. *p*-coumaric acid	total hydroxy-cinnamates
1	111.2 ± 0.7	23.0 ± 0.7	23.9 ± 2.3	25.5 ± 0.8	183.6 ± 4.3
2	42.9 ± 1.7	32.3 ± 0.6	131.9 ±0.6	35.9 ± 0.7	243.0 ± 2.5
3	95.7 ± 0.5	13.3 ± 0.4	115.6 ± 0.4	14.8 ± 0.4	239.4 ± 9.6
4	82.9 ± 1.6	15.3 ± 0.7	71.6 ± 0.6	17.0 ± 0.8	186.8 ± 0.8
5	188.0 ± 0.5	50.6 ± 0.3	32.7 ± 4.7	56.2 ± 0.3	327.5 ± 4.3
6	49.5 ± 2.7	94.2 ± 0.2	33.9 ± 0.3	131.6 ± 1.8	309.2 ± 2.2
7	89.8 ± 1.9	106.4 ± 7.0	31.7 ± 1.2	25.7 ± 1.4	253.6 ± 7.7
8	17.4 ± 0.4	74.4 ± 0.5	14.7 ± 0.2	197.6 ± 0.7	304.1 ± 1.1
9	110.0 ± 0.4	24.1 ± 0.1	16.1 ± 0.5	188.2 ± 0.5	338.4 ± 1.2
10	128.5 ± 0.9	47.6 ± 0.8	23.7 ± 0.2	319.2 ± 1.0	519.0 ± 1.9
11	117.9 ± 0.4	6.6 ± 0.2	33.9 ± 0.3	131.6 ± 1.8	290.0 ± 2.2
12	94.4 ± 1.2	101.0 ± 0.3	54.6 ± 0.2	201.5 ± 1.6	451.5 ± 3.0
13	331.8 ± 9.8	28.9 ± 0.4	26.4 ± 0.2	32.1 ± 0.4	419.2 ± 9.3
14	189.7 ± 2.2	40.9 ± 0.3	210.6 ± 0.3	462.2 ± 1.8	903.4 ± 5.5
15	110.5 ± 0.1	11.1 ± 0.2	74.7 ± 0.7	52.8 ± 1.9	249.1 ± 1.6
16	121.8 ± 0.9	30.6 ± 0.3	21.8 ± 0.8	142.0 ± 0.8	316.2 ± 1.1

Data are expressed as μM ± SE M (n=3). *caftaric acid quantified as caffeic acid equivalents. Conj., conjugated.

investigated (Table 4.4). Unlike the skin-derived stilbenes and flavonols, the hydroxycinnamates are located primarily in the flesh of the grape and as such are found in comparable levels in both red and white wines.

4.2.3.5 Flavan-3-ols

The concentrations of (+)-catechin and (-)-epicatechin are presented in Table 4.5. The highest total catechin concentrations 645.6 μM and 637.5 μM, were found in wines 5 and 7, Pinot Noirs from Chile and France respectively, which also contained high levels of total resveratrol. Wine 10, a Spanish Rioja, contained the lowest amount of total catechins, 172.6 μM, albeit it only 3.5-fold less than the highest value in wine 5. (+)-Catechin was invariably present in larger amounts than (-)-epicatechin with ratios ranging from 2.7 in the Chilean Pinot Noir, wine 5 to 16.0 in wine 14, the Minervois. The catechin levels presented in Table 4.6 are in keeping with the findings of Goldberg et al. (1998a) who also found highest concentrations in wines made from Pinot Noir grapes which appear to be constitutively higher in (+)-catechin and (-)-epicatechin than other grape varieties.

4.2.3.6 Total anthocyanins

Polymeric anthocyanins were present in all wines in larger amounts than free anthocyanins with ratios varying from 1.4 to 6.1. The highest total anthocyanin concentrations, 325.5 and 308.8 μM malvidin-3-glucoside equivalents were detected in wine 1, the Chilean Cabernet Sauvignon, and wine 14 the Minervois. The lowest level, 101.5 μM, was observed in wine 7, the Gevrey Chambertin Pinot Noir (Table 4.5).

4.2.3.7 Total phenolic content of red wines

The total phenolic content of the 16 red wines determined by the Folin-Ciocalteu colorimetric method, are presented in Table 4.1. There are almost three-fold differences in the levels present in the different wines, with concentrations ranging from 6.47 to 18.6 mM of gallic acid equivalents

Table 4.5. Flavan-3-ol and anthocyanin content of red wines (batch I).

wine	(+)-cat	(-)-epicat	total catechins	ratio	free antho	poly. pig.	total antho
1	239.8 ± 16.4	23.8 ± 0.7	263.6 ± 16.3	10.1	72.0	253.7	325.7
2	270.3 ± 16.6	47.4 ± 2.0	317.7 ± 15.6	5.7	75.0	135.3	210.3
3	468.1 ± 7.8	130.5 ± 2.0	598.6 ± 8.3	3.6	82.7	202.3	285.0
4	188.9 ± 0.6	59.4 ± 3.0	248.3 ± 3.7	3.2	15.5	94.7	110.2
5	472.6 ± 4.5	173.0 ± 5.5	645.6 ± 10.1	2.7	72.4	110.6	183.0
6	233.2 ± 1.3	91.5 ± 0.8	324.7 ± 0.6	2.5	25.3	83.9	109.2
7	490.5 ± 8.5	147.0 ± 8.1	637.5 ± 16.5	3.3	26.1	75.4	101.5
8	186.6 ± 7.0	36.6 ± 1.3	223.2 ± 7.6	5.1	80.2	154.2	234.4
9	296.8 ± 1.2	83.8 ± 0.9	380.6 ± 2.1	3.5	56.1	135.0	191.1
10	151.0 ± 2.0	21.6 ± 1.0	172.6 ± 1.3	7.0	112.7	163.3	276.0
11	299.3 ± 4.6	69.1 ± 2.2	368.4 ± 5.8	4.3	90.9	135.3	226.2
12	202.7 ± 0.9	53.5 ± 1.2	256.2 ± 1.4	3.8	41.2	121.4	162.6
13	193.8 ± 1.9	30.6 ± 1.1	224.4 ± 3.0	6.5	49.3	84.9	134.2
14	284.5 ± 1.1	17.8 ± 1.5	302.3 ± 2.6	16.0	87.3	221.2	308.5
15	198.6 ± 2.2	48.9 ± 1.1	247.5 ± 3.3	4.1	52.7	74.5	127.2
16	242.2 ± 2.3	58.4 ± 3.8	300.6 ± 6.0	4.1	46.3	120.8	167.1

Data for (+)-cat [(+)-catechin] and (-)-epicat [(-)-epicatechin] expressed as μM ± SEM n=3. Ratio, ratio of (-)-epicatechin to (+)-catechin. antho, anthocyanins. Anthocyanin data expressed as μM malvidin 3-glucoside equivalent; poly. pig., polymeric pigments.

(GAE). These figures, corresponding to 1100 to 3165 mg/L GAE, are comparable with values obtained for red wines by other investigators (Frankel et al., 1995; Sato et al. 1996; Ritchey and Waterhouse, 1999). In the current study phenolic-rich wines included the 1996 Young Vatted Bulgarian Cabernet Sauvignon (wine 3), the 1995 Minervois (wine 14) and the 1995 Gevrey Chambertain Pinot Noir (wine 7). Lowest concentrations were detected in wine 13, the 1996 Beaujolais and the 1996 Valpolicella (wine 15).

A second method was used to assess the total phenolic content of the red wines. This involved combining the figures obtained from the HPLC-based analyses of flavonols, hydroxycinnamates, (+)-catechin, (-)-epicatechin, *cis*-resveratrol, *trans*-resveratrol, *trans*-resveratrol-*O*-β-glucoside and gallic acid as well as anthocyanin values obtained with the colorimetric assay. The figures based on this method of assessment of the total phenolic content are presented in Table 4.1.

4.2.4 Relationship between antioxidant activity, vasodilation capacity and phenolic content

The statistical significance of the relationships between antioxidant activity, vasodilation capacity and the total phenolic contents of the red wines (see Table 4.1) were analysed using non-parametric Spearman rank correlation and Minitab software (Table 4.6). The ESR-based antioxidant potentials were found to correlate strongly with the Folin-Ciocalteu estimates of total phenol content ($r_S = 0.96$, $p < 0.001$). The total phenolic content based on HPLC analyses also correlated with the antioxidant potential ($r_S = 0.94$, $p < 0.001$). The pIC_{50} values for the wines were closely associated with their total HPLC derived phenolic content ($r_S = -0.81$, $p < 0.001$) and the Folin-Ciocalteu estimate of phenolic content ($r_S = -0.86$, $p < 0.001$). The vasodilation activity of the wines was found to correlate very strongly with their antioxidant activity as determined by the ESR method, ($r_S = -0.88$, $p < 0.001$). Figure 4.1 demonstrates the relationship between the ESR–based antioxidant activity, the vasodilation activity and the Folin-Ciocalteu and HPLC derived phenolic

Table 4.6. Spearman rank correlations between antioxidant activity vasodilation capacity and phenolic content of red wines (batch I).

Correlations (Spearman rank)		r_s	p
ESR antioxidant activity	F.C. phenolics	0.96	0.000***
ESR antioxidant activity	Vasodilation activity	-0.88	0.000***
ESR antioxidant activity	HPLC phenolics	0.94	0.000***
ESR antioxidant activity	Gallic acid	0.56	0.024*
ESR antioxidant activity	Total stilbenes	0.61	0.013 *
ESR antioxidant activity	Total flavan-3-ols	0.60	0.014*
Vasodilation activity	F.C. phenolics	-0.86	0.000***
Vasodilation activity	HPLC phenolics	-0.81	0.000***
Vasodilation activity	Total anthocyanins	-0.52	0.038*
F.C. phenolics	HPLC phenolics	0.93	0.000***
F.C. phenolics	Gallic acid	0.65	0.006**
F.C. phenolics	Total stilbenes	0.65	0.006**
F.C. phenolics	Total flavan-3-ols	0.57	0.021*
HPLC phenolics	Gallic acid	0.56	0.024 *
HPLC phenolics	Total stilbenes	0.58	0.024*
HPLC phenolics	Total flavan-3-ols	0.68	0.004**

F.C. phenolics, Folin-Ciocalteu total phenolics; HPLC phenolics, HPLC derived total phenolics.

*** very highly significant $p < 0.001$
** highly significant $0.001 < p < 0.01$
* significant $0.01 < p < 0.05$

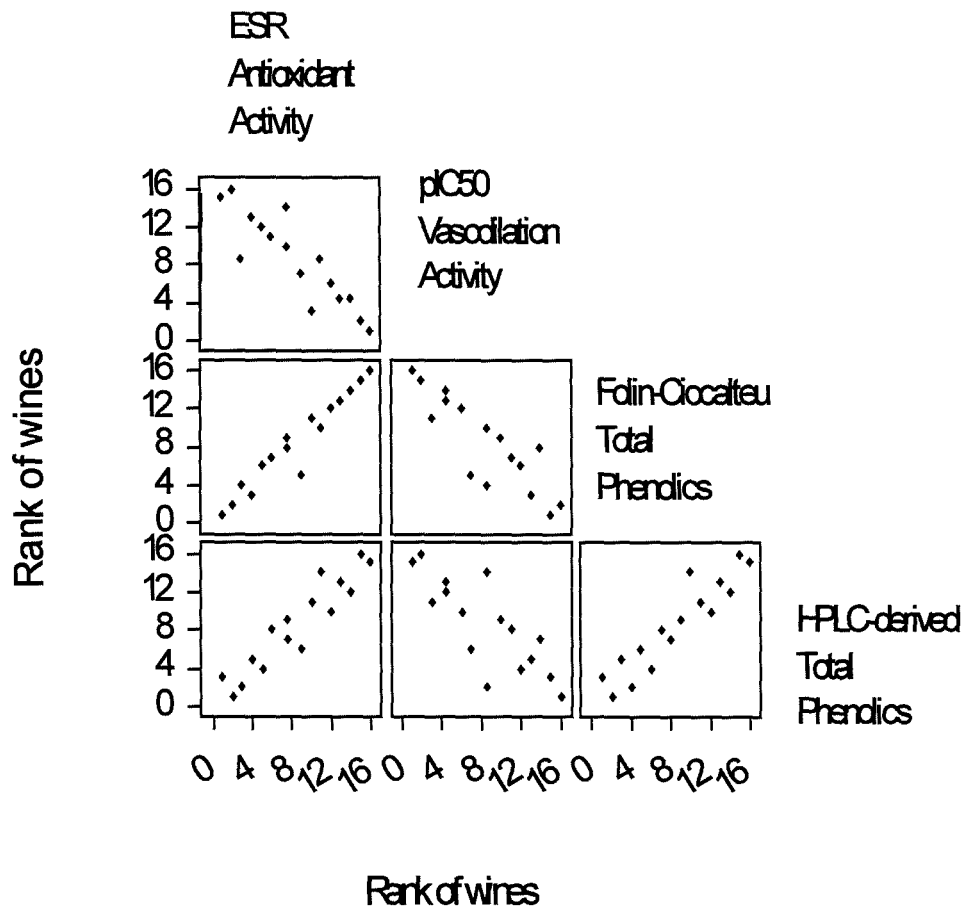


Figure 4.1. Matrix plot derived from Spearman rank correlations.

Highlighting the relationships between ESR-based antioxidant activity, vasodilation activity, and the total phenolics for wine in batch I. Antioxidant activity was determined as the number of Fremy's radicals reduced per litre of wine x 10^{21}; vasodilation activity was expressed as the concentration in μg/mL at which each extract caused 50% maximal vasodilation , pIC_{50} ; total phenolic content was determined by Folin-Ciocalteu colorimetric assay and HPLC.

contents of the wines. This close association between the ESR chemical model system and an *ex vivo* biological assay suggests that the chemical method has biological relevance.

Attempts to correlate statistically the levels of specific phenolics in the red wines (Tables 4.2–4.5) with antioxidant activity and vasodilation capacity were less successful. Although correlations with antioxidant activity were detected with gallic acid ($r_S = 0.56$, $p = 0.024$), total resveratrol ($r_S = 0.61$, $p = 0.013$) and total (+)-catechin and (-)-epicatechin ($r_S = 0.60$, $p = 0.014$), the other individual correlations were lower (total hydroxycinnamates $r_S = 0.26$, $p = 0.341$; total flavonols $r_S = 0.45$, $p = 0.08$; total anthocyanins $r_S = 0.35$, $p = 0.182$). Likewise, although the pIC_{50} values of the wines were closely correlated with their total HPLC-derived phenolic content ($r_S = -0.811$, $p < 0.001$), this association was not evident with individual phenolics with the sole exception of total anthocyanins, ($r_S = -0.52$, $p = 0.038$).

4.3 Relationship between phenolic content and antioxidant activity of wine (batch II)

The initial batch of wines looked principally at European wines, and particularly wines made from common grapes such as Cabernet Sauvignon. In order to investigate further factors influencing the phenolic content of wines the study was expanded to include lesser-known varieties and Southern Hemisphere wines.

4.3.1 Antioxidant activity

Two methods were used to determine the antioxidant activity of the 22 wines. The first method determined the antioxidant activity by measuring the ability of the wines to reduce the Fremy's salt free radical in the ESR-based assay. The values obtained ranged from 5.98 to 9.21 x 10^{21} radicals reduced per L (Table 4.7). These values are in keeping with those found for the 16 wines in

Table 4.7. Antioxidant activity and phenolic content of red wines (batch II).

wine	ESR-based antioxidant activity[a]	FRAP antioxidant activity[b]	Folin-Ciocalteu total phenolics[c]	HPLC total phenolics[d]
17	8.7 ± 0.1	21.4 ± 0.1	14.5 ± 0.1	1.0 ± 0.0
18	8.9 ± 0.1	21.9 ± 0.5	15.6 ± 0.1	1.2 ± 0.0
19	8.1 ± 0.1	17.8 ± 0.2	11.8 ± 0.1	1.2 ± 0.0
20	9.2 ± 0.1	21.2 ± 0.1	14.1 ± 0.1	1.5 ± 0.0
21	7.0 ± 0.1	15.2 ± 0.3	9.7 ± 0.1	1.0 ± 0.0
22	8.3 ± 0.1	20.7 ± 0.3	14.0 ± 0.1	1.2 ± 0.0
23	8.0 ± 0.2	20.2 ± 0.2	14.0 ± 0.2	1.2 ± 0.0
24	6.0 ± 0.1	12.9 ± 0.2	9.3 ± 0.1	0.6 ± 0.0
25	5.7 ± 0.0	13.5 ± 0.1	9.7 ± 0.3	0.9 ± 0.0
26	8.8 ± 0.0	24.2 ± 0.4	17.1 ± 0.1	1.3 ± 0.0
27	6.5 ± 0.2	15.7 ± 0.1	11.0 ± 0.1	1.0 ± 0.0
28	7.9 ± 0.1	19.3 ± 0.1	13.0 ± 0.1	1.2 ± 0.0
29	9.0 ± 0.2	24.4 ± 0.3	16.0 ± 0.1	1.6 ± 0.0
30	8.4 ± 0.1	20.6 ± 0.4	14.5 ± 0.0	1.5 ± 0.1
31	7.6 ± 0.2	18.5 ± 0.1	12.0 ± 0.0	1.3 ± 0.0
32	8.4 ± 0.1	20.2 ± 0.6	14.4 ± 0.0	1.7 ± 0.0
33	6.9 ± 0.1	15.7 ± 0.1	10.8 ± 0.0	1.4 ± 0.0
34	6.8 ± 0.1	15.7 ± 0.1	10.8 ± 0.0	1.1 ± 0.0
35	7.1 ± 0.0	16.7 ± 0.7	11.8 ± 0.0	1.2 ± 0.0
36	7.1 ± 0.0	16.7 ± 0.1	12.2 ± 0.0	1.1 ± 0.0
37	8.2 ± 0.1	20.0 ± 0.4	13.8 ± 0.1	1.0 ± 0.0
38	8.1 ± 0.1	18.1 ± 0.1	12.6 ± 0.1	1.0 ± 0.0

[a,b]Antioxidant capacity of red wines, measured by ESR spectroscopy, presented as the number of Fremy's radicals reduced by one litre of wine x 10^{21}, and by the FRAP assay expressed as concentration of Fe(II) produced as mM. [c,d]Total phenol content of red wine determined by the Folin-Ciocalteu method (mM gallic acid equivalents GAE) and from HPLC analysis of individual phenolics (mM). All data expressed as mean values ± SEM.

batch I, and in a previous study (Gardner et al., 1999). In this study the highest value was found with a high quality Burgundy (wine 20), and the lowest with wine 23, an Australian Shiraz.

The second method used to assess the antioxidant activity of the wines measured the ability of the wines reduce Fe^{3+} to form Fe^{2+}. Although there is a close relationship between both methods, the ranking of the wines differs slightly. The wine with the highest activity was the Cabernet Franc from Australia (wine 26), while the lowest was wine 21, a Côte du Rhône.

4.3.2 Analysis of individual phenolic compounds

Individual phenolics were quantified by HPLC using the same methods as used for batch 1, in addition to measuring the hydroxybenzoate, ellagic acid. Total catechins and total anthocyanins were determined using spectrophotomeric assays.

4.3.2.1 Flavonols

Free and conjugated myricetin, quercetin, kaempferol and isorhamnetin were detected as both in each wine (Table 4.8). Overall the percentage of free flavonols ranged from 9.1% in the Chilean Cabernet Sauvignon (wine 31), to 78.0% in the Australian Malbec (wine 30), with a mean value of 43.7%. The levels of total flavonols ranged almost 20-fold, from 13.86 μM in the Burgundy (wine 20) to 252.93 μM in the Chilean Syrah (wine 32). In each case myricetin and quercetin were the major contributors to the total flavonol content with kaempferol and isorhamnetin providing on average only 13.4% of total flavonols.

4.3.2.2 Stilbenes

The concentrations of *trans*-resveratrol, the isomer *cis*-resveratrol and the conjugate *trans*-resveratrol-*O*-β-glucoside are presented in Table 4.9. Levels of *trans*-resveratrol ranged 13-fold, from 6.2 μM in the Argentinean

Table 4.8. Free and conjugated flavonol content of red wines (batch II).

wine	free M	conj. M	total M	free Q	conj. Q	total Q	free K	conj. K	total K	free I	conj. I	total I	total flavonols	% free
17	6.8 ± 0.6	34.5 ± 1.8	41.3 ± 2.4	9.9 ± 0.6	23.5 ± 1.4	33.4 ± 1.9	0.8 ± 0.0	1.3 ± 0.1	2.1 ± 0.1	0.8 ± 0.0	6.7 ± 0.1	7.5 ± 0.2	84.25 ± 4.55	18.3
18	5.2 ± 0.4	17.9 ± 2.7	23.1 ± 2.3	8.8 ± 0.8	16.4 ± 3.0	25.1 ± 2.3	0.5 ± 0.0	0.6 ± 0.2	1.0 ± 0.2	0.5 ± 0.0	4.9 ± 0.8	5.4 ± 0.8	54.67 ± 5.41	21.8
19	6.0 ± 0.3	8.4 ± 2.5	14.4 ± 2.7	7.7 ± 0.3	8.7 ± 2.9	16.4 ± 3.8	0.3 ± 0.0	0.5 ± 0.2	0.8 ± 0.2	0.5 ± 0.1	3.7 ± 1.1	4.2 ± 1.1	32.81 ± 7.19	36.9
20	1.1 ± 0.3	1.3 ± 0.5	2.4 ± 0.2	4.7 ± 1.0	4.6 ± 0.4	9.3 ± 1.1	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	1.2 ± 0.3	1.5 ± 0.3	13.86 ± 1.44	23.5
21	4.7 ± 0.1	5.5 ± 1.1	10.2 ± 1.0	2.5 ± 0.23	9.0 ± 1.1	11.5 ± 1.4	0.0 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	3.4 ± 0.5	3.6 ± 0.5	25.66 ± 2.94	38.9
22	22.2 ± 0.9	9.1 ± 1.2	31.3 ± 1.4	24.8 ± 0.5	13.0 ± 2.5	37.7 ± 2.7	2.5 ± 0.1	0.5 ± 0.1	3.0 ± 0.3	5.5 ± 0.2	3.4 ± 0.5	5.9 ± 0.6	77.94 ± 4.75	65.2
23	21.0 ± 0.3	8.8 ± 2.1	29.8 ± 2.1	33.3 ± 0.5	15.5 ± 4.3	48.8 ± 4.3	3.0 ± 0.1	0.8 ± 0.4	3.8 ± 0.4	4.7 ± 0.0	4.7 ± 1.1	9.4 ± 1.1	91.84 ± 7.92	55.8
24	1.9 ± 0.3	2.1 ± 0.1	4.0 ± 0.2	5.4 ± 0.6	4.6 ± 0.5	9.9 ± 0.7	0.1 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	3.1 ± 0.3	3.3 ± 0.3	17.50 ± 1.30	24.1
25	7.6 ± 1.4	3.8 ± 1.7	11.3 ± 0.4	9.3 ± 1.2	11.2 ± 1.3	20.5 ± 0.7	0.2 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	4.2± 0.2	5.1 ± 0.1	37.62 ± 1.24	43.9
26	13.1 ± 3.3	10.7 ± 1.3	23.8 ± 2.0	27.6 ± 6.4	25.7 ± 1.2	53.3 ± 5.2	2.6 ± 0.4	1.3 ± 0.6	3.9 ± 0.8	1.8 ± 0.3	2.8 ± 1.4	4.6 ± 1.6	86.03 ± 10.24	36.9
27	14.0 ± 0.3	1.4 ± 0.2	15.3 ± 0.1	22.1 ± 0.3	5.1 ± 0.0	27.2 ± 0.3	1.2 ± 0.0	03 ± 0.1	1.5 ± 0.1	1.8 ± 0.0	1.7 ± 0.3	3.5 ± 0.2	47.74 ± 0.08	67.3
28	15.5 ± 1.1	14.8 ± 1.4	30.2 ± 1.6	3.4 ± 0.2	18.1 ± 1.2	21.5 ± 1.2	0.2 ± 0.0	0.9 ± 0.1	1.1 ± 0.1	0.5 ± 0.1	5.3 ± 0.5	5.8 ± 0.5	58.60 ± 3.21	55.3
29	9.9 ± 0.8	2.7 ± 0.3	12.6 ± 0.6	18.4 ± 0.2	0.9 ± 0.3	19.3 ± 0.1	1.5 ± 0.0	0.3 ± 0.1	1.8 ± 0.1	1.1 ± 0.0	1.7 ± 0.1	2.8 ± 0.1	36.85 ± 0.99	63.4
30	13.5 ± 0.3	6.9 ± 1.2	20.5 ± 1.1	2.4 ± 0.1	6.1 ± 0.5	8. 6 ± 0.5	0.1 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	0.3 ± 0.0	5.5 ± 0.2	5.8 ± 0.2	35.59 ± 1.80	78.0
31	4.4 ± 0.3	37.1 ± 5.3	41.4 ± 5.2	6.4 ± 0. 2	46.2 ± 8.4	52.6 ± 8.4	0.7 ± 0.0	6.0 ± 1.2	6.7 ± 1.2	0.4 ± 0.0	9.2 ± 2.0	9.6 ± 2.0	110.31 ± 16.81	9.1
32	13.0 ± 1.2	63.6 ± 11.7	77.3 ± 10.7	34.5 ± 2.6	84.8 ± 19.0	119.3 ± 16.8	5.4 ± 0.3	7.5 ± 2.2	12.9 ± 2.0	4.4 ± 0.4	39.1 ± 7.0	43.5 ± 6.8	252.93 ± 36.19	15.0
33	14.0 ± 0.5	9.8 ± 1.3	23.7 ± 1.3	17.2 ± 0.7	14.4 ± 1.7	31.6 ± 2.0	1.3 ± 0.1	1.4 ± 0.2	2.7 ± 0.1	2.7 ± 0.1	4.5 ± 0.8	7.2 ± 0.8	64.19 ± 4.34	50.1
34	15.8 ± 0.5	4.8 ± 1.1	20.7 ± 0.7	10.2 ± 0.2	10.0 ± 0.1	20.2 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	1.7 ± 0.0	2.3 ± 0.0	44.02 ± 0.57	75.5
35	16.6 ± 0.3	8.9 ± 1.6	25.4 ± 1.3	18.0 ± 0.6	10.0 ± 2.3	28.0 ± 2.0	0.7 ± 0.1	0.4 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	3.8 ± 0.6	4.8 ± 0.6	59.24 ± 3.93	60.5
36	16.4 ± 0.7	3.9 ± 2.2	20.3 ± 1.6	14.1 ± 0.6	13.3 ± 2.9	27.5 ± 2.3	0.2 ± 0.0	1.2 ± 0.2	1.5 ± 0.2	0.8 ± 0.1	6.8 ± 1.1	7.6 ± 1.1	56.77 ± 5.10	61.5
37	7.4 ± 0.3	8.8 ± 0.8	16.2 ± 0.6	14.0 ± 0.8	13.5 ± 1.1	27.5 ± 0.5	1.0 ± 0.1	0.7 ± 0.1	1.7 ± 0.0	0.3 ± 0.0	2.7 ± 0.1	3.0 ± 0.1	48.41 ± 0.97	39.4
38	6.7 ± 0.3	6.4 ± 2.7	13.1 ± 2.5	27.0 ± 1.1	17.6 ± 9.6	44.6 ± 8.6	1.4 ± 0.1	1.4 ± 0.7	2.8 ± 0.6	0.3 ± 0.0	2.0 ± 0.7	2.3 ± 0.7	73.31 ± 9.50	21.0

Data are expressed as μM ± SEM, (n=3). M, myricetin; Q, quercetin; K, kaempferol; I, isorhamnetin. % free, free flavonols as % of total. Conj., conjugated.

Table 4.9. Stilbene content of red wines (batch II).

wine	*trans*-resveratrol	*cis*-resveratrol	*trans*-resveratrol glucoside	total resveratrol
17	36.8 ± 1.9	8.2 ± 0.2	3.7 ± 0.1	48.7 ± 1.9
18	18.0 ± 0.2	3.3 ± 0.8	5.0 ± 0.1	24.7 ± 2.2
19	36.4 ± 0.3	8.8 ± 0.1	5.0 ± 0.5	50.1 ± 0.7
20	35.5 ± 0.5	8.2 ± 0.5	12.7 ± 0.0	56.4 ± 1.0
21	49.7 ± 0.8	8.0 ± 0.3	7.0 ± 0.2	64.7 ± 1.0
22	9.4 ± 0.4	1.9 ± 0.6	3.7 ± 0.0	15.1 ± 1.0
23	16.9 ± 1.4	2.3 ± 0.4	8.9 ± 0.1	28.1 ± 1.7
24	49.7 ± 0.4	23.0 ± 0.2	1.3 ± 0.1	74.0 ± 0.5
25	8.0 ± 0.3	1.7 ± 0.1	3.0 ± 0.0	12.7 ± 0.3
26	10.4 ± 0.1	n.d.	1.1 ± 0.1	11.5 ± 0.0
27	23.5 ± 0.4	n.d.	1.1 ± 0.0	24.2 ± 0.3
28	45.9 ± 0.1	4.3 ± 0.1	2.5 ± 0.0	52.6 ± 0.1
29	6.2 ± 0.2	1.7 ± 0.2	1.1 ± 0.0	9.0 ± 0.3
30	41.2 ± 0.1	9.0 ± 0.3	6.8 ± 0.1	57.0 ± 0.4
31	6.3 ± 0.2	n.d.	4.9 ± 0.0	11.2 ± 0.2
32	10.3 ± 0.2	4.0 ± 0.2	2.9 ± 0.0	17.3 ± 0.4
33	81.8 ± 0.8	18.5 ± 0.3	6.1 ± 0.1	106.4 ± 1.0
34	17.2 ± 0.3	2.1 ± 0.4	3.2 ± 0.0	22.5 ± 0.3
35	69.6 ± 2.9	n.d.	1.7 ± 0.0	71.3 ± 2.9
36	29.3 ± 1.1	2.0 ± 0.1	21.8 ± 0.2	53.2 ± 1.2
37	26.9 ± 0.6	4.3 ± 0.1	9.6 ± 0.2	40.8 ± 1.0
38	19.0 ± 1.4	n.d.	8.8 ± 0.1	27.8 ± 1.3

Data expressed as μM *trans*-resveratrol ± SEM (n=3). n.d., not detected.

Tempranillo (wine 29) to over 80 μM in the South African Cinsault (wine 33). *Cis*-resveratrol was undetected in five wines and was highest, at 23.0 μM, in the Australian Pinot Noir (wine 24). *Trans*-resveratrol-*O*-β-glucoside was detected in all wines with the highest levels of 21.8 μM being found in the Portuguese Ribbatejo (wine 36), and the lowest 1.1 μM in three wines (wines 26, 27 and 29).

4.3.2.3 Hydroxybenzoates

Information on the levels of gallic acid and ellagic acid are presented in Table 4.10. Levels of gallic acid ranged almost 8-fold from 68.8 μM in the South African Cinsault (wine 33) to 537.1 μM in the Australian Cabernet Franc (wine 26). Ellagic acid was found only in low levels, ranging from 1μM (wines 33 and 34) to 6.8 μM in the Australian Cabernet Sauvignon (wine 22).

4.3.2.4 Hydroxycinnamates

The concentrations of caftaric acid, caffeic acid, *p*-coumaric acid and conjugated *p*-coumaric acid are shown in Table 4.11. Total hydroxycinnamates ranged 10-fold from 68.2 μM in the Australian Pinot Noir (wine 24) to 660.3 μM in the Chilean Syrah (wine 32). In most wines caffeic acid is found primarily as the tartrate conjugate. The major hydroxycinnamates in wines are the *p*-coumaric acid conjugates, with the major conjugates being tartrate derivatives (Singleton, 1982).

4.3.2.5 Flavan-3-ols

(+)-Catechin and (-)-epicatechin were determined by HPLC, and the results are shown in Table 4.12. Levels of (+)-catechin were higher than (-)-epicatechin in every wine bar one, the Australian Pinot Noir, wine 24. (+)-Catechin ranged from 22.3 μM in wine 24 to 675.8 μM in the Burgundy, while (-)-epicatechin was found in levels varying from 38.1 μM in the Portuguese Ribbatejo (wine 36) to 240.0 μM again in the Burgundy. Total

Table 4.10. Hydroxybenzoate content of red wines (batch II).

wine	gallic acid	ellagic acid	total hydroxybenzoates
17	210.8 ± 9.0	2.3 ± 0.0	213.1 ± 9.0
18	350.5 ± 7.1	2.3 ± 0.1	352.8 ± 7.0
19	162.8 ± 3.3	1.1 ± 0.0	163.9 ± 3.3
20	309.2 ± 2.6	2.5 ± 0.1	311.6 ± 2.5
21	146.2 ± 2.2	1.5 ± 0.0	147.7 ± 2.2
22	294.9 ± 6.3	6.8 ± 0.1	301.7 ± 6.4
23	232.6 ± 4.5	6.6 ± 0.3	239.2 ± 4.4
24	199.1 ± 1.2	3.0 ± 0.0	202.0 ± 1.2
25	194.8 ± 0.5	5.0 ± 0.1	199.8 ± 0.5
26	537.1 ± 9.3	5.1 ± 0.2	542.2 ± 9.2
27	271.8 ± 3.7	3.5 ± 0.0	275.2 ± 3.7
28	221.4 ± 4.4	3.8 ± 0.0	225.2 ± 4.4
29	458.3 ± 3.1	2.7 ± 0.1	461.0 ± 3.1
30	343.5 ± 0.7	1.6 ± 0.1	345.1 ± 0.8
31	112.9 ± 0.9	2.4 ± 0.1	115.3 ± 1.0
32	151.3 ± 0.7	3.5 ± 0.3	154.8 ± 0.4
33	68.8 ± 0.6	1.0 ± 0.0	69.8 ± 0.6
34	132.5 ± 0.7	1.0 ± 0.1	133.4 ± 0.8
35	142.1 ± 0.2	1.7 ± 0.1	143.7 ± 0.3
36	164.6 ± 4.7	2.9 ± 0.0	167.5 ± 4.7
37	317.6 ± 11.8	3.2 ± 0.1	320.8 ± 11.7
38	318.6 ± 1.0	1.9 ± 0.0	320.5 ± 1.0

Data expressed as μM gallic or ellagic acid ± SEM, n=3.

Table 4.11. Hydroxycinnamate content of red wines (batch II).

wine	caftaric acid	free caffeic acid	free *p*-coumaric acid	total *p*-coumaric acid	total hydroxy-cinnamates
17	10.1 ± 0.1	37.5 ± 0.9	14.0 ± 0.5	191.7 ± 0.9	240.0 ± 0.1
18	35.2 ± 2.2	16.3 ± 0.4	16.6 ± 1.0	210.9 ± 0.2	262.4 ± 1.6
19	37.6 ± 1.2	30.0 ± 1.4	19.5 ± 0.2	261.4 ± 1.2	328.3 ± 1.6
20	68.6 ± 1.5	7.4 ± 0.1	4.1 ± 0.1	16.8 ± 3.2	94.1 ± 3.7
21	81.9 ± 2.5	23.9 ± 1.7	25.1 ± 1.3	213.0 ± 1.5	319.5 ± 1.3
22	6.7 ± 0.1	54.3 ± 1.3	34.5 ± 0.3	283.4 ± 0.4	344.5 ± 0.8
23	25.8 ± 0.3	27.3 ± 0.5	28.1 ± 0.1	398.9 ± 3.2	449.4 ± 2.9
24	33.5 ± 1.0	7.2 ± 0.9	13.8 ± 0.0	27.0 ± 0.5	68.2 ± 1.8
25	17.5 ± 0.0	17.4 ± 0.0	43.6 ± 0.4	302.4 ± 1.4	338.2 ± 1.7
26	53.6 ± 2.2	17.3 ± 3.8	19.6 ± 0.2	223.7 ± 1.2	295.6 ± 5.1
27	55.1 ± 1.8	12.0 ± 2.5	25.5 ± 0.5	216.9 ± 0.8	284.5 ± 1.8
28	40.6 ± 1.6	24.4 ± 1.2	23.1 ± 0.4	192.4 ± 1.6	260.2 ± 0.2
29	18.4 ± 0.2	12.4 ± 0.1	38.8 ± 0.1	315.4 ± 6.8	351.0 ± 6.6
30	29.8 ± 1.4	13.0 ± 0.5	77.2 ± 0.4	419.2 ± 1.6	462.8 ± 3.0
31	44.0 ± 0.8	7.8 ± 0.2	10.6 ± 1.1	199.4 ± 1.2	251.2 ± 1.5
32	6.1 ± 0.4	75.7 ± 1.3	105.7 ± 0.1	575.7 ± 15.9	660.3 ± 21.6
33	71.5 ± 0.4	23.6 ± 0.6	70.7 ± 0.1	398.1 ± 1.9	494.0 ± 1.2
34	60.8 ± 1.9	87.3 ± 3.8	62.8 ± 0.1	232.2 ± 0.6	375.2 ± 0.6
35	54.7 ± 0.5	16.8 ± 0.9	51.5 ± 0.1	251.3 ± 0.5	321.4 ± 1.1
36	80.0 ± 1.9	16.0 ± 1.3	35.3 ± 0.9	278.8 ± 2.5	372.4 ± 0.4
37	63.4 ± 0.9	9.5 ± 1.9	12.1 ± 0.3	134.3 ± 2.2	206.6 ± 4.6
38	64.0 ± 1.4	23.8 ± 2.1	17.7 ± 0.1	107.2 ± 2.7	195.4 ± 4.7

Data expressed as μM of caftaric, caffeic or p-coumaric acid ± SEM, n=3

Table 4.12. Flavan-3-ol content of red wines (batch II).

wine	(+)-catechin	(-)-epicatechin	total flavan-3-ol	ratio
17	133.5 ± 10.7	86.9 ± 4.4	220.4 ± 14.7	1.5
18	193.8 ± 2.3	101.8 ± 0.6	292.2 ± 4.7	1.9
19	220.7 ± 1.6	150.4 ± 0.8	371.1 ± 1.2	1.5
20	675.8 ± 8.5	240.0 ± 7.1	911.1 ± 14.1	2.8
21	205.0 ± 1.4	75.7 ± 0.8	280.7 ± 0.6	2.7
22	81.9 ± 0.8	52.7 ± 1.5	134.7 ± 1.3	1.5
23	49.1 ± 2.1	42.1 ± 0.3	91.2 ± 2.2	1.2
24	22.2 ± 2.2	73.9 ± 1.0	96.2 ± 3.2	0.3
25	58.8 ± 2.0	59.8 ± 0.4	118.7 ± 1.6	1.0
26	118.1 ± 13.1	71.6 ± 1.0	189.7 ± 13.9	1.6
27	63.7 ± 5.8	49.0 ± 1.0	112.7 ± 5.3	1.3
28	147.5 ± 3.0	88.3 ± 1.2	235.8 ± 4.2	1.7
29	221.6 ± 3.7	110.1 ± 3.0	331.7 ± 6.7	2.0
30	186.5 ± 6.8	115.4 ± 2.7	301.9 ± 9.4	1.6
31	109.5 ± 3.1	62.5 ± 0.7	172.1 ± 3.8	1.8
32	78.2 ± 4.2	50.7 ± 0.9	128.8 ± 4.8	1.5
33	102.2 ± 3.5	88.5 ± 0.6	190.7 ± 3.9	1.2
34	103.7 ± 3.4	50.0 ± 1.1	153.7 ± 4.4	1.9
35	80.9 ± 7.3	48.4 ± 0.5	129.3 ± 7.8	1.7
36	92.9 ± 1.8	38.1 ± 0.4	131.0± 2.0	2.4
37	136.8 ± 3.4	72.7 ± 0.9	209.4 ± 4.3	1.9
38	131.1 ± 8.0	88.9 ± 6.4	219.9 ± 14.4	1.5

Data expressed as μM (+)-catechin ± SEM, n=3. Ratio, ratio of (-)-epicatechin to (+)-catechin.

HPLC catechins ranged 10-fold from 91.2 μM in the Australian Shiraz to 911.2 μM in the Pinot Noir containing Burgundy (wine 20). The Burgundy contained approximately 2.5-fold higher levels of total catechins than any other wine, with a (-)-epicatechin:(+)-catechin ratio of 1:2.8. Red wines from Burgundy are traditionally made from Pinot noir grapes, which have been shown to be particularly rich in catechins (Goldberg et al., 1998a).

4.3.2.6 Total catechins

A spectral assay was also used to estimate total catechins, which includes the procyanidins and catechin esters as well as the monomers. A close relationship was observed between levels of total catechins determined by spectral and HPLC methods (r_p = 0.776, p = 0.000). Spectral levels of total catechins ranged from a high of 2561 μM once again in the Burgundy, to a low of 772 and 776 μM in wines 25 and 24 respectively (Table 4.13). After the wines had been reacted to determine the ESR-based antioxidant activity the catechin content was determined again using the spectral method. The concentration measured this time corresponds to those catechins that were not oxidised by the radical in the antioxidant assay. By subtracting this value from the catechin content of the untreated wine a value was obtained for the oxidised catechins, i.e. the catechins which reacted with the radical. The wine with the highest level of reactive catechins was wine 20, the Burgundy, and the lowest was the Australian Cabernet Franc (wine 26).

4.3.2.7 Total anthocyanins

A spectral method was used to estimate levels of free and total anthocyanins as well as polymeric pigments (Figure 4.14). Highest levels of total anthocyanins, 601 μM were found in wine 31, the Chilean Cabernet Sauvignon, while lowest levels were in the Australian Pinot Noir (wine 24). The ratio of free anthocyanins:polymeric pigments varied between the wines. Young wines are more likely to still have mainly free anthocyanins (wine 31), however as a wines ages the anthocyanins form larger polymeric complexes

Table 4.13. Spectrophotomeric determination of total catechin content of red wines (batch II).

wine	total catechins	catechins after Fremys	oxidised catechins
17	1961.0 ± 66.5	752.3 ± 20.3	1209.0 ± 183.6
18	2054.2 ± 21.4	862.0 ± 5.2	1192.2 ± 23.4
19	1865.1 ± 16.9	540.6 ± 7.2	1324.5 ± 19.6
20	2569.9 ± 22.4	894.2 ± 6.9	1675.7 ± 25.1
21	1420.2 ± 3.8	345.7 ± 6.2	1074.4 ± 7.9
22	1433.5 ± 38.9	543.3 ± 14.5	890.2 ± 44.8
23	1016.4 ± 9.3	382.1 ± 5.2	634.3 ± 11.4
24	775.7 ± 5.9	148.8 ± 2.8	626.9 ± 7.2
25	772.3 ± 4.8	183.7 ± 0.7	588.7 ± 5.2
26	1843.0 ± 5.2	907.6 ± 24.5	246.5 ± 26.9
27	1197.0 ± 8.6	361.0 ± 10.3	836.7 ± 14.5
28	1524.3 ± 16.9	585.0 ± 11.7	939.3 ± 22.0
29	2429.2 ± 13.1	1266.0 ± 40.3	1163.2 ± 45.5
30	1657.5 ± 13.4	636.6 ± 11.0	1020.8 ± 18.9
31	1284.9 ± 16.2	460.5 ± 1.7	824.3 ± 17.6
32	1114.9 ± 19.6	389.9 ± 7.2	724.9 ± 22.7
33	965.3 ± 10.7	276.2 ± 11.0	689.2 ± 16.2
34	1282.1 ± 7.6	376.0 ± 11.0	906.1 ± 14.5
35	1111.7 ± 1.7	40.9 ± 6.9	726.3 ± 7.6
36	1134.2 ± 8.6	382.1 ± 5.5	752.1 ± 10.7
37	1432.4 ± 17.9	566.8 ± 10.0	865.6 ± 22.4
38	1524.9 ± 5.2	515.7 ± 9.6	1009.2 ± 12.1

Data expressed as μM (+)-catechin ± SEM, n=3

Table 4.14. Total anthocyanin content of red wines (batch II).

wine	free anthocyanins	polymeric pigments	total anthocyanins
17	5.1	238.8	243.90
18	20.6	226.7	247.31
19	n.d.	328.3	325.77
20	19.7	168.1	187.75
21	3.4	242.6	245.98
22	197.2	175.3	372.47
23	142.7	223.7	366.42
24	30.8	91.7	122.52
25	97.8	120.1	217.81
26	96.8	171.5	268.29
27	170.2	92.1	262.24
28	230.7	137.1	367.74
29	257.5	141.0	398.56
30	243.0	177.5	420.50
31	441.5	160.0	601.44
32	270.2	218.6	488.75
33	385.9	133.7	519.57
34	250.7	88.3	339.01
35	298.0	147.9	445.83
36	220.3	127.8	348.08
37	104.2	117.8	221.97
38	86.6	70.7	157.31

Data expressed as μM Malvidin 3-glucoside equivalents. n.d., not detected.

(wine 17). A striking feature of the wines is that the New World wines have significantly higher levels of anthocyanins compared with the European wine.

4.3.2.8 Total phenolic content of red wines

Results of the total phenolic content of the 22 wines, determined by the Folin-Ciocalteu method are reported in Table 4.6. They range from 9.31 μM GAE in the Australian Pinot Noir (wine 24) to 17.08 μM GAE in the Australian Cabernet Franc (wine 26). These values correspond to 1642.03 and 2905.94 mg/L GAE respectively. This 2-fold range is less than the range observed with batch I, although comparable to published results. The highest phenolic containing wine, the Australian Cabernet Franc also had the highest levels of antioxidant activity as determined by the FRAP assay. HPLC derived phenolics were determined by combining the levels of individual phenolics. Levels varied from 566 μM in the Australian Pinot Noir (wine 24) to 1694.38 μM in the Chilean Syrah, which had the highest flavonol content.

4.4.3 Relationship between antioxidant activity and phenolic content

Non-parametric Spearman rank correlations were carried out to determine the relationship between the antioxidant activity and the phenolic content of the 22 red wines, and the results are presented in Table 4.15.

Two methods were used to determine the antioxidant activity of the wines, the ESR-based assay and FRAP assay. Although they are assessing the ability of the wine to act as an antioxidant by two different mechanisms they show a very strong correlation ($r_s = 0.95$, $p < 0.001$). Both the ESR and the FRAP assays of antioxidant activity yielded values which were closely correlated with Folin-Ciocalteu-derived total phenolics ($r_s = 0.93$, $p < 0.001$ and $r_s = 0.98$, $p < 0.001$ respectively). They were also found to be significantly correlated with the HPLC-derived total phenol content of the wine ($r_s = 0.62$, $p < 0.002$ and $r_s = 0.60$, $p < 0.03$ respectively).

Table 4.15. Spearman rank correlations between antioxidant activity and phenolic content of red wines (batch II).

Correlations (Spearman rank)		r_s	p
ESR antioxidant activity	FRAP antioxidant activity	0.95	0.000***
ESR antioxidant activity	F.C. phenolics	0.93	0.000***
ESR antioxidant activity	HPLC phenolics	0.62	0.002**
ESR antioxidant activity	Gallic acid	0.61	0.002**
ESR antioxidant activity	Total flavan-3-ol	0.60	0.003**
ESR antioxidant activity	Polymeric pigments	0.52	0.014*
FRAP antioxidant activity	F.C. phenolics	0.98	0.000***
FRAP antioxidant activity	HPLC phenolics	0.60	0.003**
FRAP antioxidant activity	Gallic acid	0.63	0.002**
FRAP antioxidant activity	(+)-catechin	0.48	0.025*
FRAP antioxidant activity	*trans*-resveratrol	-0.47	0.028*
FRAP antioxidant activity	Polymeric pigments	0.47	0.028*
F.C. phenolics	Gallic acid	0.67	0.001***
F.C. phenolics	(+)-catechin	0.44	0.043*
F.C. phenolics	*trans*-resveratrol	-0.45	0.034*
F.C. phenolics	Polymeric pigments	0.44	0.043*
HPLC phenolics	Total *p*-coumaric acid	0.48	0.028*
HPLC phenolics	Total anthocyanins	0.65	0.001***

F.C. phenolics, Folin-Ciocalteu total phenolics; HPLC phenolics, HPLC derived total phenolics.

*** very highly significant $p < 0.001$
** highly significant $0.001 < p < 0.01$
* significant $0.01 < p < 0.05$

The correlations between both the ESR and the FRAP assays, and the levels of individual phenolics (Table 4.8–4.12) were also investigated. Significant correlations with ESR-based antioxidant activity were found only with gallic acid ($r_s = 0.61$, $p < 0.002$), total flavan-3-ols ($r_s = 0.60$, $p < 0.003$) and to a lesser extent with anthocyanin polymeric pigments ($r_s = 0.52$, $p < 0.014$). Similar relationships were observed with FRAP antioxidant activity and individual phenolics. Significant correlations were found with gallic acid ($r_s = 0.63$, $p < 0.002$), (+)-catechin ($r_s = 0.48$, $p < 0.025$), *trans*-resveratrol ($r_s = -0.47$, $p < 0.028$) and anthocyanin polymeric pigments ($r_s = 0.47$, $p < 0.028$).

4.4 Discussion

The varying capacities of the red wines to act both as *in vitro* antioxidants and, in the case of batch I, *ex vivo* vasodilators appears to be associated with the phenolic content of the wines, whether determined by the Folin-Ciocalteu assay or by the summation of the levels of individual phenolics analysed primarily by HPLC. These relationships become more evident in Figures 4.2 and 4.3. In Figure 4.2 the vasodilation pIC_{50} figures are plotted as inverse values x 10^3 and the wines (batch I) are ranked to visualise the concomitant reductions in vasodilation and antioxidant activity that are paralleled by decreasing phenolic content. While in Figure 4.3 the ESR-based antioxidant activity of the wines (batch II) is plotted against their total phenolic content.

Two methods were used to determine the total phenol content of the red wines, the Folin-Ciocalteu assay, and the sum of the individual HPLC phenolics. While showing a similar trend, there is a ca. 10-fold between the values. There are two likely reasons for this discrepancy. Firstly, the analysis of the individual components in the wines did not include the condensed tannins, the oligomers and polymers of (+)-catechin and (-)-epicatechin, so their contribution to the total phenolic content of the wines was not determined using the HPLC methods. However, according to Soleas et al. (1997c) and Singleton (1982) these components comprise only ca. 20% of the total phenolics in red wines. Secondly, and probably the main cause of the

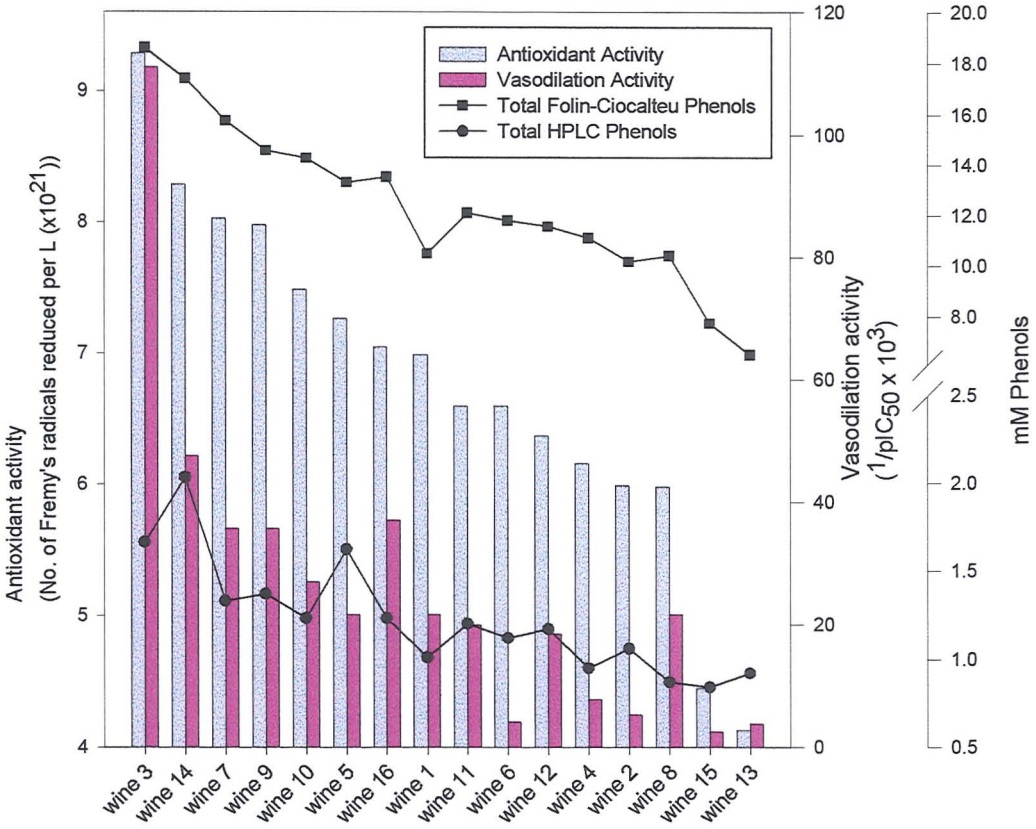


Figure 4.2. Relationship between antioxidant activity, vasodilation capacity, and phenolic content of red wines (batch I)

Antioxidant activity was determined as the number of Fremy's radicals reduced per litre of wine x 10^{21}; vasodilation activity was expressed as $1/pIC_{50}$ x 10^3, where pIC_{50} is the concentration in μg/mL of wine extract at which there is 50% maximal contraction of aortic rings. Total phenolic content was determined by two methods, the Folin-Ciocalteu colorimetric assay [results expressed as mM gallic acid equivalents (GAE)] and HPLC (results expressed as mM of individual phenolics as presented in Tables 4.2-4.5). All data are expressed as mean values ± SEM.

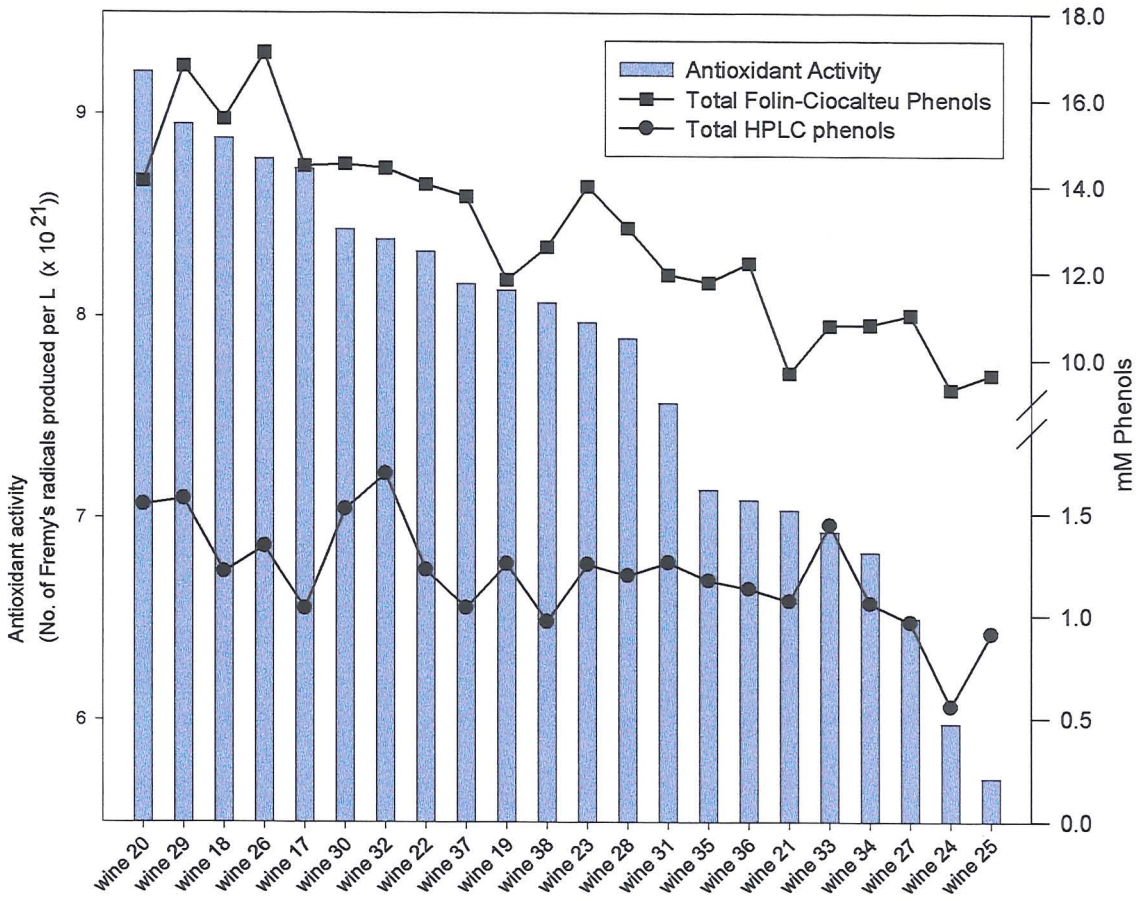


Figure 4.3. Relationship between antioxidant activity and the phenolic content of red wines (batch II)

Antioxidant activity was determined as the number of Fremy's radicals reduced per Litre of wine x 10^{21}. The total phenolic content was determined by two methods, the Folin-Ciocalteu colorimetric assay (results expressed as mM GAE) and HPLC (results expressed as mM of individual phenolics as presented in Tables 4.8-4.14). All data are expressed as mean values ± SEM.

difference between figures obtained by the two methods, is the fact that the Folin-Ciocalteu method does not provide a specific assay for phenolics as it reacts positively with many easily oxidisable non-phenolic compounds present in red wines and other matrixes (Singleton, 1982). In addition, as different phenolic have widely varying reaction stoichiometries, expressing the Folin-Ciocalteu results as gallic acid equivalents may cause an over-estimation in the total phenolic content of the wines.

4.4.1 Discussion of batch I results

Although the wines all showed activity there is a large spread in the antioxidant capacity of the individual wines and likewise in their ability to relax pre-contracted aorta. The phenolic-rich Bulgarian Young Vatted Cabernet Sauvignon (wine 3) has high antioxidant and vasodilation capacities while at the other end of the scale the low phenolic content of the Beaujolais (wine 13) is characterised by a markedly lower vasodilation and antioxidant activity. The Bulgarian Young Vatted Cabernet Sauvignon (wine 3) underwent extensive skin extraction using a rotary extractor during vinification, facilitating the release of more phenolics than would have otherwise have been possible using more traditional methods. In contrast, the Beaujolais (wine 13) was produced conventionally from thin-skinned Gamy grapes that undergo carbonic maceration and are, therefore, only lightly extracted.

4.4.1.1 Phenolic profile

It is well accepted that the health benefits that are attributed to red wine and not to white wine are due to the presence of skin-derived phenolics in the former. Of the individual phenolics quantified in this study the stilbenes, the flavonols, the anthocyanins and the flavan-3-ols are found principally in grape skins. When each of these families is represented as a percentage of total skin-derived phenolics (Fig. 4.4) it is apparent that the flavan-3-ols and the anthocyanins are quantitatively the major components in the skin. Total

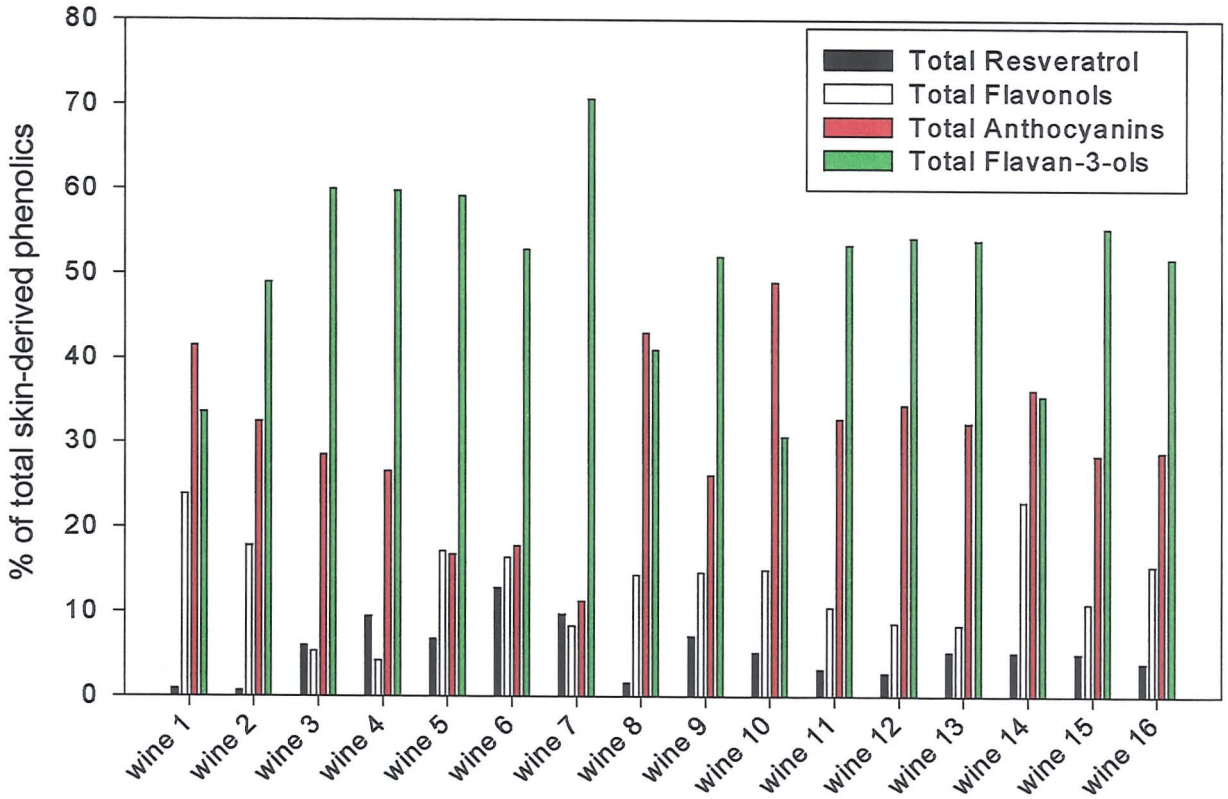


Figure 4.4. Selected phenolics, quantified as μM, as a percentage of the total skin-derived phenolics

flavonols and stilbenes are only minor contributors to the phenolic content of grape skins. Statistical analysis showed that of the skin-derived phenolics only the total flavan-3-ols were significantly correlated with antioxidant activity.

4.4.1.2 Vasodilation activity

A number of studies have previously investigated the vasodilation activity of a range of plant extracts, including red wine, grape juice and grape skin extract (Fitzpatrick et al., 1993, 1995; Andriambeloson et al., 1998). There is clear evidence that the most active compounds are skin-derived, supported by the low vasodilation activity of white wine as opposed to red wine where there is extensive grape skin extraction. Recent studies have attempted to identify the vasoactive agent. Investigations using the flavonols, apigenin, isorhamnetin, kaempferol, luteolin, myricetin, quercetin and rutin, showed that apart from rutin they all induced significant relaxation of rat pulmonary arteries (MacLean et al., 1997). In contrast, Andriambeloson et al. (1998) found that only the anthocyanin and oligomeric condensed tannin containing fractions of red wine showed the same activity as the original red wine polyphenolic fraction. The present study found that while there is a strong correlation between vasodilation activity and total phenolics quantified by either by the Folin-Ciocalteu assay or by HPLC, the only correlation with a phenolic family was with the total anthocyanins.

4.4.2 Discussion of batch II results

The relationship between the antioxidant activity and the phenolic content of a second batch of wines was investigated for two reasons. The initial sample set of only 16 wines was quite small and slightly skewed towards old world wines, making it hard to draw any firm conclusions. In addition a number of other assays were available at this point, including the FRAP assay for antioxidant activity, spectral assay for total catechins and an HPLC method for the separation of ellagic acid.

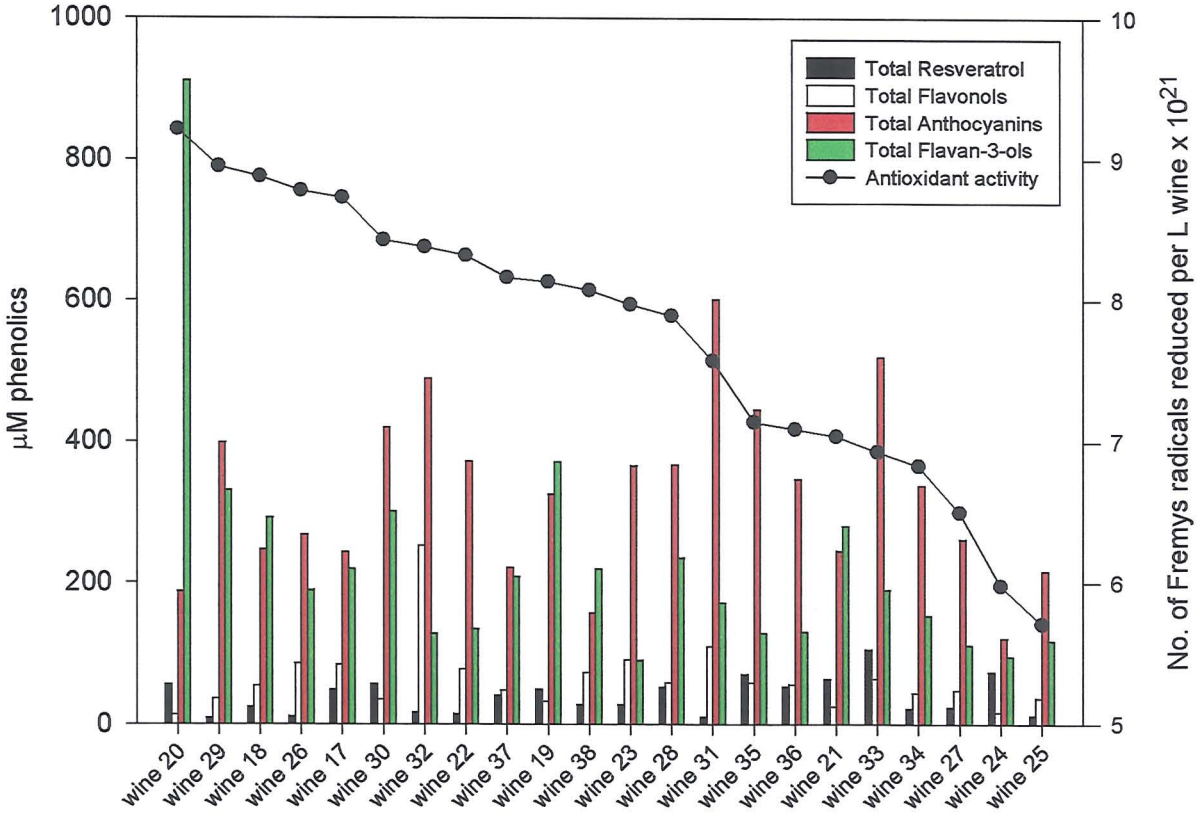


Figure 4.5. Phenolic content and antioxidant activity of skin-derived phenolics (batch II)

The wine with the highest ESR-antioxidant activity was the Pommard Premier Cru from Burgundy (wine 20). Wines from this region have traditionally been made from Pinot Noir grapes. Generally such wines are light in style as Pinot Noir is a thin-skinned grape. However this particular wine is an extremely well made premium wine and may be expected to have undergone the extensive maceration required to provide a wine with significant tannins to age well. Surprisingly the wines with the lowest ESR-antioxidant activity are both from Australia (wines 24 and 25). Conventionally it is assumed that the sunny growing conditions in Australia would favour ripe grapes with high levels of antioxidant phenolic compounds (Price et al., 1996). However comparing the antioxidant activities for these two wines, with the values obtained for the low activity Beaujolais and Valpollicella wines in batch I shows that the Australian wines are significantly higher. It is likely that the Australian wines, while having the lowest antioxidant activities in batch II, are not significantly lower than other wines when viewed in the correct context, i.e. compared with batch I wines. This highlights the importance of analysing a reasonable number and range of wines before drawing conclusions.

4.4.2.1 Phenolic profile

Once again a strong correlation was observed between the total phenolic content and the antioxidant activity of a wine. On average the total phenolic content (derived by either the Folin-Ciocalteu assay or by HPLC) of the wines in batch II is higher than those found in batch I. The second batch of wines is sourced primarily from the Southern Hemisphere where wine-making has become more of a science than an art. With the combination of hot sunny climates, and modern efficient extraction equipment such regions could be expected to produce wines that contained higher levels of phenolic compounds.

Another important feature of batch II wines is the domination of anthocyanins in the skin (Fig. 4.5). Whereas in batch I the major skin-derived phenolic were the flavan-3-ols, in batch II it was the anthocyanins. This could be a function of the sunny climate in many of the Southern Hemisphere wine-making

countries. The grapes have an opportunity to ripen fully and continue their production of anthocyanins. The one exception to the dominance of anthocyanins in the skin is wine 20, the Premier Cru from Burgundy. This wine also recorded the highest antioxidant activity.

4.5 Conclusion

Although both the antioxidant activity and the vasodilation activity of the wines were found to correlate strongly with total phenol content, determined by either the Folin-Ciocalteu assay or by HPLC, the relationship with individual phenolics was less clear. The antioxidant or vasodilation activity did not appear to be due significantly to the presence of a particular compound or family of compounds. Further studies are required to identify which phenolic compound(s) are responsible for the antioxidant activity of red wines.

Chapter 5 Extraction of phenolics during the vinification of red wine

5.1 Introduction

Phenolics contained within the skin, seeds and flesh of black grapes are extracted into red wines during the processes of vinification. The traditions of vinification differ between countries, regions and wine-makers. It is not a static process and it will vary with the vintage, grape variety and grape quality.

The processes of viticulture and vinification determine the content and profile of phenolic compounds in wine. Vineyard factors such as grape variety, climate, geographical origin and disease pressure affect the phenolic compounds produced in grapes (Siemann and Creasy, 1992; McDonald et al., 1998). During vinification the length of skin contact, temperature, presence of seeds, stems, and enzymes have all been shown to effect the extraction of phenolics (Ramey et al., 1986; Mattivi et al., 1995; Kovac et al., 1995) and are discussed in greater detail in section 1.4.

This study sets out to investigate the influence of four different approaches to vinification on the extraction of phenolics into wine. Two varieties were studied, Cabernet Sauvignon and Merlot. The phenolic content of grapes and the resultant wine will be compared. This will provide information on the nature and the extent of the extraction of phenolic compounds from grapes into wine. Measurements were made of changes in the antioxidant activity of wines throughout the study. This will allow the relationship between the extraction of individual phenolics into a wine, and any change in the antioxidant activity to be examined. It is hypothesised that this approach will provide information on the contribution that phenolic families make to the antioxidant activity of a wine, and will subsequently enable the identification of the major contributors.

5.2 Extraction of phenolics during red wine vinification

Grape and wine samples were collected as detailed in Table 5.1. In brief, grapes were weighed and kept at -20 °C until they could be frozen in liquid nitrogen and kept at -80 °C. Wines were filtered and the addition of ethanol to a final concentration of 30% halted fermentation. Samples were purged with carbon dioxide, bottled and stored in the dark until analysed. Details of the grapes and wines analysed are given in Table 5.2.

Table 5.1. Schedule of sample collection

sample	wine A	wine B	wine C	wine D
Grapes	15/3/1999	15/3/1999	15/3/1999	17/3/1999
Juice - day 0	15/3	15/3	16/3	17/3
day 1	16/3	16/3	17/3	18/3
day 2	17/3	17/3	18/3	19/3
day 3	18/3	18/3	19/3	20/3
day 4	19/3	19/3	20/3	21/3
day 5	20/3	20/3	21/3	22/3
day 6	21/3	21/3	22/3	23/3
day 7	22/3	22/3	23/3	24/3
day 8	23/3	23/3	24/3	-
day 9	24/3	24/3	-	-

Each wine was analysed for a range of phenolics using the HPLC methods described previously (Section 2.3). These were the free and conjugated flavonols, myricetin, quercetin, kaempferol and isorhamnetin; the flavan-3-ols (+)-catechin and (-)-epicatechin; the free anthocyanins malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-acetyl)glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside; gallic acid; the hydroxycinnamates caftaric, caffeic and *p*-coumaric acids, and the stilbenes, *trans*-resveratrol and *trans*-resveratrol-*O*-β-glucoside. The total phenol content was determined by the Folin-Ciocalteu assay and also by the summation of the HPLC-derived individual phenolic

Table 5.2. Details of grapes and wines from Viña san Pedro

wine	grape	comment
A	Cabernet Sauvignon (Basic quality. Supplied to Safeway Stores plc as their own label Chilean Cabernet Sauvignon)	• Large, pale redish-purple grapes. • Grapes collected on 15/3/1999 crushed and de-stemmed. • Juice collected (day 0 sample) then 1 h in the rotor-vat • 16/3/2000 traditional fermentation initiated (day 1). Wine pumped-over 2 x day for 15 min. Fermentation in medium-sized steel tanks. Racked and transferred to steel large tanks on 22/3/1999 (day 7). Mixed with other similar wine musts.
B	Merlot (Basic quality. Undergoes thermovinification. This extracts colour and tannins. Used to add to less tannic wines to ensure vintage to vintage consistency.)	• Large, pale red grapes. • Grapes were collected on 15/3/1999 crushed and de-stemmed. Juice collected (day 0 sample). • Must heated to 60-65 °C in rotor-vat for 1 h. Juice drained. Skins pressed and pressing added back to juice. Pectolytic enzymes added to juice at 7 °C. Stored in medium sized steel tank. • Racked and transferred to large steel tanks on 22/3/1999 (day 7). Mixed with other similar wine musts. • Fermentation initiated after day 9.
C	Cabernet Sauvignon (Resverva quality. This is the highest quality of wine produced by Viña San Pedro. Bottled and sold in Chile under the label 'Cabo de Horno'.)	• Small, dense grapes. Deep red/purple colour. • Grapes collected 15/3/1999. De-stemmed. Placed in vat immediately. • 16/3/1999 juice collected (day 0). • Fermentation initiated 17/3/1999 (day 1). Fermentation in very small concrete tank. Wine mixed using pump out-pump in method 2 x day.
D	Merlot (Varietal quality. Although not top quality, sufficient to be bottled without blending. Produced by Viña San Pedro and supplied to Safeway Stores labeled as '35 Sur Merlot'.)	• Medium-sized grapes. Deep red colour. • Grapes collected 17/3/1999 along with juice (day 0). De-stemmed and placed in rotor-vat for 1 h. • Fermentation initiated 18/3/1999 (day 1). • Fermentation in medium-sized steel tank. Wine pumped-over 2 x day for 15 min.

Table 5.3. Total phenolic content and antioxidant activity of Chilean wines.

Sample	Folin-Ciocalteu total phenolics[a]	HPLC derived total phenolics[b]	ESR-based antioxidant activity[c]
Wine A day 0	2.8 ± 0.2	50.5 ± 2.0	1.2 ± 2.3
day 1	2.9 ± 0.0	85.0 ± 0.5	2.0 ± 0.4
day 2	4.5 ± 0.1	228.9 ± 1.4	7.0 ± 2.2
day 3	7.7 ± 0.2	330.0 ± 4.3	8.2 ± 0.6
day 4	8.4 ± 0.1	571.1 ± 10.9	16.1 ± 0.9
day 5	8.2 ± 0.1	631.8 ± 14.3	17.8 ± 0.3
day 6	9.8 ± 0.3	694.9 ± 2.7	22.1 ± 1.5
day 7	7.9 ± 0.3	538.7 ± 71.7	22.4 ± 1.4
day 8	8.3 ± 0.3	604.4 ± 3.6	22.7 ± 0.0
day 9	8.2 ± 0.1	610.7 ± 1.9	20.1 ± 0.0
Wine B day 0	6.5 ± 0.2	426.1 ± 3.3	18.4 ± 2.0
day 1	9.4 ± 0.1	634.1 ± 3.0	27.4 ± 2.7
day 2	7.1 ± 0.0	524.4 ± 2.0	19.4 ± 0.6
day 3	7.1 ± 0.1	534.9 ± 2.1	24.3 ± 1.1
day 4	8.4 ± 1.0	527.5 ± 2.2	19.3 ± 1.5
day 5	6.5 ± 0.1	473.3 ± 7.6	18.0 ± 2.0
day 6	7.5 ± 0.0	546.1 ± 5.5	20.5 ± 1.1
day 7	6.4 ± 0.1	537.1 ± 0.7	18.7 ± 0.7
day 8	7.3 ± 0.1	515.7 ± 32.4	19.2 ± 0.7
day 9	7.0 ± 0.0	671.0 ± 6.6	18.9 ± 0.0
Wine C day 0	2.1 ± 0.0	303.2 ± 1.4	n.d.
day 1	3.3 ± 0.0	513.5 ± 3.5	3.2 ± 1.2
day 2	5.9 ± 0.2	450.6 ± 1.6	6.7 ± 1.4
day 3	6.8 ± 0.0	549.0 ± 3.7	21.0 ± 1.5
day 4	8.7 ± 0.3	643.3 ± 11.2	23.7 ± 1.3
day 5	9.1 ± 0.2	611.4 ± 9.6	25.8 ± 0.4
day 6	9.5 ± 0.2	873.7 ± 3.0	25.6 ± 1.0
day 7	9.7 ± 0.3	794.1 ± 11.6	30.0 ± 0.8
day 8	10.5 ± 0.3	920.0 ± 21.7	36.4 ± 0.1
Wine D day 0	3.4 ± 0.1	28.2 ± 5.0	0.3 ± 0.0
day 1	4.5 ± 0.1	153.4 ± 21.2	8.5 ± 0.6
day 2	6.1 ± 0.1	284.9 ± 13.7	14.5 ± 1.1
day 3	8.8 ± 0.1	341.4 ± 10.6	29.1 ± 1.7
day 4	11.4 ± 0.3	420.3 ± 5.1	27.5 ± 1.2
day 5	10.9 ± 0.2	547.0 ± 14.7	37.0 ± 1.2
day 6	11.2 ± 0.3	428.0 ± 11.3	28.3 ± 1.0
day 7	11.4 ± 0.1	397.7 ± 9.2	29.5 ± 0.1

[a,b]Total phenolic content of wine quantified by the Folin-Ciocalteu assay [mM gallic acid equivalents (GAE)] and from HPLC analysis of individual phenolics (μM). Results are expressed as mean values ± SEM, n=3. [c]Antioxidant capacity of wines, measured by ESR spectroscopy, presented as the number of Fremy's radicals reduced by one litre of wine x 10^{20} ± SEM x 10^{20}. n.d, not detected.

compounds (Table 5.3). While a summary of the phenolic contents of the grapes and wines is presented in Tables 5.4 to 5.7, the individual results are found in Appendix Tables 1 to 15. The extraction of the various phenolics from the grapes into the wines is followed graphically in Figures 5.1 to 5.8.

5.2.1 Wine A – Traditional fermentation basic Cabernet Sauvignon

This wine will eventually be the Chilean Cabernet Sauvignon wine that is used in the fractionation (Chapter 6) study. It has consistently recorded high flavonol levels over a number of years (McDonald et al., 1998) and has a higher than average total phenolic content and antioxidant capacity (see Chapter 4 wines 1 and 31). This wine is made on a very large scale in steel vats. Because the buyers require a consistent product, the wine is removed from the vats where it is fermented and transferred to an even larger tank where it is mixed with other tanks of the same type of wine.

5.2.1.1 Flavonols

Free and conjugated myricetin, quercetin, kaempferol and isorhamnetin are found within the grapes, reaching an average total flavonol content of 84.6 ± 3.2 nmol/g grape tissue. Nearly 23% of the flavonols in grapes were found as the aglycone, compared with the juice (day 0) which contained only 9% (Appendix Table 1). This was unexpected, as flavonols are usually found conjugated in grapes. The high level of aglycones may be due to the extraction procedure.

The major flavonols in the wine were myricetin and quercetin, however myricetin was not detected until day 2. The total flavonol content increased from 6.5 ± 0.2 μM in the juice to over 90 μM by day 9 (Table 5.4). Total flavonols remain relatively steady from day 5 to day 9 (Fig. 5.1 [A]). The ratio of free to conjugated flavonols also remains quite regular over this period. It fluctuates from 10.3% to 21.3%, with a mean value of 16.3%.

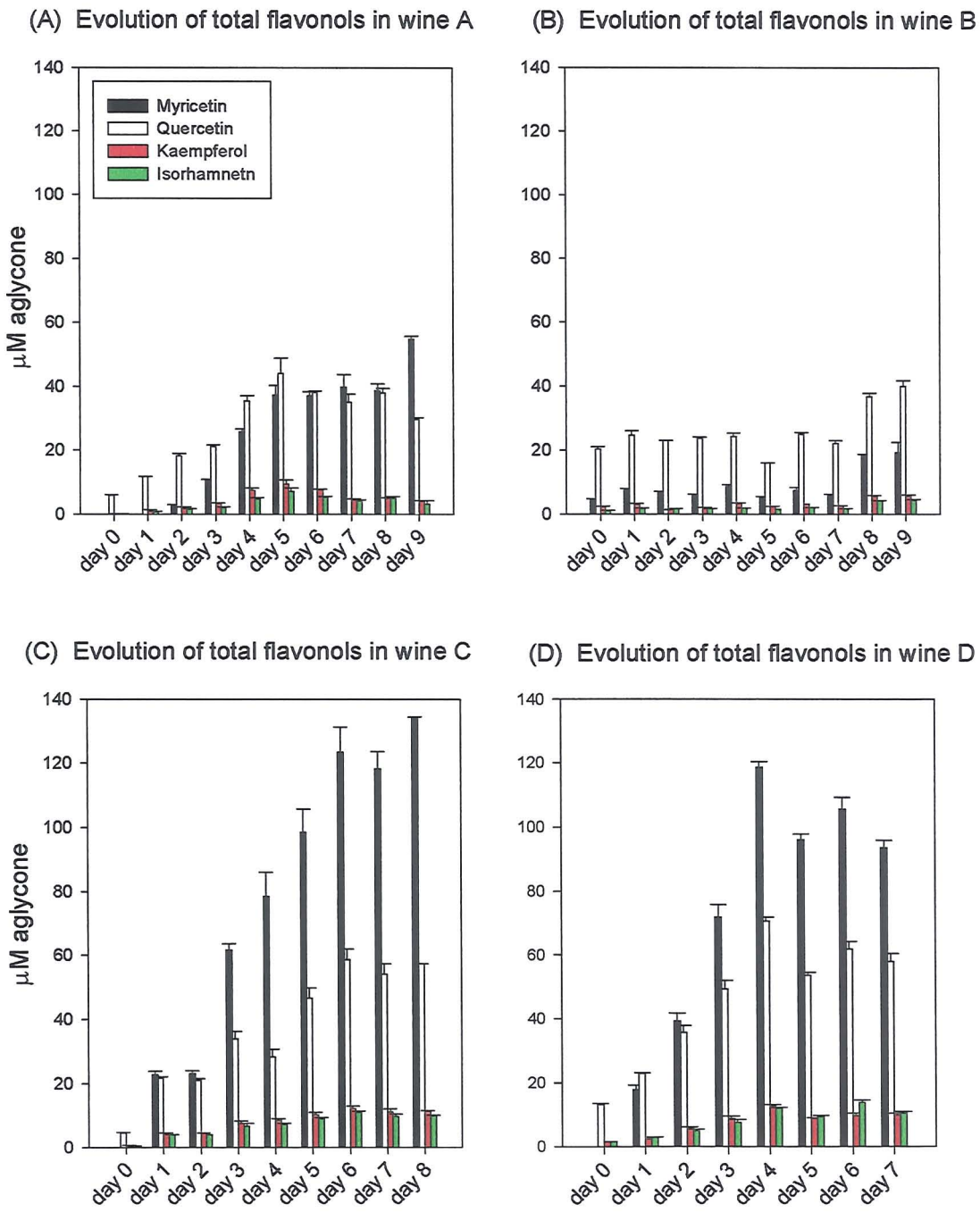


Figure 5.1. Comparison of the extraction of total myricetin, quercetin, kaempferol and isorhamnetin into wines A-D

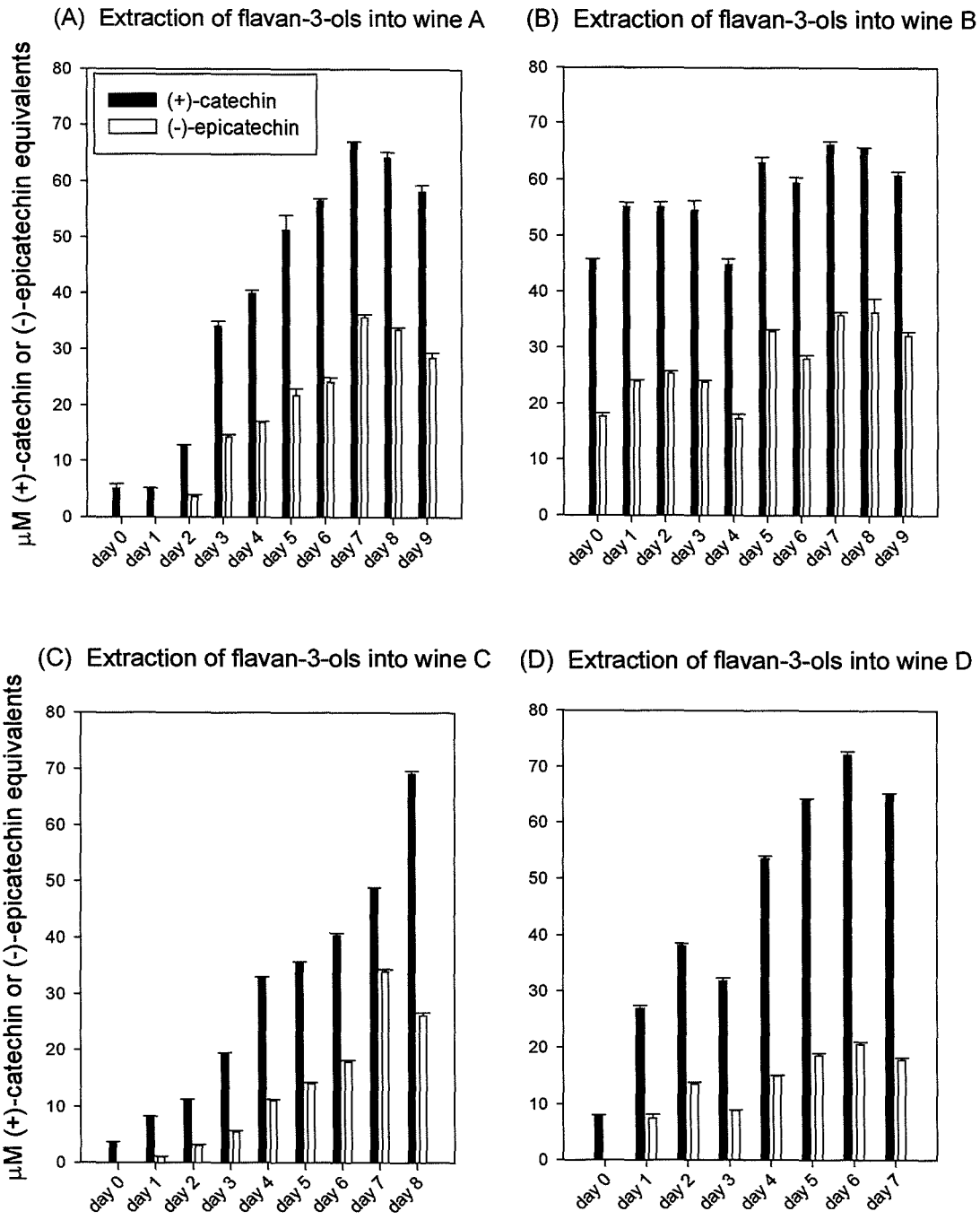


Figure 5.2. Comparison of the extraction of (+)-catechin and (-)-epicatechin into wines A-D

Table 5.4. Summary of the HPLC phenolic content of wine and grapes A.

sample	total flavonols	total flavan-3-ols	total anthocyanins	gallic acid	total hydroxy-cinnamates	total stilbenes	total phenolics
grape*	84.6 ± 3.2	900 ± 0.0	2500 ± 10.0	27.7 ± 0.7	345.2 ± 2.9	12.3 ± 0.3	3883.1 ± 114.6
juice/day 0	6.5 ± 0.2	5.1 ± 0.8	n.d.	5.4 ± 0.6	33.0 ± 0.1	0.5 ± 0.1	50.5 ± 2.0
day 1	13.9 ± 0.3	4.9 ± 0.3	4.5 ± 0.1	8.2 ± 0.0	51.2 ± 0.4	2.2 ± 0.1	85.0 ± 0.5
day 2	25.1 ± 0.4	16.3 ± 0.6	28.2 ± 0.2	22.4 ± 0.2	129.7 ± 0.8	5.5 ± 0.1	228.9 ± 1.4
day 3	37.9 ± 0.8	48.4 ± 1.4	26.8 ± 0.2	28.3 ± 0.2	180.8 ± 0.4	4.8 ± 0.2	330.0 ± 4.3
day 4	73.9 ± 3.4	56.8 ± 0.8	29.7 ± 2.5	60.3 ± 0.3	336.9 ± 3.3	7.0 ± 0.1	571.1 ± 10.9
day 5	98.0 ± 10.2	73.1 ± 3.9	36.0 ± 0.8	72.7 ± 0.5	335.7 ± 0.9	8.0 ± 0.5	631.8 ± 14.3
day 6	88.5 ± 1.5	80.6 ± 0.8	27.5 ± 0.2	79.8 ± 0.5	401.2 ± 4.2	9.9 ± 0.2	694.9 ± 2.7
day 7	83.7 ± 7.0	102.6 ± 0.5	28.8 ± 0.3	80.8 ± 0.4	265.7 ± 0.8	5.5 ± 0.5	538.7 ± 71.7
day 8	86.70 ± 3.1	97.8 ± 1.2	35.8 ± 1.5	87.6 ± 1.3	282.4 ± 1.4	7.5 ± 0.9	604.4 ± 3.6
day 9	91.9 ± 2.2	86.7 ± 2.1	73.7 ± 0.9	80.7 ± 1.4	271.4 ± 0.8	6.7 ± 0.3	610.7 ± 1.9

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected.

5.2.1.2 Flavan-3-ols

Although grapes A contained almost equi-molar levels of (+)-catechin and (-)-epicatechin (Appendix Table 5), (+)-catechin was present in ca. 2-fold higher concentration in each of the wine samples. In fact (-)-epicatechin was undetected in juice (day 0) and in the day 1 samples.

Total (+)-catechin and (-)-epicatechin levels ranged from 5.1 ± 0.8 μM in the juice (day 0) to over 100 μM by day 7 (Table 5.4). Levels of total flavan-3-ols decreased slightly after day 7 (Fig. 5.2 [A]).

5.2.1.3 Anthocyanins

Six different anthocyanins were detected in grapes A (Appendix Table 9), with a total anthocyanin content of 2.5 ± 0.1 μmol/g. Peonidin-3-glucoside could not be quantified as it formed a shoulder on the large malvidin-3-glucoside peak (Fig. 5.3).

Only three anthocyanins could be detected in the wine sample. These were all conjugates of malvidin; malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-acetyl)glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside (Appendix Table 10). The extraction of anthocyanins into wine A can be followed in Figure 5.4 (A). Although no anthocyanins could be detected in the juice, by day 9 maximum levels of 73.7 ± 0.9 μM total anthocyanins had been attained.

5.2.1.4 Gallic acid

The extraction of gallic acid into wine A is shown in Figure 5.5. Levels of gallic acid increased from 5.4 ± 0.6 μM in the juice (day 0) to a high of 87.6 ± 1.3 μM by day 8 (Table 5.4). A sharp increase in the content of gallic acid was observed between day 3 and day 4, with values plateauing by day 6.

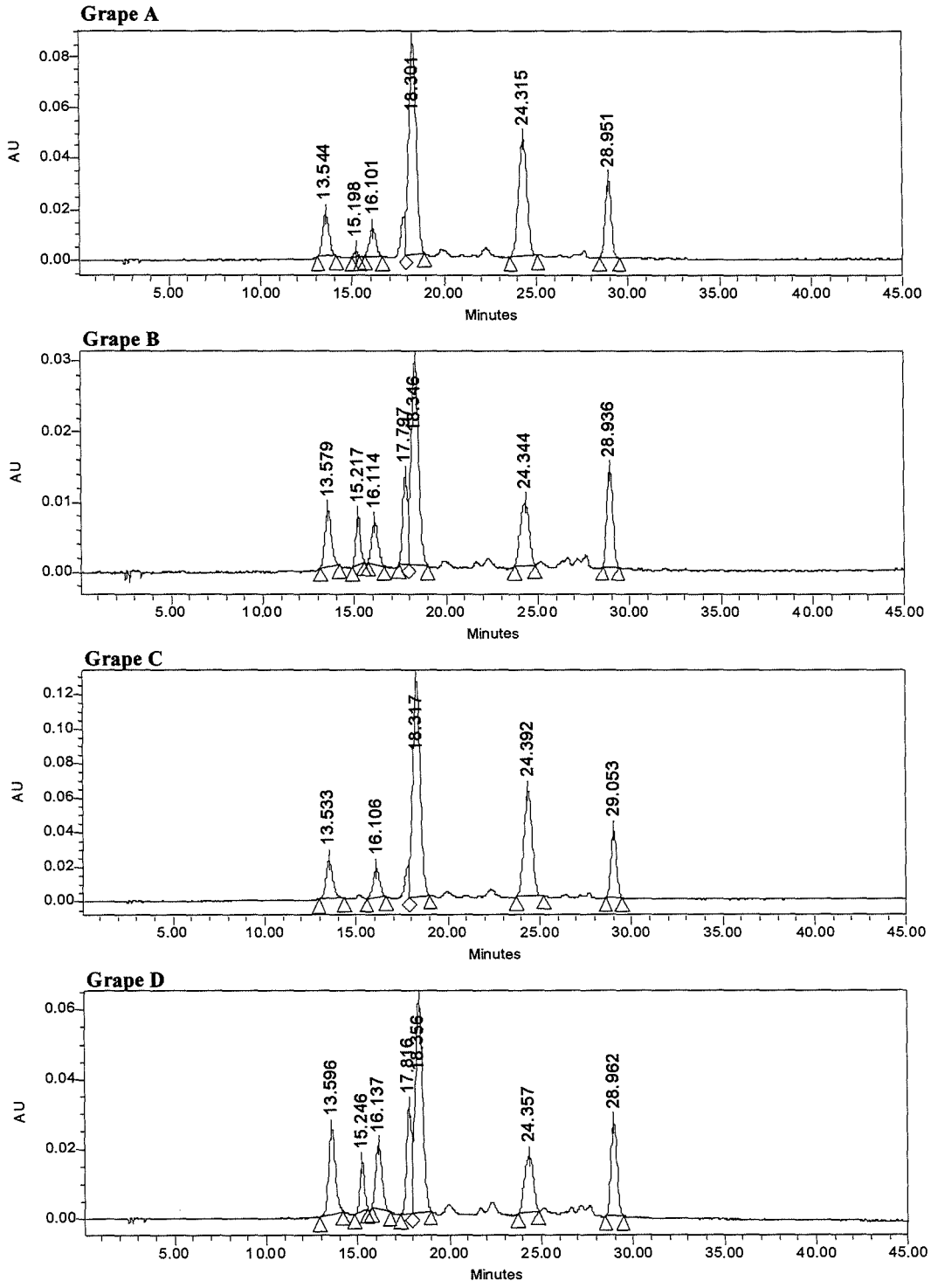


Figure 5.3. Comparison of the anthocyanin profile of grapes A, B, C and D

Column: 240 x 4.6 mm i.d. 4 µm Novapak C_{18}. Flow: I mL/min. Gradient: 5 to 30% ACN in 5% aqueous formic acid, over 40 min. Detection: Absorbance at 520 nm. Peaks 13.5 min, delphinidin-3-glucoside; 15.2 min cyanidin-3-glucoside; 16.1 min, petunidin-3-glucoside; 17.8 min, peonidin-3-glucoside; 18.3 min, malvidin-3-glucoside; 24.3 min malvidin-3-acetylglucoside; 28.9 min, malvidin-3-*p*-coumaroyl glucoside.

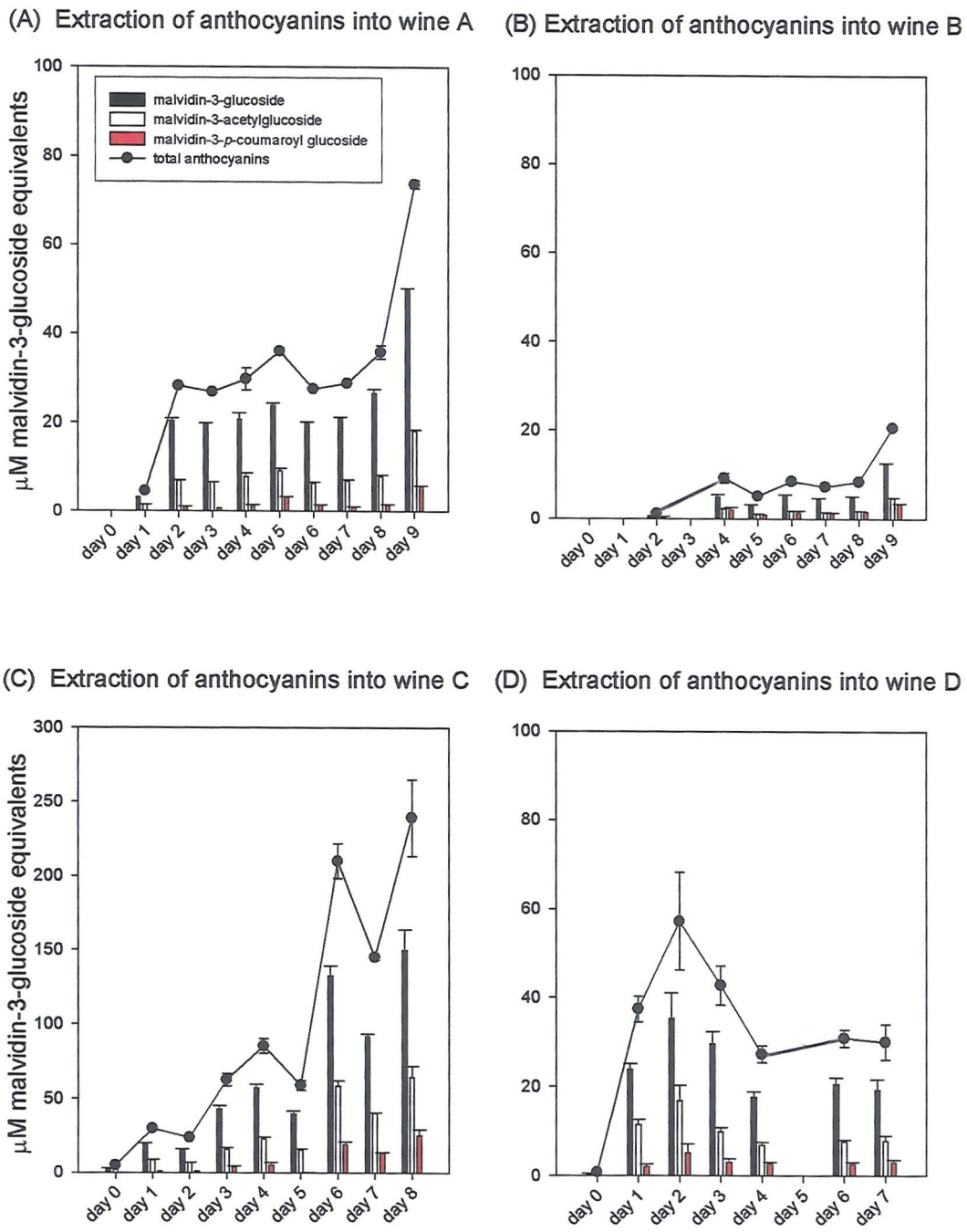


Figure 5.4. Comparison of the extraction of anthocyanins into wines A-D

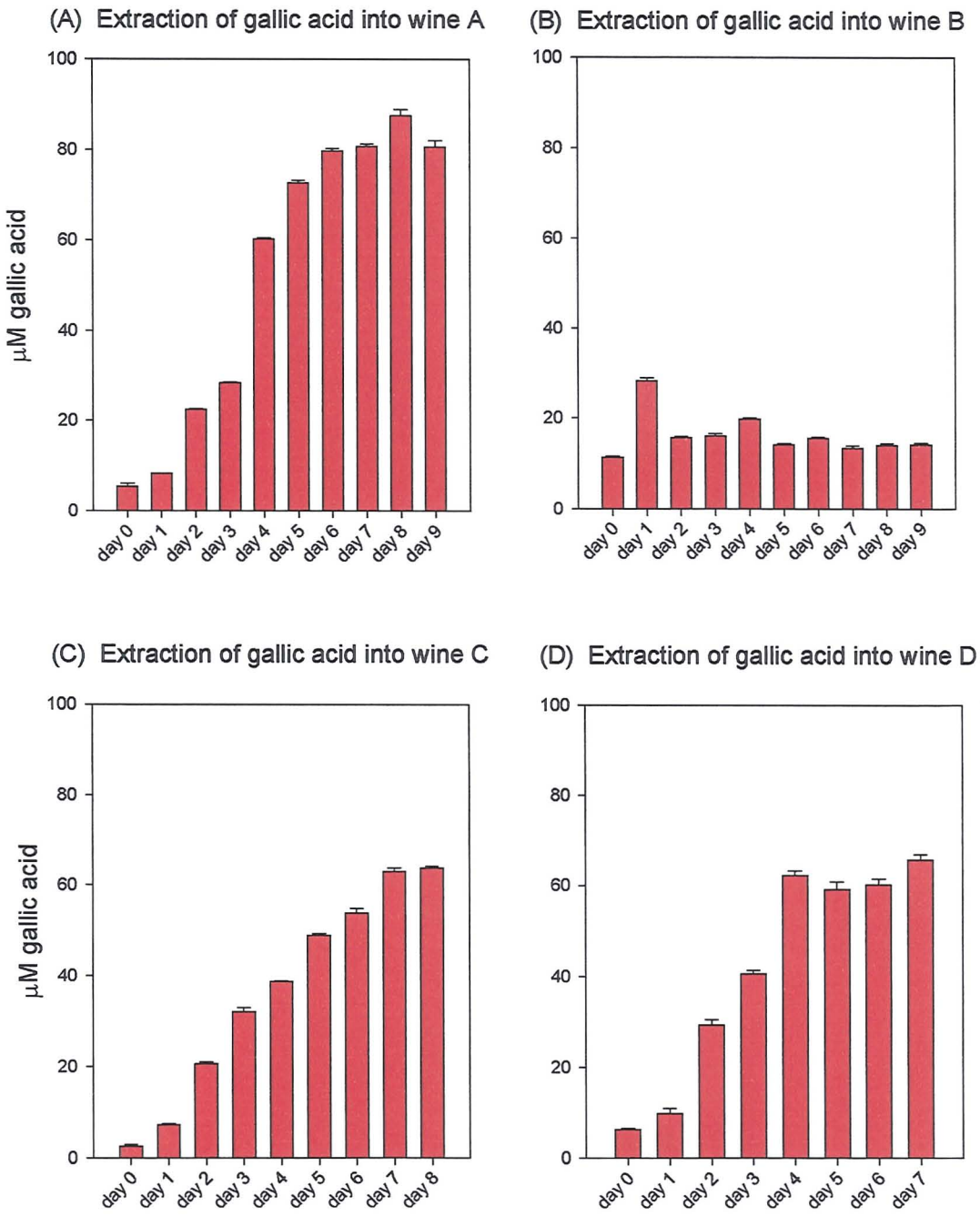


Figure 5.5. Comparison of the extraction of gallic acid into wines A-D

5.2.1.5 Hydroxycinnamates

Only caftaric acid and conjugated *p*-coumaric acid were detected in grapes A, with a total content of 345.2 ± 2.9 nmol/g (Appendix Table 14). Similar patterns were observed with the wine samples where only very low levels of free caffeic and *p*-coumaric acids were detected, accounting for less than 5% of the total hydroxycinnamate content at day 9. Levels of total caffeic and *p*-coumaric acid shown in Figure 5.6 can be taken to be caftaric and conjugated *p*-coumaric acids.

In all samples bar the juice (day 0), total *p*-coumaric acid was present in higher levels than total caffeic acid. Maximum levels of 401.2 ± 4.2 μM were reached by day 6, and then fell to around 270 μM by day 9.

5.2.1.6 Stilbenes

Only *trans*-resveratrol and its glucoside were detected in grapes A, with *trans*-resveratrol glucoside contributing 78% of the total stilbene content (Appendix Table 18). Levels of *trans*-resveratrol in wine ranged from 0.1 ± 0.0 μM in the juice (day 0) to a maximum of 1.8 ± 0.1 μM (Fig. 5.7 [A]). The glucoside was the major stilbene present in the wine samples. Maximum levels of 8.2 ± 0.2 μM were obtained by day 6 from a minimum of 0.4 ± 0.1 μM in the juice at day 0 (Fig. 5.8 [A]).

5.2.2 Wine B – Thermovinified Merlot

This wine underwent thermovinification treatment whereby it was heated to over 60 °C for 1 h. It did not undergo fermentation until after day 9 when no further samples could be collected. Thermovinification allows much of the colour to be quickly extracted from the grapes and into the wine. In a poor vintage such wines can be added to those wines which do not have sufficient body. This will add colour and tannins, and help improve the original wine. Thermovinified wines are not bottled without prior blending with wines produced with traditional fermentation.

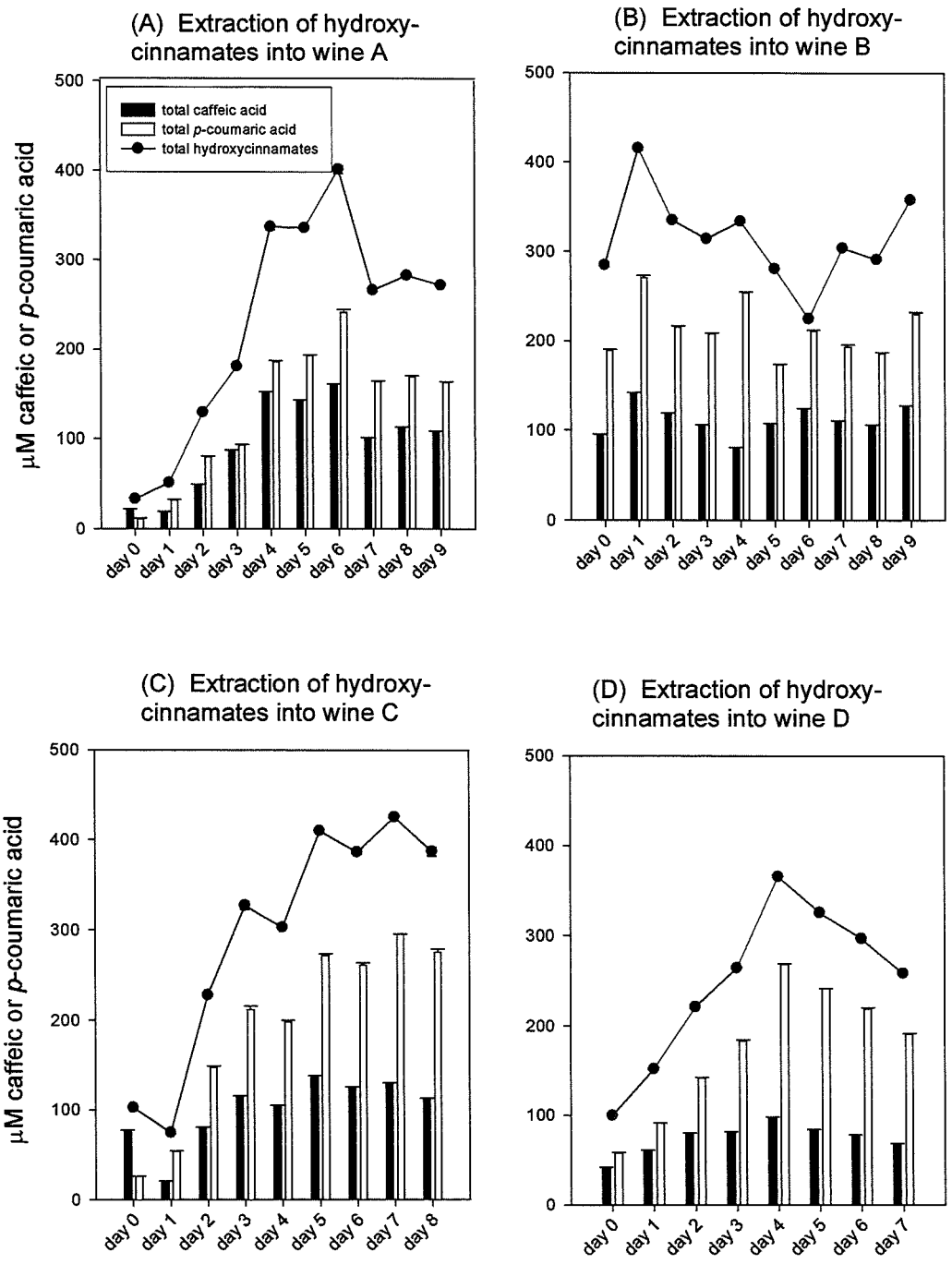


Figure 5.6. Comparison of the extraction of caffeic and *p*-coumaric acids into wines A-D

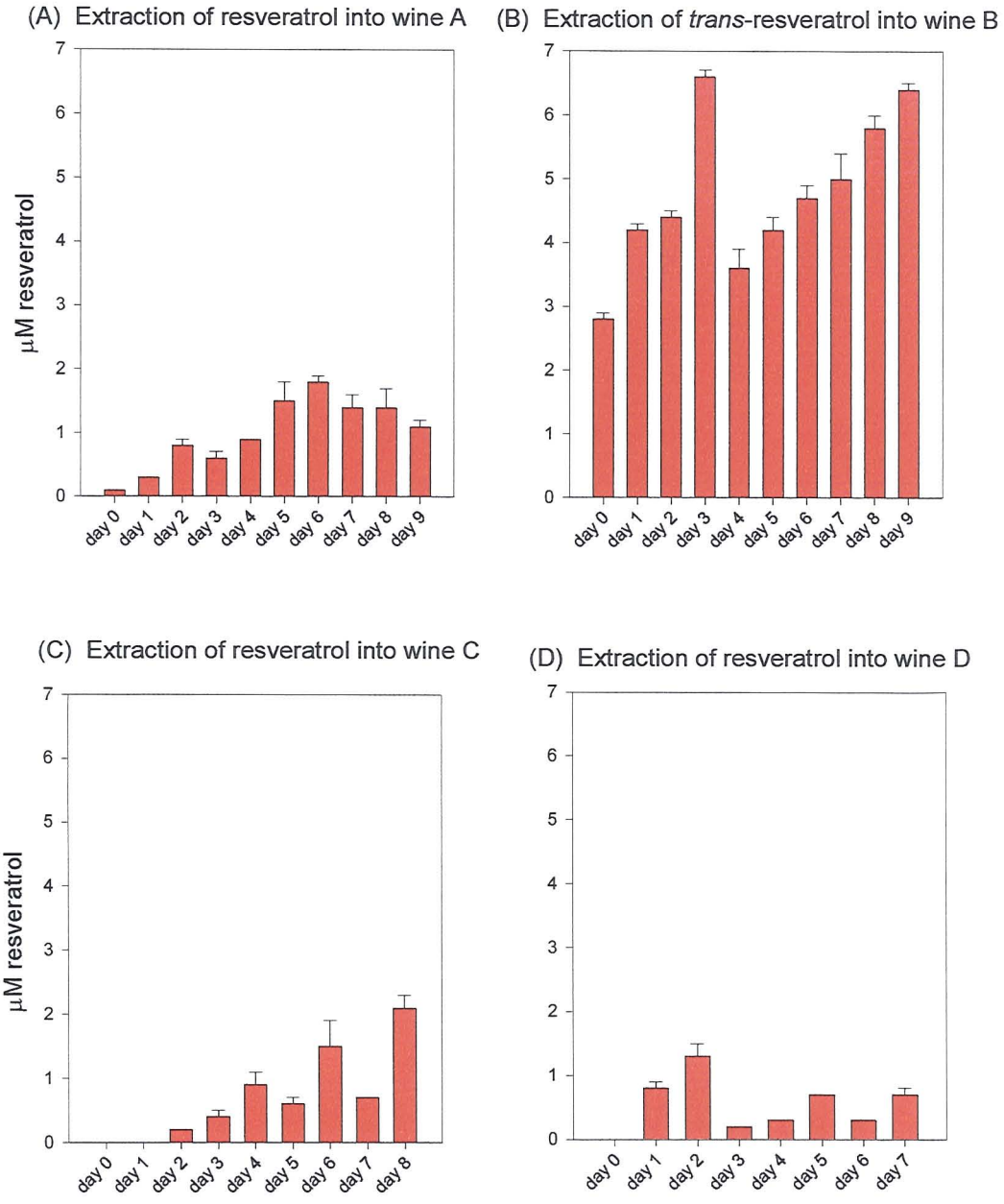


Figure 5.7. Comparison of the extraction of *trans*-resveratrol into wines A-D

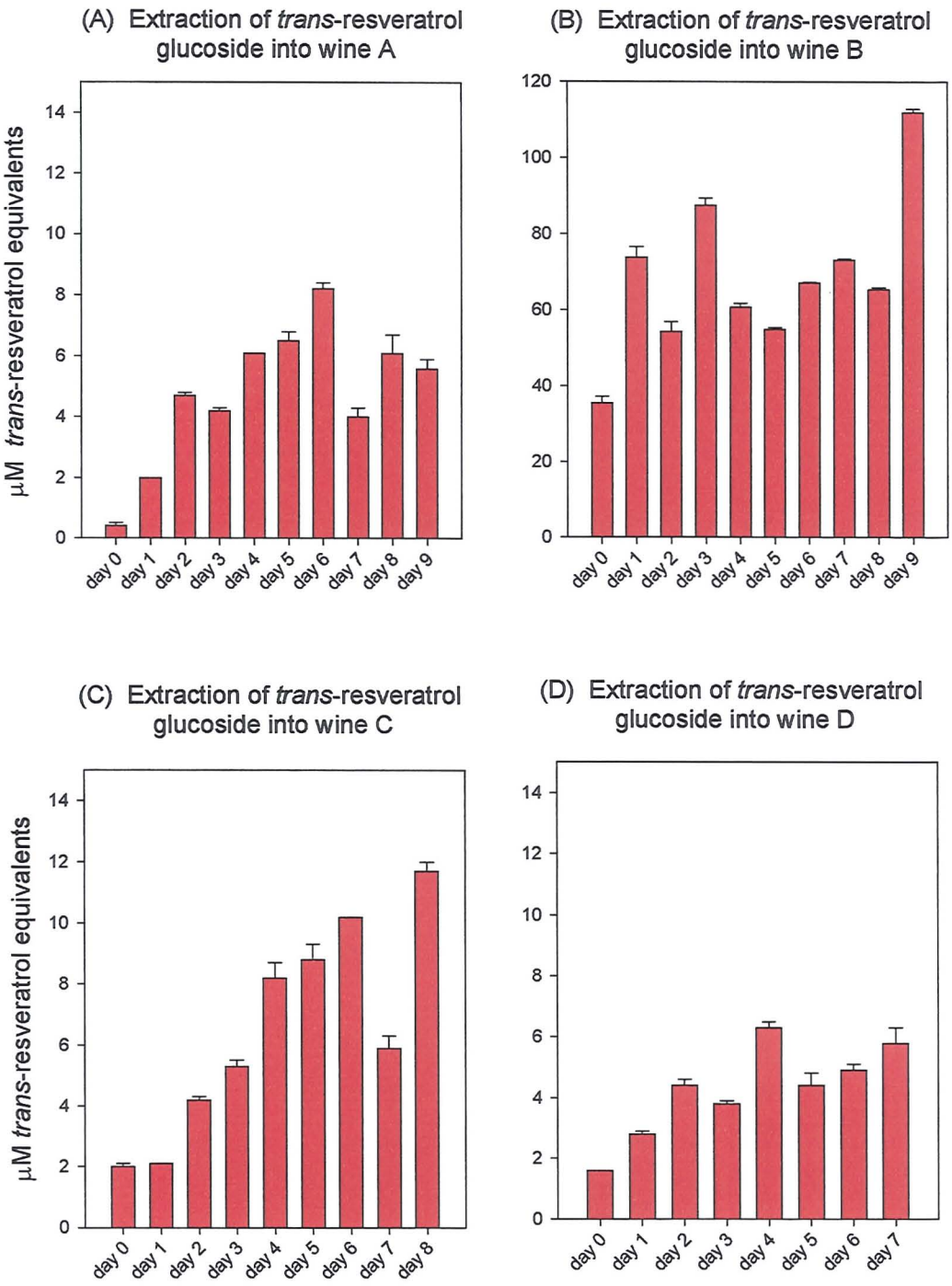


Figure 5.8. Comparison of the extraction of trans-resveratrol glucoside into wines A-D

5.2.2.1 Flavonols

With the exception of conjugated myricetin, the Merlot grapes contained free and conjugated myricetin, quercetin, kaempferol and isorhamnetin. Grapes contained an average of 93.3 ± 3.5 nmol/g total flavonols, 16.5% of which were free. Quercetin was noted to account for over 60% of the total flavonol content of the grapes (Appendix Table 2).

Quercetin was also the major flavonol extracted into wine (Fig. 5.1 [B]), with myricetin, kaempferol and isorhamnetin found in relatively low levels. The total flavonol concentration remained very steady from the juice, day 0, (28.2 ± 0.9 μM) to day 7 (32.4 ± 1.2 μM). The samples taken at day 8 and day 9 had significantly higher total flavonol levels of 65.1 ± 1.3 and 69.4 ± 5.4 μM (Table 5.5). A similar pattern was observed with the levels of free flavonols. Although they were around 50% in the juice and stayed at that level until day 7, they then dropped dramatically to between 15 and 16%.

5.2.2.2 Flavan-3-ols

Equi-molar concentrations of (+)-catechin and (-)-epicatechin were found in grapes B, with an average total concentration of 0.7 ± 0.0 μmol/g (Appendix Table 6).

In wine samples, however, (+)-catechin was found in levels almost 2-fold higher than (-)-epicatechin. Total flavan-3-ol levels increased slightly from 63.5 ± 0.7 μM in the juice (day 0) to between 90 and 100 μM by days 7, 8 and 9 (Table 5.5). On average levels of (+)-catechin and (-)-epicatechin did not vary much over the 9 days of sampling (Fig. 5.2 [B]).

5.2.2.3 Anthocyanins

Although they are present in only low levels, seven anthocyanins were found in grapes B (Fig. 5.3 and Appendix Table 9). The total anthocyanin content of the grapes was found to be approximately 1.0 ± 0.0 μmol/g. This is 2- to 3-

fold lower than the other grapes investigated. It was noted that these grapes were relatively large and fleshier than the other grapes analysed, i.e. had a lower ratio of skin to volume. As anthocyanins are found within the skin, the presence of considerable flesh had a diluting influence.

Very low levels of anthocyanins were found in all the samples of wine B (Fig. 5.4 [B]). No anthocyanins could be detected until day 2, and maximum levels only reached 20.6 ± 0.4 μM total anthocyanins (Table 5.5). Once again only the malvidin conjugates could be detected in the wine samples.

5.2.2.4 Gallic acid

Comparing the extraction profile of the extraction of gallic acid into wine B with those of the other wines A, C, and D, it is apparent that little is extracted with thermovinification. The gallic acid content remains under 20 μM (with the exception of day 1), which is comparable to the content of gallic acid at day 3 of wine A (Fig. 5.5 [B]).

5.2.2.5 Hydroxycinnamates

Grapes B contained the conjugates of caffeic and *p*-coumaric acids, with a total hydroxycinnamate content of 154.6 ± 4.2 nmol/g (Appendix Tables 15). No free caffeic acid was detected in any of the wine samples, and free *p*-coumaric acid was always less than 2% of the total *p*-coumaric acid quantified. Very high levels of total hydroxycinnamates were found in the juice (day 0), 284.4 ± 1.7 μM. Although levels fluctuated throughout the sampling period (Fig. 5.6 [B]), the final concentration of total hydroxycinnamates reached only 357.6 ± 2.0 μM (Table 5.5). Conjugated *p*-coumaric acid was the major hydroxycinnamate present, with total caffeic acids contributing on average only one third of the total hydroxycinnamate content.

Table 5.5. Summary of the HPLC phenolic content of wine and grapes B.

sample	total flavonols	total flavan-3-ols	total anthocyanins	gallic acid	total hydroxy-cinnamates	total stilbenes	total phenolics
grape*	93.3 ± 3.5	700 ± 0.0	1000 ± 0.0	19.3 ± 0.8	154.6 ± 4.2	34.3 ± 1.6	2048 ± 23.4
juice/day 0	28.2 ± 0.9	63.5 ± 0.7	n.d.	11.3 ± 0.3	284.4 ± 1.7	38.3 ± 1.8	426.1 ± 3.3
day 1	37.2 ± 1.8	79.2 ± 1.1	n.d.	28.3 ± 0.7	412.5 ± 2.6	78.0 ± 2.8	634.1 ± 3.0
day 2	32.9 ± 0.2	80.6 ± 1.3	1.2 ± 0.1	15.6 ± 0.3	334.8 ± 1.1	58.6 ± 2.6	524.4 ± 2.0
day 3	33.5 ± 0.3	78.4 ± 1.9	n.d.	16.0 ± 0.6	313.9 ± 1.1	94.2 ± 1.6	534.9 ± 2.1
day 4	38.2 ± 1.6	62.2 ± 1.9	9.2 ± 1.1	19.8 ± 0.3	333.6 ± 1.5	64.4 ± 0.6	527.5 ± 2.2
day 5	25.3 ± 0.3	96.1 ± 1.2	5.2 ± 0.0	14.1 ± 0.3	280.2 ± 0.8	59.1 ± 0.6	473.3 ± 7.6
day 6	36.8 ± 0.6	87.5 ± 1.6	8.5 ± 0.6	15.6 ± 0.2	334.5 ± 2.2	71.8 ± 0.0	546.1 ± 5.5
day 7	32.4 ± 1.2	102.1 ± 0.3	7.3 ± 0.4	13.4 ± 0.6	303.6 ± 1.9	78.1 ± 0.6	537.1 ± 0.7
day 8	65.1 ± 1.3	99.5 ± 0.6	8.4 ± 0.3	14.0 ± 0.3	290.7 ± 1.0	71.1 ± 0.4	515.7 ± 32.4
day 9	69.4 ± 5.4	93.0 ± 1.3	20.6 ± 0.4	14.2 ± 0.3	357.6 ± 2.0	118.3 ± 0.8	671.0 ± 6.6

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected.

5.2.2.6 Stilbenes

High levels of *trans*-resveratrol glucoside were found in grapes B, 31.8 ± 1.5 nmol/g, with *trans*-resveratrol present on average at a concentration of only 2.4 ± 0.1 nmol/g (Appendix Table 18). Similar high levels were observed with the wines (Fig. 5.7 [B] and 5.8 [B]). Levels of *trans*-resveratrol ranged from 2.8 ± 0.1μM in the juice (day 0) to 6.4 ± 0.1 μM by day 8. Apart from a rogue point on day 3, *trans*-resveratrol appears to be steadily extracted into the wine.

5.2.3 Wine C – Quality Cabernet Sauvignon

The grapes used for this wine were characterised by being very small in size, with a deep purple hue. They had been grown on old vines and in an area of the vineyard known to produce grapes with a concentrated flavour. Due to the high quality of the Cabernet Sauvignon grapes this wine was to be made into a premium reserva wine. This means that it will be aged in for three years, at least a year of which must be in oak. Vinification took place in small concrete vats that would allow more contact between the skins and the juice. To further encourage this mixing of the skins and the juice the wine was pumped-over. In this case the wine was completely emptied from the vat, allowing the skins to fall to the bottom. The wine was then added back and sprayed over the top of the skins.

5.2.3.1 Flavonols

The small dense Cabernet Sauvignon berries had an average total flavonol content of 143.9 ± 7.4 nmol/g grape tissue, almost 1.5 times higher than that observed with grapes A and B (Appendix Table 3).

Although no myricetin was detected in the juice (day 0), it was rapidly extracted from the grapes into the wine (Fig. 5.1 [C]). The total flavonol content of the wine increased from 5.7 ± 0.1 μM in the juice to over 200 μM by day 6, at which point levels remained steady (Table 5.6). On average myricetin accounted for over half of the grape total flavonol content.

Table 5.6. Summary of the HPLC phenolic content of wine and grapes C.

sample	total flavonols	total flavan-3-ols	total anthocyanins	gallic acid	total hydroxy-cinnamates	total stilbenes	total phenolics
grape*	143.9 ± 7.4	600 ± 0.0	3300 ± 10	21.5 ± 0.2	368.3 ± 42.2	6.8 ± 0.3	4413.9 ± 79.5
juice/day 0	5.7 ± 0.1	3.8 ± 0.0	4.9 ± 0.0	2.6 ± 0.4	102.5 ± 0.2	2.0 ± 0.1	303.2 ± 1.4
day 1	53.0 ± 1.2	9.0 ± 0.2	29.6 ± 0.2	7.3 ± 0.2	74.4 ± 0.4	2.1 ± 0.0	513.5 ± 3.5
day 2	53.0 ± 0.6	14.1 ± 0.2	23.6 ± 0.1	20.6 ± 0.5	227.8 ± 0.8	4.4 ± 0.1	450.6 ± 1.6
day 3	110.1 ± 5.8	24.9 ± 0.2	62.3 ± 4.1	32.0 ± 0.9	326.7 ± 4.2	5.7 ± 0.3	549.0 ± 3.7
day 4	132.8 ± 10.5	43.9 ± 0.2	85.2 ± 4.9	38.8 ± 0.1	302.6 ± 1.4	9.1 ± 0.7	643.3 ± 11.2
day 5	164.7 ± 11.4	49.6 ± 0.2	58.7 ± 3.1	48.9 ± 0.5	409.7 ± 2.0	9.2 ± 0.3	611.4 ± 9.6
day 6	205.5 ± 12.3	58.2 ± 0.4	209.9 ± 11.7	53.8 ± 1.0	386.1 ± 2.5	11.7 ± 0.4	873.7 ± 3.0
day 7	193.3 ± 10.1	82.5 ± 0.5	145.0 ± 2.1	63.0 ± 0.8	425.2 ± 0.4	6.6 ± 0.5	794.1 ± 11.6
day 8	213.3 ± 0.5	95.3 ± 0.4	239.1 ± 25.7	63.7 ± 0.4	386.7 ± 4.9	13.8 ± 0.1	920.0 ± 21.7

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3.

Although quercetin was responsible for just under half of the grape total flavonol content, it reached levels of only around a quarter of the total flavonol content of the wine from day 6 onwards.

5.2.3.2 Flavan-3-ols

Grapes C had an average of 0.6 μmol/g total flavan-3-ols, with a ratio (-)-epicatechin to (+)-catechin of approximately 1:1 (Appendix Table 7). The flavan-3-ol content is significantly lower than those observed for grapes A, B and D.

Maximum flavan-3-ol levels in wine C were found to be similar to those of wines A, B and D (Fig. 5.2 [C]). A maximum of 95.3 μM total flavan-3-ols was recorded on day 8, after an almost linear extraction from the juice over the sampling period (Table 5.5). (+)-Catechin was present, on average, in a 2- to 3-fold excess compared with (-)-epicatechin.

5.2.3.3 Anthocyanins

As with the Cabernet Sauvignon grapes A, not all of the expected anthocyanins could be detected in grapes C (Fig. 5.3). Five anthocyanins were quantified, with a total anthocyanin content of 3.3 ± 0.1 μmol/g. This was the highest concentration of anthocyanins of the grapes analysed. This is most likely to be attributable to the high skin:volume ratio of the small, dense berries.

Compared with the other wines analysed, high levels of anthocyanins were extracted into the wine (Fig. 5.3 [C]). Note that the y-axis is 3-fold greater than the other graphs. Although levels of anthocyanins fluctuated during the extraction period, the maximum total anthocyanin levels of 239.1 ± 25.7 μM were recorded on day 8 (Table 5.6).

Table 5.7. Summary of the HPLC phenolic content of wine and grapes D.

sample	total flavonols	total flavan-3-ols	total anthocyanins	gallic acid	total hydroxy-cinnamates	total stilbenes	total phenolics
grape*	327.9 ± 6.7	1100 ± 0.0	2200 ± 0.0	40.0 ± 0.9	323.0 ± 5.6	24.3 ± 1.2	3998.6 ± 30.0
juice/day 0	16.5 ± 0.4	7.7 ± 0.3	0.8 ± 0.1	6.3 ± 0.3	99.4 ± 0.4	1.6 ± 0.0	28.2 ± 5.0
day 1	47.4 ± 1.8	34.3 ± 0.8	37.4 ± 2.9	9.9 ± 1.2	151.3 ± 0.9	3.6 ± 0.1	153.4 ± 21.2
day 2	85.9 ± 5.5	51.6 ± 0.7	57.2 ± 11.1	29.3 ± 1.1	220.8 ± 0.8	5.8 ± 0.3	284.9 ± 13.7
day 3	137.8 ± 8.3	40.7 ± 0.6	42.7 ± 4.4	40.6 ± 0.8	262.3 ± 1.5	4.0 ± 0.1	341.4 ± 10.6
day 4	214.2 ± 3.5	68.7 ± 0.4	27.3 ± 1.9	62.2 ± 1.1	365.4 ± 1.3	6.6 ± 0.2	420.3 ± 5.1
day 5	166.1 ± 2.8	82.6 ± 0.5	n.d.	59.2 ± 1.6	325.3 ± 0.3	5.1 ± 0.4	547.0 ± 14.7
day 6	191.3 ± 7.2	92.7 ± 0.9	30.9 ± 1.9	60.2 ± 1.3	296.8 ± 1.8	5.3 ± 0.2	428.0 ± 11.3
day 7	171.9 ± 2.0	83.6 ± 0.5	30.0 ± 4.0	65.7 ± 1.1	258.4 ± 0.7	6.5 ± 0.5	397.7 ± 9.2

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected.

5.2.3.4 Gallic acid

The content of gallic acid increased at a steady rate from 2.6 ± 0.4 μM in the juice to 63.7 ± 0.4 μM by day 8 (Table 5.6). The gallic acid content begins to plateau by days 7 and 8 (Fig. 5.5 [C]).

5.2.3.5 Hydroxycinnamates

The total hydroxycinnamate content of grapes C was found to be on average 368.3 ± 42.2 nmol/g (Appendix Table 16). In contrast to the other grapes, 45% of the total *p*-coumaric acid was found as the free aglycone. This pattern was not observed in the wine samples where free caffeic and *p*-coumaric acids only contributed around 2% of the total hydroxycinnamates present.

The total hydroxycinnamate content of the wine samples increased from 102.5 ± 0.2 μM in the juice (day 0), to 425.2 ± 0.4 μM by day 8. As with the other wines, *p*-coumaric acid was the major hydroxycinnamate present.

5.2.3.6 Stilbenes

Trans-resveratrol was not detected in grapes C, and *trans*-resveratrol glucoside was found at a concentration of only 6.8 nmol/g (Appendix Table 18). *Trans*-resveratrol was found in very low levels in wine C. It was undetected in the juice and day 1 samples, and reached a maximum of only 2.1 ± 0.1 μM on day 8 (Fig. 5.7 [C]). The glucoside however was steadily extracted into the wine. Levels of 2.0 ± 0.1 μM were found in the juice (day 0), increasing to 11.7 ± 0.3 μM by day 8 (Fig. 5.8 [C]).

5.2.4 Wine D – Traditional fermentation varietal Merlot

The grapes used to make this wine were of a deep red hue and were medium-sized. They were of sufficient quality to merit a 'varietal' status. They did not require blending prior to bottling. The wine underwent a traditional

fermentation after the grapes were extracted in the rotor-vat. This was to maximise the colour and tannins of the wine.

5.2.4.1 Flavonols

The total flavonol content of the Merlot grapes used for this wine is found to be 327.9 ± 6.7 nmol/g, 4-fold higher than grapes A and B, and 2-fold higher than grapes C. Around 13% of the flavonols in the grape were free, a value similar to the other grapes (Appendix Table 4).

Although the grapes contained twice the flavonol content of grapes C, the final total flavonol content of the wine was 171.9 ± 2.0 μM, slightly less than the final value recorded for wine C (Tables 5.6 and 5.7). Once again the major flavonol in the wine was myricetin, ranging from being undetected in the juice (day 0) to accounting for over 50% of the total flavonols by day 7. The maximum flavonol content occurred at day 4 and remained relatively steady from that point onwards (Fig. 5.1 [D]).

5.2.4.2 Flavan-3-ols

Total flavan-3-ol levels of 1.1 ± 0.0 μmol/g were recorded in grape D, with (+)-catechin present in excess to (-)-epicatechin (Appendix Table 8). This pattern continued, with (+)-catechin found in the wines at levels over 3-fold higher than (-)-epicatechin. Maximum flavan-3-ol levels of 92.7 ± 0.9 μM were attained on day 6, nearly 78% of which was due to (+)-catechin (Table 5.7). The extraction of flavan-3-ols into the wine from the grape can be seen in Figure 5.2 (D). Compared to wines A and C, quite high levels of flavan-3-ols were extracted into the wines in day 1 and day 2. However, although grapes D had higher flavan-3-ol levels than the others quantified, the maximum levels attained were similar to those found in wines A, B and C.

5.2.4.3 Anthocyanins

Once again seven anthocyanins could be quantified in the Merlot grapes D (Fig. 5.3). Total anthocyanin levels of 2.2 ± 0.0 μmol/g were recorded with malvidin-3-glucoside contributing around 37 % to this total.

An unusual extraction profile was observed with wine D anthocyanins (Fig. 5.4 [D]). Maximum total anthocyanin levels were obtained by day 2 (57.2 ± 11.1 μM), and then decreased to only 30.0 ± 4.0 μM by day 7. Anthocyanin values are not reported for day 5 due to sample deterioration.

5.2.4.4 Gallic acid

Although wine D reaches a similar maximum gallic acid concentration to the other wines, it is attained at an earlier stage (Fig. 5.5 [D]). By day 4 gallic acid had reached levels of over 60 μM (Table 5.7). This is compared to only 6.3 ± 0.3 μM in the juice (day 0).

5.2.4.5 Hydroxycinnamates

Free hydroxycinnamic acids were not detected in grapes D. Conjugated *p*-coumaric acids were responsible for over 80% of the total hydroxycinnamate content (Appendix Table 15). In the wine samples, the total hydroxycinnamic content ranged from 99.4 ± 0.4 μM in the juice (day 0) to a maximum of 365.4 ± 1.3 μM by day 4. At this point the hydroxycinnamate content decreased to 258.4 ± 0.7 μM by day 7. Once again conjugated *p*-coumaric acids were the major hydroxycinnamates present, with levels of total caffeic acids remaining relatively steady over the sampling period (Fig. 5.6 [D]).

5.2.4.6 Stilbenes

While *trans*-resveratrol glucoside was found in grapes D at levels of approximately 24.0 ± 1.2 nmol/g, the aglycone was not detected (Appendix Table 18). Very low levels of *trans*-resveratrol were detected in wine D, on

average they were less than 1μg/g (Fig. 5.7 [D]). *Trans*-resveratrol glucoside was steadily extracted into the wine. From 1.6 ± 0.0 μM in the juice (day 0), levels reached 5.8 ± 0.5 μM by day 8.

5.3 Changes in antioxidant activity during vinification

In addition to analysing the phenolic content of each sample, the ESR-derived antioxidant capacity was also determined. The correlation between the antioxidant activity and the extraction of each phenolic was assessed statistically using Pearson correlations (Tables 5.8 to 5.12).

As with the analyses of bottled wines, a very close relationship was observed between the Folin-Ciocalteu total phenolic content and the antioxidant activity of each wine (Fig. 5.9).

5.3.1 Wine A

The total phenol content ranged from 2.8 ± 0.2 mM GAE in the juice (day 0) to 8.2 ± 0.1 mM GAE by day 9. The day 9 value is in the range found with finished wines (Tables 4.1 and 4.7). The ESR-based antioxidant activity rises concomitantly with the total phenol content, however the antioxidant activities of the unfinished wines are almost 4-fold less than a finished wine.

The antioxidant activity peaks on day 6, along with the total phenol content, derived by either the Folin-Ciocalteu assay or HPLC. This wine was racked on day 7 and moved to a larger mixing vat. The decrease in total phenolic and antioxidant activity at this point could be due to dilution with less well-extracted wines.

The antioxidant activity was very highly significantly or highly significantly correlated with all of the individual phenolic families with the exception of the total anthocyanins, $r_p = 0.674$, $p = 0.033$ (Table 5.8).

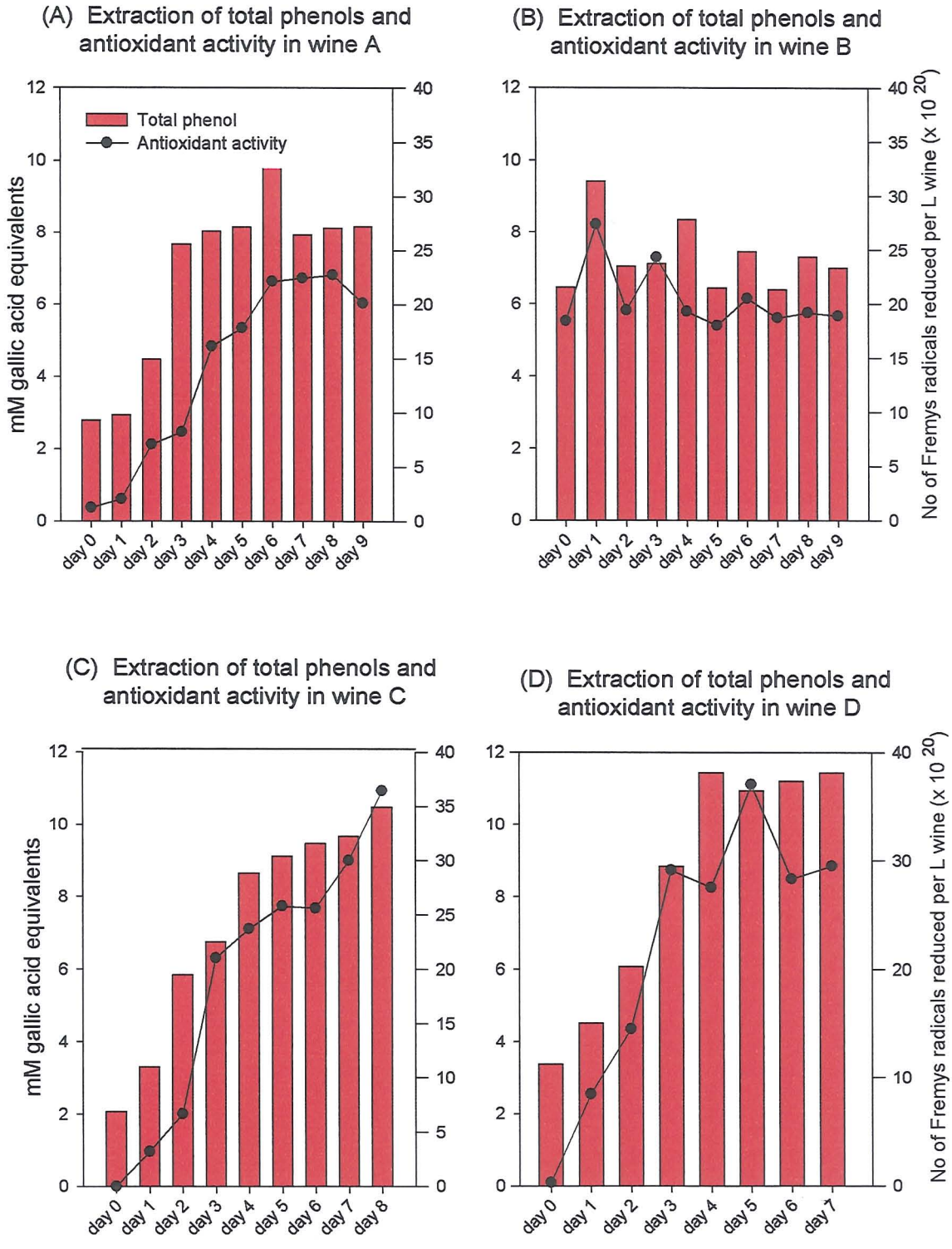


Figure 5.9. Comparison of the extraction of total phenols and antioxidant activity in wines A-D

Table 5.8. Pearson correlations between antioxidant activity and phenolic content of Chilean wine A.

Correlations (Pearson)		r_p	p
ESR antioxidant activity	F.C. phenolics	0.896	0.000***
ESR antioxidant activity	HPLC phenolics	0.959	0.000***
ESR antioxidant activity	Total flavan-3-ols	0.970	0.000***
ESR antioxidant activity	Total flavonols	0.963	0.000***
ESR antioxidant activity	Total anthocyanins	0.674	0.033*
ESR antioxidant activity	Gallic acid	0.995	0.000***
ESR antioxidant activity	Total hydroxycinn.	0.900	0.000***
ESR antioxidant activity	Total stilbenes	0.848	0.002**
F.C. phenolics	HPLC phenolics	0.957	0.000***
F.C. phenolics	Total flavan-3-ols	0.883	0.001**
F.C. phenolics	Total flavonols	0.905	0.000***
F.C. phenolics	Total anthocyanins	0.651	0.042*
F.C. phenolics	Gallic acid	0.886	0.001**
F.C. phenolics	Total hydroxycinn.	0.953	0.000***
F.C. phenolics	Total stilbenes	0.897	0.000***
HPLC phenolics	Total flavan-3-ols	0.911	0.000***
HPLC phenolics	Total flavonols	0.905	0.000***
HPLC phenolics	Total anthocyanins	0.704	0.023*
HPLC phenolics	Gallic acid	0.962	0.000***
HPLC phenolics	Total hydroxycinn.	0.976	0.000***
HPLC phenolics	Total stilbenes	0.926	0.000***

F.C. phenolics, Folin-Ciocalteu total phenolics; HPLC phenolics, HPLC derived total phenolics. Total hydroxycinn, total hydroxycinnamates.

*** very highly significant $p < 0.001$
** highly significant $0.001 < p < 0.01$
* significant $0.01 < p < 0.05$

Table 5.9. Pearson correlation between antioxidant activity and phenolic content of Chilean wine B.

Correlations (Pearson)		r_p	p
ESR antioxidant activity	F.C. phenolics	0.720	0.019*
ESR antioxidant activity	HPLC phenolics	0.466	0.175
ESR antioxidant activity	Total flavan-3-ols	-0.206	0.569
ESR antioxidant activity	Total flavonols	-0.094	0.795
ESR antioxidant activity	Total anthocyanins	-0.440	0.203
ESR antioxidant activity	Gallic acid	0.811	0.004**
ESR antioxidant activity	Total hydroxycinn.	0.488	0.152
ESR antioxidant activity	Total stilbenes	0.287	0.422
F.C. phenolics	HPLC phenolics	0.501	0.140
F.C. phenolics	Total flavan-3-ols	-0.371	0.292
F.C. phenolics	Total flavonols	0.106	0.770
F.C. phenolics	Total anthocyanins	-0.121	0.739
F.C. phenolics	Gallic acid	0.946	0.000***
F.C. phenolics	Total hydroxycinn.	0.477	0.163
F.C. phenolics	Total stilbenes	0.110	0.763
HPLC phenolics	Total flavan-3-ols	0.248	0.489
HPLC phenolics	Total flavonols	0.577	0.081
HPLC phenolics	Total anthocyanins	0.519	0.124
HPLC phenolics	Gallic acid	0.529	0.116
HPLC phenolics	Total hydroxycinn.	-0.323	0.363
HPLC phenolics	Total stilbenes	0.840	0.002**

F.C. phenolics, Folin-Ciocalteu total phenolics; HPLC phenolics, HPLC derived total phenolics. Total hydroxycinn, total hydroxycinnamates.

*** very highly significant $p < 0.001$
** highly significant $0.001 < p < 0.01$
* significant $0.01 < p < 0.05$

5.3.2 Wine B

Although the phenolic profile of wine B was erratic, the antioxidant activity was significantly correlated with the Folin-Ciocalteu derived total phenol content, $r_p = 0.720$, $p = 0.019$. This relationship is shown in Figure 5.9 (B). Even though this wine did not undergo alcoholic fermentation, its final total phenolic content and antioxidant activity are similar to those of wine A (Table 5.3).

Very few significant relationships were observed between the phenolic components and antioxidant activity of wine B (Table 5.9). The ESR antioxidant activity and the Folin-Ciocalteu total phenol content were both highly significantly correlated to the gallic acid content of the wine ($r_p = 0.811$, $p = 0.004$ and $r_p = 0.946$, $p = 0.000$ respectively). This was surprising as gallic acid is only a minor component of wine B.

5.3.3 Wine C

The total phenol content and the antioxidant activity of the wine increased steadily throughout the sampling period. Both the final Folin-Ciocalteu total phenol content and antioxidant activity were significantly higher than those achieved for wines A and B (Fig. 5.9 [C]). The antioxidant activity was undetected at day 0, and rose to $36.4 \pm 0.1 \times 10^{20}$ Fremys radicals reduced per L wine, while the Folin-Ciocalteu total phenolic content increased from 2.1 ± 0.0 mM GAE to 10.5 ± 0.3 mM GAE (Table 5.3).

The antioxidant activity was at least highly significantly correlated to all of the major phenolic families. Similar close relationships were found with Folin-Ciocalteu and HPLC total phenolic contents and each individual family (Table 5.10).

Table 5.10. Pearson correlation between antioxidant activity and phenolic content of Chilean wine C.

Correlations (Pearson)		r_p	p
ESR antioxidant activity	F.C. phenolics	0.964	0.000***
ESR antioxidant activity	HPLC phenolics	0.892	0.001**
ESR antioxidant activity	Total flavan-3-ols	0.890	0.001**
ESR antioxidant activity	Total flavonols	0.961	0.000***
ESR antioxidant activity	Total anthocyanins	0.826	0.006**
ESR antioxidant activity	Gallic acid	0.972	0.000***
ESR antioxidant activity	Total hydroxycinn.	0.931	0.000***
ESR antioxidant activity	Total stilbenes	0.886	0.001**
F.C. phenolics	HPLC phenolics	0.886	0.001**
F.C. phenolics	Total flavan-3-ols	0.857	0.003**
F.C. phenolics	Total flavonols	0.958	0.000***
F.C. phenolics	Total anthocyanins	0.798	0.01*
F.C. phenolics	Gallic acid	0.975	0.000***
F.C. phenolics	Total hydroxycinn.	0.951	0.000***
F.C. phenolics	Total stilbenes	0.897	0.001**
HPLC phenolics	Total flavan-3-ols	0.905	0.001**
HPLC phenolics	Total flavonols	0.958	0.000***
HPLC phenolics	Total anthocyanins	0.960	0.000***
HPLC phenolics	Gallic acid	0.915	0.001**
HPLC phenolics	Total hydroxycinn.	0.779	0.013*
HPLC phenolics	Total stilbenes	0.872	0.002**

F.C. phenolics, Folin-Ciocalteu total phenolics; HPLC phenolics, HPLC derived total phenolics. Total hydroxycinn, total hydroxycinnamates.

*** very highly significant $p < 0.001$
** highly significant $0.001 < p < 0.01$
* significant $0.01 < p < 0.05$

Table 5.11. Pearson correlation between antioxidant activity and phenolic content of Chilean wine D.

Correlations (Pearson)		r_p	p
ESR antioxidant activity	F.C. phenolics	0.939	0.001**
ESR antioxidant activity	HPLC phenolics	0.965	0.000***
ESR antioxidant activity	Total flavan-3-ols	0.833	0.01*
ESR antioxidant activity	Total flavonols	0.899	0.002**
ESR antioxidant activity	Total anthocyanins	-0.065	0.878
ESR antioxidant activity	Gallic acid	0.922	0.001**
ESR antioxidant activity	Total hydroxycinn.	0.897	0.003**
ESR antioxidant activity	Total stilbenes	0.333	0.420
F.C. phenolics	HPLC phenolics	0.928	0.001**
F.C. phenolics	Total flavan-3-ols	0.833	0.01*
F.C. phenolics	Total flavonols	0.983	0.000***
F.C. phenolics	Total anthocyanins	-0.044	0.918
F.C. phenolics	Gallic acid	0.993	0.000***
F.C. phenolics	Total hydroxycinn.	0.923	0.001**
F.C. phenolics	Total stilbenes	0.318	0.442
HPLC phenolics	Total flavan-3-ols	0.905	0.002**
HPLC phenolics	Total flavonols	0.905	0.002**
HPLC phenolics	Total anthocyanins	-0.014	0.975
HPLC phenolics	Gallic acid	0.924	0.001**
HPLC phenolics	Total hydroxycinn.	0.935	0.001**
HPLC phenolics	Total stilbenes	0.375	0.360

F.C. phenolics, Folin-Ciocalteu total phenolics; HPLC phenolics, HPLC derived total phenolics. Total hydroxycinn, total hydroxycinnamates.

*** very highly significant $p < 0.001$
** highly significant $0.001 < p < 0.01$
* significant $0.01 < p < 0.05$

5.3.4 Wine D

The highest Folin-Ciocalteu total phenol content was found in wine D, 11.4 ± 0.1 mM GAE, however the corresponding antioxidant activity was only 29.5 ± 0.1 x 10^{20} Fremys radicals reduced per L. Indeed the relationship between the total phenolic content and the antioxidant activity was not as close as was found with the other traditionally fermented wines A and C, r_p = 0.939, p = 0.001 (Table 5.11).

The antioxidant activity appeared to plateau around day 3, with day 5 recording an aberrant value. Indeed the anthocyanin data for this time point was abnormal and was omitted.

The antioxidant activity was significantly correlated with total flavan-3-ols, flavonols, gallic acid and total hydroxycinnamates. Similar patterns were found with both Folin-Ciocalteu and HPLC total phenolics and the individual phenolics.

5.4 Phenolic profile of Chilean wines

Several factors were investigated in this study; two grape varieties, Cabernet Sauvignon and Merlot were used, varying qualities were analysed and traditional and thermovinification techniques were studied. With so many variables it is difficult to draw definitive conclusions, but several patterns are apparent.

5.4.1 Vinification

The major influence on the phenolic content of wine appears to be the vinification method. While wines A, C and D have similar patterns for the extraction of phenolics from grapes into wine, wine B is dramatically different.

Wine B underwent thermovinification in which the wine is heated to around 60 °C for 1 h to encourage the extraction of the phenolics. In wines A, C and D

myricetin is the predominant flavonol by days 7, 8 and 9, even though quercetin is found in higher levels in grapes. However in wine B quercetin is the major flavonol. It is noted that the day 0 samples of wines A, C and D contain more quercetin than myricetin suggesting that it is the increasing alcohol content during fermentation that encourages the extraction of myricetin. The ability to selectively extract a particular flavonol could be of importance as it has been shown that certain quercetin conjugates appear to be absorbed into the bloodstream more efficiently than others (Aziz et al., 1998).

Wines A, C and D underwent alcoholic fermentation that was started on day 1. This would cause the alcohol to increase steadily until a maximum was obtained which inhibited yeast metabolism, or the wine was racked to remove the yeast. However alcoholic fermentation of wine B was not initiated during the sampling period. As there was no alcohol to increase the extraction of many compounds, the phenolic profile of the wine was relatively static over the 9 days. The only exception to this was *trans*-resveratrol. Apart from a rogue point at day 3, levels of the stilbene increased steadily from day 4 to day 9. Compared to the other wines, *trans*-resveratrol is found in much higher levels, particularly at day 0 (Fig. 5.7). This suggests that the high levels are a result of the thermovinification. This pattern was much more evident with *trans*-resveratrol glucoside (Fig. 5.8). In general levels of the glucoside are 10-fold higher in wine B than in other wines. Grapes B had a *trans*-resveratrol glucoside concentration 3-fold higher than wines A and C, but this alone cannot explain such elevated levels. Increasing the temperature during winemaking is known to encourage phenolic extraction (Ramey et al., 1986). The elevated temperatures during thermovinification may encourage the subsequent extraction of particular phenolic compounds from grape skins.

The levels of gallic acid in wine B are also considerably less than in wines A, C and D. Although the day 0 levels in wine B are significantly higher compared with the other wines, the absence of alcohol retards their extraction.

Although thermovinification is believed to encourage the extraction of colour into the wines, this colour is not provided by free anthocyanins (Fig. 5.4 [B]).

Wine B contained less of the free anthocyanins than the other wines. While wines A, C and D maintained a vibrant red colour, wine B was a dull reddish brown throughout. The vibrant hues of a young wine are attributed to free anthocyanins, however as a wine ages these compounds form larger condensation products. These complexes cause the wine to become duller and browner in colour. The methodology presently available does not allow for the identification of these condensation products, so it is not possible to confirm whether thermovinification encourages their formation and the premature chemical ageing of the wine.

5.4.2 Quality

Three levels of wine were analysed, basic, A and B, varietal, D, and reserva, C, the quality being determined by the grapes used.

Large fleshy grapes (A and B) were used for the basic wines. Their large volume ensured that a significant quantity of wines could be made, the bulk of the flesh supplying the juice. Much of the flavour and texture of a wine is due to skin-derived compounds. These basic wines would require blending with either a more concentrated wine, or one that had undergone thermovinification. Wine A had 3-fold lower levels of the skin-derived flavonols than either wines C or D. The same pattern was observed with the other skin-derived phenolics, the anthocyanins. Indeed wine C had 3-fold higher levels of total anthocyanins than wine A.

As the size of a grape decreases, its flavours become more intense. The skin:volume ratio decreases so more concentrated wines are produced (wine D). At the top end of the scale are the 'reserva' wines. The grapes had very little flesh and relatively thick skins.

5.4.3 Variety

Cabernet Sauvignon and Merlot grape varieties were investigated, however because of other influencing factors, only general patterns can be described,

and no firm conclusions can be drawn. It was noted however that in both the Merlot grapes seven anthocyanins could be quantified, compared with only 6 and 5 for grapes A and C respectively (Fig. 5.3). The anthocyanin profiles of grapes and wine have been used to detect adulteration (Mazza, 1995). The relative levels of the anthocyanins are very similar within varieties, and characteristic between varieties.

5.5 Contribution of phenolic compounds to the antioxidant activity of wine

The antioxidant activity of a wine is due to polyphenolic compounds (Sato et al., 1996; Paquay et al., 1997; Simonetti et al., 1997; Gardner et al., 1999). Removing polyphenols by precipitating them with PVPP (polyvinylpolypyrrolidone) abolishes the antioxidant activity of a wine (Paquay et al., 1997).

Studies to identify the particular phenolics responsible for the antioxidant activity have highlighted the anthocyanin and proanthocyanidin classes (Simonetti et al., 1997; Ghiselli et al., 1998). This study has found that using traditional fermentation (wines A, C and D) the content of the majority of phenolic families was correlated with the ESR-derived antioxidant activity. Contrary to other studies (Ghiselli et al., 1998) total anthocyanin levels were not found to be related to the antioxidant activity in wines A and D. The extraction of anthocyanins was rather erratic and it may be that this obscured a relationship that might have been apparent at a later date. Previous investigations of finished wines, presented in Chapter 4, found that with batch I wines there was no correlation between antioxidant activity and spectral anthocyanin content. However, with batch II wines, a significant relationship was observed between the polymeric pigment content and the antioxidant activity of New World wines ($r_s = 0.52$, $p = 0.014$).

Total flavan-3-ols, (+)-catechin and (-)-epicatechin were correlated to antioxidant activity in each of the traditionally fermented wines (A, C and D).

This is in line with other work that has shown that the catechin/proanthocyanidin fraction of a wine is responsible for its antioxidant activity (Simonetti et al., 1997).

Although the fermenting wines attained a Folin-Ciocalteu total phenolic content comparable with a finished wine, the final antioxidant activity was significantly lower than a finished wine. Finished wines ranged from 5.7 ± 0.0 and $9.2 \pm 0.0 \times 10^{21}$ Fremys radicals reduced per L wine (batch II), compared with final values of 2.0 ± 0.0, 1.9 ± 0.0, 3.6 ± 0.0 and $3.0 \pm 0.0 \times 10^{21}$ Fremys radicals reduced per L wine for wines A, B, C and D respectively.

This anomaly has been attributed to the young chemical age of the fermenting wines. While the samples may have their full complement of phenolics, they lack the complexes and condensation products that appear over time.

There has been conflicting evidence on the effect of ageing on the antioxidant activity of wines. As wine ages anthocyanins and other compounds complex and with the proanthocyanidins they contribute to the formation of tannins. A study of ageing in Spanish red wines found that older wines had a greater antioxidant activity (Larrauriet et al., 1999). This was attributed to the increase in tannins during ageing. Indeed an increase in the degree of polymerisation of polyphenols from grape seed extracts has resulted in an increase in their superoxide radical scavenging activity (Yamaguchi et al., 1999). However a comparison of young Italian wines and aged counterparts found that the young wines made with carbonic maceration, and to be consumed within three months, had a higher antioxidant activity than wine made for ageing (Pelligrini et al., 2000). There was no difference in the total phenol or flavan-3-ol content of the wines and the authors speculated that the phenolics lose antioxidant activity as they age. However the young and aged wines were vinified differently and may have had significantly altered phenolic profiles, which would contribute to differences in their antioxidant activity.

5.6 Conclusion

The phenolic content of a wine is the result of a number of viticultural and vinification factors including grape variety and quality, length and temperature of vinification and alcohol content.

Phenolic compounds are extracted grapes during traditional fermentation resulting in an increase in the antioxidant activity of the wine. It is proposed that the difference in antioxidant activity between young and old wines be due to the absence of larger condensed tannin complexes. To investigate this hypothesis further would require precise fractionation of a wine to chromatographically isolate the active compounds.

Chapter 6 Identification of the major phenolic antioxidants in fractionated red wine

6.1 Introduction

It is well established that the antioxidant activity of a wine is strongly associated with its total phenolic content (Sato et al., 1996; Paquay et al., 1997; Simonetti et al., 1997; Gardner et al., 1997). The association has been observed when the total phenolic content was determined by the Folin-Ciocalteu assay and also the combined results of individual phenolic quantified by HPLC (Chapter 4, Burns et al., 2000).

Spectral determination of the total catechin and total anthocyanin contents of wine has also been shown to be correlated with antioxidant activity (Sato et al., 1996; Simonetti et al., 1997). The spectral assays provide an estimate of the content of catechins or anthocyanins, however this approach lacks accuracy and specificity. Many of the phenolic compounds are structurally and chemically very alike and will have similar reactions, this may result in an over estimation of the content of the phenolic family in question. In addition the active compound could be a minor component of the wine and its presence could be obscured by that of a more abundant compound. These inaccuracies can be avoided by separating the phenolic compounds in a wine.

The technique of fractionation has been used to separate the major families from wine. Both solid phase extraction and liquid-liquid extraction have been utilised. Liquid-liquid extraction of wine with ethyl acetate was used to separate wine into neutral and acidic fractions (Salgoîty-Auguste and Bertrand, 1984). Poor extraction efficiency was reported for the procyanidins. This approach was adapted to separate wine into three fractions in order to investigate the antioxidant activity of a wine (Ghiselli et al., 1998). The three

fractions were reported to contain phenolic acids and quercetin-3-glucuronide; the catechins and quercetin-3-glucoside, and the anthocyanins, respectively.

Jaworski and Lee (1987) fractionated grape juice into neutral and acidic fractions using solid phase extraction. Good recoveries were obtained for *trans*-caftaric acid and *cis*- and *trans*-coutaric acids in the acidic fraction, and (+)-catechin, (-)-epicatechin and procyanidins B_2 and B_3 in the neutral fraction. However the neutral fraction was eluted with methanol which is not selective for the catechins, and the fraction also contained flavonols and anthocyanins.

This method was optimised by Oszmianski et al. (1988). Dealcoholised wine was separated into four fractions using SEP-PAK C_{18} cartridges. Fraction I contained the phenolic acids, while the neutral phenolics were selectively eluted to separate procyanidins, catechins and anthocyanin monomers (Fraction II), flavonols (Fraction III), and the polymeric anthocyanins (Fraction IV). High recoveries were observed with this method, and subsequent HPLC analysis of the fractions confirmed that the fractionation was successful.

Because of differences in polarity between phenolics within a family, fractionation, either with liquid or solid phase supports lacks selectivity. Anthocyanin monomers elute with procyanidins from SEP-PAK C_{18} cartridges (Oszamianski et al., 1988), and quercetin conjugates are found in the phenolic acid and catechin fractions after liquid-liquid extraction (Ghiselli et al., 1998).

This chapter describes the use of a novel large-scale preparative HPLC approach to separate wine phenolics on the basis of their polarity. A previous study separated the phenolic compounds from a 25 μL aliquot of 10-fold concentrated Petit Syrah red wine using an analytical HPLC column (Teissedre et al., 1996). Ten fractions were collected, corresponding to six minutes of run time. The phenolic content of each fraction was described. The three major contributors to the antioxidant activity of wine were fractions 2, 3 and 4, which contained the catechins and procyanidins.

6.2 Fractionation of wine concentrate

Prior to concentration, the content of the major phenolics in the Chilean Cabernet Sauvignon wine (1999) was quantified using the methods described in Section 2.3. The results are presented in Table 6.1.

Concentrated wine was loaded onto a preparative HPLC C_{18} column and eluted with a 40 min gradient of 5 to 40% ACN in 0.1% formic acid. The eluent was collected into sixty fractions. The phenolic content of each fraction was analysed by gradient elution HPLC as described in Section 2.3.4.

The Folin-Ciocalteu phenolic content, anthocyanin content and ESR-derived antioxidant of each fraction was determined. In addition the content of catechins of selected fraction was also quantified.

6.3 HPLC analysis of fractions

The phenolic profile of each fraction collected, and aliquots of the wash were determined by gradient elution HPLC analysis. The major phenolics in the main fractions are described in Table 6.2. Where possible the major phenolics present in each fraction were identified by comparison of retention time and spectral characteristics with an authentic standard.

If identification of the phenolics present was not possible, their likely phenolic class is described. For example fraction 29 contained four major phenolics, two of which had similar absorption spectra to anthocyanins, while two were identified as flavonol glycosides on the same basis.

Gallic acid, caffeic and caftaric acids were eluted in the early fractions along with (+)-catechin and (-)-epicatechin. Compounds with catechin-like spectra, perhaps including the procyanidins, were found in fractions 15 through to 20. Anthocyanins began to elute at this stage. The major anthocyanin present in wine is malvidin-3-glucoside, which was identified in fractions 21-28, reaching a maximum in fraction 21. Fractions 29 to 37 contained mainly

Table 6.1. Phenolic composition of Chilean Cabernet Sauvignon, Lontúe 1999.

Phenolic	Concentration	Phenolic	Concentration
Flavonols (μM aglycone)		*HPLC Anthocyanins (μM aglycone)*	
Free myricetin	21.5 ± 0.6	Del-3-glucoside	23.6 ± 0.7
Conj. myricetin	107.2 ± 7.6	Pet-3-glucoside	21.1 ± 0.6
Total myricetin	128.7 ± 8.0	Mv-3-glucoside	231.1 ± 1.9
Free quercetin	11.9 ± 0.1	Mv-3-acetylG	80.7 ± 0.3
Conj. quercetin	43.1 ± 3.0	Mv-3-*p*-coumG	18.9 ± 0.6
Total quercetin	55.1 ± 3.1	TOTAL	**375.3 ± 2.0**
Free kaempferol	1.6 ± 0.0	*Spectral anthocyanins**	
Conj. kaempferol	5.2 ± 0.3	Free anthocyanins	282.9 ± 8.6
Total kaempferol	6.8 ± 0.4	Polymeric pigments	77.1 ± 8.6
Free isorhamnetin	1.4 ± 0.1	TOTAL	**360.0 ± 0.0**
Conj. isorhamnetin	15.9 ± 1.1	*Hydroxycinnamates (μM aglycone)*	
Total isorhamnetin	17.3 ± 1.2	Caftaric acid	37.0 ± 0.4
TOTAL	**207.9 ± 12.4**	Free caffeic acid	20.0 ± 1.0
Flavan-3-ols (μM aglycone)		Free *p*-coumaric acid	27.2 ± 1.4
(+)-catechin	104.3 ± 4.8	Conj. *p*-coumaric acid	62.1 ± 1.3
(-)-epicatechin	63.9 ± 2.3	Total *p*-coumaric acid	89.3 ± 1.2
TOTAL	168.3 ± 7.1	TOTAL	**126.3 ± 1.5**
Gallic acid (μM aglycone)	**215.4 + 1.1**		
Stilbenes (μM aglycone)		*HPLC total phenolics*	**1120.3 ± 14.1**
trans-resveratrol	13.6 ± 0.4		
cis-resveratrol	13.6 ± 0.4	*Folin-Ciocalteu total phenolics (mM GAE)*	**10.52 ± 0.12**
TOTAL	**27.2 ± 0.8**		

All data expressed as μM aglycone ± SEM except * quantified as μM malvidin-3-glucoside equivalents. Del-3-G, delphinidin-3-glucoside; Pet-3-glucoside, petunidin-3-glucoside; Mv-3-glucoside, malvidin-3-*O*-glucoside; Mv-3-acetylG, malvidin-3-*O*-(6-*O*-acetyl)glucoside; Mv-3-*p*-coumG, malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside.

Table 6.2. Major phenolics in main fractions.

fraction	Rt (minutes)	λmax (nm)	ABU† @ λ	putative phenolic
2	3.93	310	0.6 @ 313	UNK
3 / 4	5.98	270	1.6 @ 280	gallic acid
5	13.41	327.8	0.6 @ 313	UNK
6	14.47	325	2.5 @ 313	UNK
7	14.47	329	0.28 @ 313	* caftaric acid
	15.04	278	0.46 @ 280	* (+)-catechin
8 / 9 /10	14.47	329	0.20 @ 313	* caftaric acid
/11	14.96	276	0.30 @ 280	(+)-catechin
	18.12	314.7	0.25 @ 313	UNK
12	18.45	275.6	0.09 @ 280	* (-)-epicatechin
13	17.79	324.3	0.20 @ 313	* caffeic acid
14	16.63	523.7	0.25 @ 520	anthocyanin
	17.80	324.3	0.80 @ 313	* caffeic acid
	18.12	314.7	0.40 @ 313	UNK
15	16.63	523.7	0.40 @ 520	anthocyanin
	17.80	324.3	0.30 @ 313	* caffeic acid
	18.87	275.6	0.32 @ 280	catechin like
16	16.66	523.7	0.12 @ 520	anthocyanin
18	18.61	515.2	0.50 @ 520	anthocyanin
	19.28	526.7	0.17 @ 520	anthocyanin
	23.00	272.1	0.22 @ 280	UNK
19	19.27	524.9	0.50 @ 520	anthocyanin
	21.22	278.0	0.80 @ 280	catechin like
20	19.29	526.0	0.15 @ 520	anthocyanin
	19.90	526.0	0.05 @ 520	" "
	21.28	516.4	0.21 @ 520	" "
	21.23	278.0	1.00 @ 280	catechin like
	21.71	527.0	0.20 @ 520	anthocyanin
21 / 22	21.44	524.9	1.60 @ 520	* mv 3 glucoside
	23.01	308.8	0.24 @ 313	* *p*-coumaric acid
23 / 24 /	21.47	526.0	1.40 @ 520	* mv 3 glucoside
25 / 26	21.70	355	0.35 @ 365	flavonol glycoside
27	21.31	509.1	0.14 @ 520	* mv 3 glucoside
	21.58	527.3	0.08 @ 520	anthocyanin
				* *trans*-resveratrol
	24.31	317.0	0.12 @ 313	glucoside
28	21.32	509	0.14 @ 520	* mv 3 glucoside
	22.63	527	0.06 @ 520	anthocyanin
	24.32	317.1	0.12 @ 313	* *trans*-resveratrol
				glucoside
	27.24	529	0.03 @ 520	anthocyanin

Table 6.2 cont. Major phenolics in main fractions.

fraction	Rt (minutes)	λmax (nm)	ABU† @ λ	putative phenolic
29	22.62	527	0.12 @ 520	anthocyanin
	24.58	355.2	0.07 @ 365	flavonol glycoside
	24.98	354.0	0.19 @ 365	flavonol glycoside
	27.25	531	0.02 @ 520	anthocyanin
30	23.26	511.6	0.08 @ 520	anthocyanin
	24.59	354.0	0.05 @ 365	flavonol glycoside
	24.97	354.0	0.70 @ 365	flavonol glycoside
31	24.97	354.0	0.80 @ 365	flavonol glycoside
	23.26	511.6	0.60 @ 520	anthocyanin
32	24.99	354	0.44 @ 365	flavonol glycoside
	30.39	279.2	0.14 @ 280	catechin like
33	25.02	528.6	0.14 @ 520	anthocyanin
35	27.12	n.d.		double anthocyanin peak
	27.30			
36	27.56	529.8	0.80 @ 520	anthocyanin
	27.86	346.9	0.05 @ 365	flavonol glycoside
	28.28	356.4	0.30 @ 365	flavonol glycoside
37	27.64	527.3	0.70 @ 520	anthocyanin
	28.36	357.6	0.14 @ 365	flavonol glycoside
	29.27	285.1	0.50 @ 280	UNK
38 / 39	27.64	529.8	0.40 @ 520	anthocyanin
40	32.98	315.9	0.05 @ 313	* *trans*-resveratrol
41	33.62	315.9	0.07 @ 313	* *trans*-resveratrol
	36.24	265	0.05 @ 280	UNK
46 / 47	32.39	531	0.50 @ 520	anthocyanin
48 / 49	32.22	533	0.06 @ 520	anthocyanin
	33.32	503	0.02 @ 520	anthocyanin
	36.91	369.3	0.04 @ 365	flavonol glycoside
53	35.55	505.5	0.025 @ 520	anthocyanin

† Maximum absorbance at λ in absorbance units. Where more than one fraction is included the highest maximum absorbance will be shown; * Peak assignment on the basis of retention time and spectra characteristics; mv-3-glucoside, malvidin-3-*O*-glucoside. n.d., not detected.

flavonol glycosides (the aglycones eluted after fraction 50), and anthocyanins. *Trans*-resveratrol was identified in fractions 40 and 41, with its glucoside eluting earlier in fractions 27 and 28.

A number of significant peaks were observed which has unfamiliar absorption spectra. In this case the λ max is reported and the compound(s) described as unknown.

6.4 Total phenolic content of fractions

The total phenolic content of the fractions was determined using the Folin-Ciocalteu assay (Table 6.3). Levels ranged from 0.3 ± 0.1 mM GAE in the second wash, to a maximum of 33.3 ± 1.3 mM GAE in fraction 6. The distribution of phenolics is shown in Figure 6.1. Fractions 5, 6 and 7 contain considerably higher levels of phenolic compounds than the other fractions. A second peak of phenolic content can be observed around fraction 18, with levels decreasing steadily in the following fractions.

6.5 Spectral anthocyanin content of fractions

Free anthocyanins, polymeric pigments and total anthocyanins were quantified using a spectral assay previously described (Section 2.5.2). The results are presented in Table 6.4 and shown in Figure 6.2 (A).

Anthocyanins were detected in every fraction, but not in the two organic washes. In the early fractions (1-30) the anthocyanins are found principally as free conjugates. With the exception of fractions 35 to 40 the remainder of the fractions contain mainly polymeric pigments.

Maximum levels of 357.7 ± 25.7 μM malvidin-3-glucoside equivalents were found in fraction 5. Three areas of particularly high anthocyanin are noted, fractions 19-26, 35-38 and 46+47. This pattern closely resembles the distribution of the three major malvidin conjugates found in wine, malvidin-3-

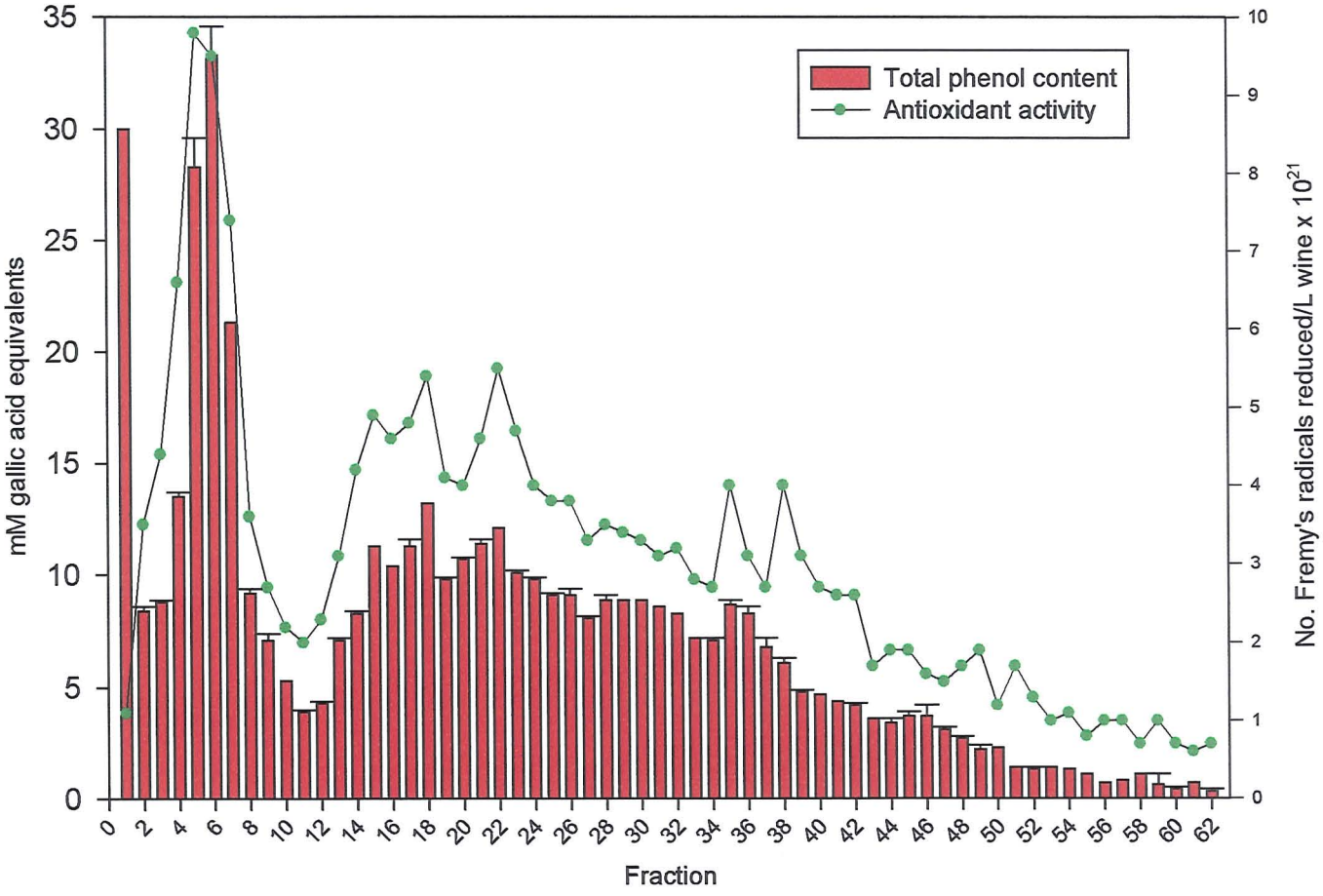


Figure 6.1. Total phenol content and antioxidant activity of fractions

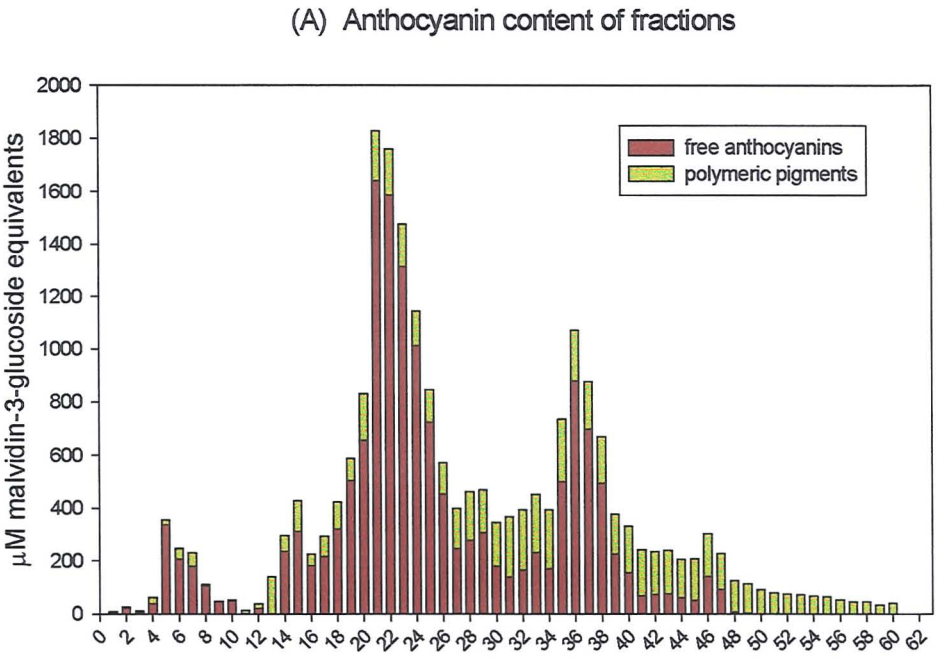
Table 6.3. Total phenol content and antioxidant activity of fractions 1-60.

fraction	Folin-Ciocalteu total phenolics[a]	ESR-derived antioxidant activity[b]
1	3.0 ± 0.0	1.1 ± 0.2
2	8.4 ± 0.2	3.5 ± 0.0
3	8.8 ± 0.1	4.4 ± 0.2
4	13.5 ± 0.2	6.6 ± 0.2
5	28.3 ± 1.3	9.8 ± 0.1
6	33.3 ± 1.3	9.5 ± 0.1
7	21.3 ± 0.0	7.4 ± 0.8
8	9.2 ± 0.2	3.6 ± 0.0
9	7.1 ± 0.3	2.7 ± 0.3
10	5.3 ± 0.0	2.2 ± 0.2
11	3.9 ± 0.1	2.0 ± 0.1
12	4.3 ± 0.1	2.3 ± 0.2
13	7.1 ± 0.1	3.1 ± 0.0
14	8.3 ± 0.1	4.2 ± 0.2
15	11.3 ± 0.0	4.9 ± 0.0
16	10.4 ± 0.0	4.6 ± 0.2
17	11.3 ± 0.3	4.8 ± 0.1
18	13.2 ± 0.0	5.4 ± 0.1
19	9.8 ± 0.1	4.1 ± 0.0
20	10.7 ± 0.1	4.0 ± 0.1
21	11.4 ± 0.2	4.6 ± 0.2
22	12.1 ± 0.0	5.5 ± 0.2
23	10.1 ± 0.1	4.7 ± 0.5
24	9.8 ± 0.1	4.0 ± 0.3
25	9.1 ± 0.1	3.8 ± 0.5
26	9.1 ± 0.3	3.8 ± 0.1
27	8.1 ± 0.1	3.3 ± 0.4
28	8.9 ± 0.2	3.5 ± 0.3
29	8.9 ± 0.0	3.4 ± 0.1
30	8.9 ± 0.0	3.3 ± 0.8

Table 6.3 cont. Total phenol content and antioxidant activity of fractions 1-60.

fraction	Folin-Ciocalteu total phenolics[a]	ESR-derived antioxidant activity[b]
31	8.6 ± 0.0	3.1 ± 0.7
32	8.3 ± 0.0	3.2 ± 0.8
33	7.2 ± 0.1	2.8 ± 0.4
34	7.1 ± 0.2	2.7 ± 0.3
35	8.7 ± 0.3	4.0 ± 0.4
36	8.3 ± 0.4	3.1 ± 0.2
37	6.8 ± 0.2	2.7 ± 0.3
38	6.1 ± 0.1	4.0 ± 0.4
39	4.8 ± 0.0	3.1 ± 0.2
40	4.7 ± 0.0	2.7 ± 0.5
41	4.4 ± 0.1	2.6 ± 0.2
42	4.2 ± 0.0	2.6 ± 0.0
43	3.6 ± 0.2	1.7 ± 0.5
44	3.4 ± 0.2	1.9 ± 0.5
45	3.7 ± 0.5	1.9 ± 0.1
46	3.7 ± 0.1	1.6 ± 0.4
47	3.1 ± 0.1	1.5 ± 0.5
48	2.7 ± 0.2	1.7 ± 0.4
49	2.2 ± 0.0	1.9 ± 0.3
50	2.3 ± 0.0	1.2 ± 0.4
51	1.4 ± 0.1	1.7 ± 0.7
52	1.3 ± 0.0	1.6 ± 0.1
53	1.4 ± 0.0	1.0 ± 0.2
54	1.3 ± 0.0	1.1 ± 0.0
55	1.1 ± 0.0	0.8 ± 0.4
56	0.7 ± 0.0	1.0 ± 0.4
57	0.8 ± 0.0	1.0 ± 0.0
58	1.1 ± 0.5	0.7 ± 0.0
59	0.6 ± 0.1	1.0 ± 0.0
60	0.4 ± 0.0	0.7 ± 0.0
wash 1	0.7 ± 0.0	0.6 ± 0.0
wash 2	0.3 ± 0.1	0.7 ± 0.0

[a]Data expressed as mM gallic acid equivalents ± SEM, n=2; [b]Antioxidant activity expressed as no. Fremy's radicals reduced/L x 10^{21} ± SEM.



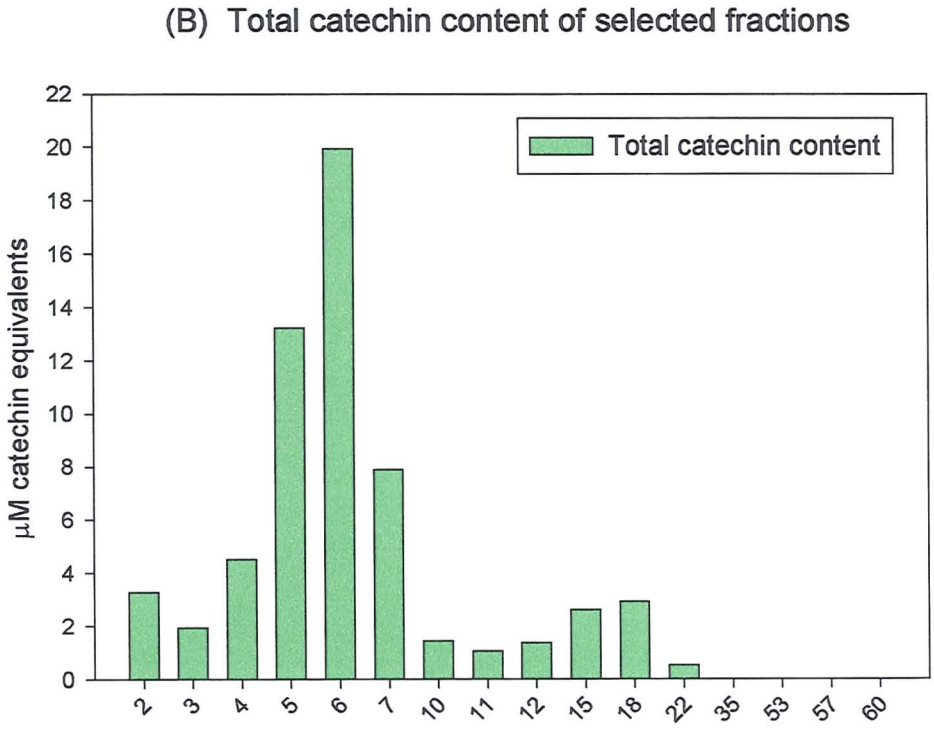


Figure 6.2. Total anthocyanin content of sixty fractions and total catechin content of selected fractions

O-glucoside, malvidin-3-*O*-(6-*O*-acetyl)glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside.

6.6 Total catechin content of selected fractions

The total catechin content of selected fractions was determined using a spectral assay previously described (Section 2.7.4). The fractions to be analysed were chosen on the basis of a combination of the total phenol content and phenolic profile. These were the fractions most likely to contain catechins.

Of the fractions analysed the highest catechin content (19.9 ± 1.0 mM (+)-catechin equivalents) was fraction 6, with fractions 5 and 7 also recording high levels (Table 6.5 and Fig. 6.2 [B]).

6.7 Total antioxidant activity

Electron-spin resonance-derived antioxidant activity was determined for each fraction (Table 6.3). The highest activity was recorded in fraction 5 (9.8 ± 0.1 x 10^{21} Fremys radicals reduced/L), while the lowest activity of 0.7 ± 0.0 x 10^{21} was found in fractions 58, 60 and wash 2.

Fractions 4 to 7 had a significantly higher antioxidant activity than the other fractions (Fig. 6.1). A second peak of antioxidant activity was observed around fraction 22. The antioxidant activity decreased steadily from fraction 22 to the final wash fraction.

6.8 Relationship between phenolic content and antioxidant activity

Analysis of whole wines has repeatedly confirmed that the antioxidant activity of a wine is closely related to its phenolic content (Sato et al., 1996; Simonetti et al., 1997; Gardner et al., 1999). This study extents the above observation to a fractionated wine. The total phenolic content of each fraction is closely

Table 6.4. Summary of total anthocyanin content in fractions 1-60.

fraction	free anthocyanins	polymeric pigments	total anthocyanins
1	4.3 ± 4.3	4.3 ± 4.3	8.6 ± 0.0
2	23.6 ± 2.1	2.1 ± 2.1	25.7 ± 0.0
3	6.4 ± 2.1	4.3 ± 0.0	10.7 ± 1.1
4	40.7 ± 2.1	21.4 ± 0.0	62.1 ± 2.1
5	338.6 ± 25.7	17.1 ± 0.0	355.7 ± 25.7
6	207.9 ± 23.6	38.6 ± 4.3	246.4 ± 19.3
7	182.1 ± 10.7	47.1 ± 0.0	229.3 ± 10.7
8	105.0 ± 6.4	6.4 ± 2.1	111.4 ± 4.3
9	47.1 ± 0.0	0.0 ± 0.0	47.1 ± 0.0
10	51.4 ± 12.9	2.1 ± 2.1	53.6 ± 10.7
11	4.3 ± 0.0	10.7 ± 2.1	15.0 ± 2.1
12	23.6 ± 10.7	15.0 ± 2.1	38.6 ± 8.6
13	n.d.	139.3 ± 2.1	117.9 ± 2.1
14	235.7 ± 12.9	60.0 ± 12.9	295.7 ± 0.0
15	310.7 ± 15.0	117.9 ± 4.3	428.6 ± 8.6
16	184.3 ± 4.3	40.7 ± 2.1	225.0 ± 2.1
17	218.6 ± 4.3	75.0 ± 2.1	293.6 ± 6.4
18	321.4 ± 4.3	100.7 ± 2.1	422.1 ± 2.1
19	505.7 ± 8.6	81.4 ± 0.0	587.1 ± 8.6
20	657.9 ± 2.1	173.6 ± 2.1	831.4 ± 0.0
21	1643.6 ± 27.9	184.3 ± 4.3	1827.9 ± 23.6
22	1587.9 ± 70.7	173.6 ± 2.1	1761.4 ± 68.6
23	1317.9 ± 27.9	158.6 ± 0.0	1476.4 ± 27.9
24	1013.6 ± 6.4	132.9 ± 0.0	1146.4 ± 6.4
25	726.4 ± 19.3	117.9 ± 2.1	844.3 ± 17.2
26	454.3 ± 4.3	117.9 ± 2.1	572.1 ± 2.1
27	246.4 ± 2.1	152.1 ± 2.1	398.6 ± 0.0
28	278.6 ± 8.6	184.3 ± 4.3	462.9 ± 4.3
29	306.4 ± 6.4	162.9 ± 0.0	469.3 ± 6.4
30	182.1 ± 2.1	165.0 ± 2.1	347.1 ± 0.0

Table 6.4 cont. Summary of total anthocyanin content in fractions 1-60.

fraction	free anthocyanins	polymeric pigments	total anthocyanins
31	139.3 ± 2.1	229.3 ± 2.1	368.6 ± 4.3
32	167.1 ± 4.3	227.1 ± 0.0	394.3 ± 4.3
33	231.4 ± 0.0	220.7 ± 2.1	452.1 ± 2.1
34	171.4 ± 47.2	222.9 ± 42.9	394.3 ± 4.3
35	501.4 ± 12.9	233.6 ± 2.1	735.0 ± 10.7
36	880.7 ± 2.1	190.7 ± 2.1	1071.4 ± 4.3
37	698.6 ± 0.0	177.9 ± 2.1	876.4 ± 2.1
38	497.1 ± 8.6	173.6 ± 2.1	670.7 ± 10.7
39	227.1 ± 4.3	150.0 ± 4.3	377.1 ± 0.0
40	156.4 ± 10.7	173.6 ± 2.1	330.0 ± 8.6
41	70.8 ± 1.1	171.4 ± 0.0	315.0 ± 2.1
42	75.0 ± 1.1	158.6 ± 0.0	310.7 ± 2.1
43	76.1 ± 0.0	162.9 ± 0.0	317.1 ± 0.0
44	63.4 ± 0.0	141.4 ± 4.3	270.0 ± 4.3
45	53.9 ± 7.4	154.3 ± 0.0	263.6 ± 15.0
46	141.6 ± 6.4	160.7 ± 6.4	447.9 ± 6.4
47	95.1 ± 2.1	132.9 ± 8.6	325.7 ± 4.3
48	8.5 ± 8.5	117.9 ± 15.0	135.0 ± 2.1
49	4.2 ± 2.1	109.3 ± 2.1	117.9 ± 2.1
50	n.d.	92.1 ± 2.1	83.6 ± 2.1
51	n.d.	79.3 ± 2.1	70.7 ± 2.1
52	n.d.	75.0 ± 6.4	57.9 ± 2.1
53	n.d.	72.9 ± 0.0	62.1 ± 2.1
54	n.d.	66.4 ± 2.1	57.9 ± 10.7
55	n.d.	64.3 ± 0.0	49.3 ± 2.1
56	n.d.	53.7 ± 2.1	36.4 ± 2.1
57	n.d.	45.0 ± 2.1	34.3 ± 4.3
58	n.d.	45.0 ± 2.1	17.1 ± 0.0
59	n.d.	34.3 ± 4.3	27.9 ± 15.0
60	n.d.	40.7 ± 2.1	36.4 ± 2.1
wash 1	n.d.	n.d.	n.d.
wash 2	n.d.	n.d.	n.d.

Data expressed as μM malvidin-3-glucoside equivalents ± SEM, n=2; n.d., not detected.

Table 6.5. Total catechin content of selected fractions.

fraction	total catechin
2	3.3 ± 0.0
3	1.9 ± 0.2
4	4.5 ± 0.0
5	13.2 ± 0.2
6	19.9 ± 1.0
7	7.9 ± 0.0
10	1.4 ± 0.0
11	1.0 ± 0.0
12	1.4 ± 0.0
15	2.6 ± 0.1
18	2.9 ± 0.0
22	0.5 ± 0.0
35	n.d.
53	n.d.
57	n.d.
60	n.d.

Data expressed as mM catechin equivalents ± SD, n=3. n.d., not detected.

correlated with the corresponding antioxidant activity (r_p = 0.967, p = 0.000). This close relationship is shown in Figure 6.1. The profile of phenolic content closely mirrors the antioxidant activity observed for each fraction. Although each fraction recorded antioxidant activity, fractions 3 to 8 had a particularly high activity and also significantly higher phenolic content compared with other wines.

The catechin content of fractions 2 to 7 was noted to be closely related to both the phenolic content (r_p = 0.954, p = 0.000) and the antioxidant activity (r_p = 0.865, p = 0.000) of each fraction (Fig. 6.3). Free anthocyanins were also reported in fractions 5, 6 and 7 using the spectral assay, however no significant anthocyanins were detected by HPLC-PDA.

The major phenolics present in the fractions were investigated by gradient HPLC analysis (Table 6.2). While gallic acid is reported in fractions 3 and 4, and caftaric acid and (+)-catechin in fractions 7 through to 11, the identity of the compound(s) in fractions 5 and 6 was uncertain

6.8.1 Identification of the major compounds in active fractions

Fractions 5 and 6 had the greatest antioxidant activity. Spectral analysis of these fractions confirmed the presence of catechins and the identity of the major compounds in the active fractions was investigated by LC-MS.

6.8.1.1 Fraction 5

The total PDA scan of fraction 5 showed the presence of only one major peak (Fig. 6.4 [A]). Its absorption spectra had a peak maximum at 321 nm but did not resemble a known compound (Fig. 6.5 [A]). The mass spectra of the unknown compound contained only one ion at 618 a.m.u which again was not recognisable (Fig 6.5 [B]). MS-MS of the main peak showed that it consisted of four major ions at 263.9, 265.9, 490.9, and 545.0 a.m.u.

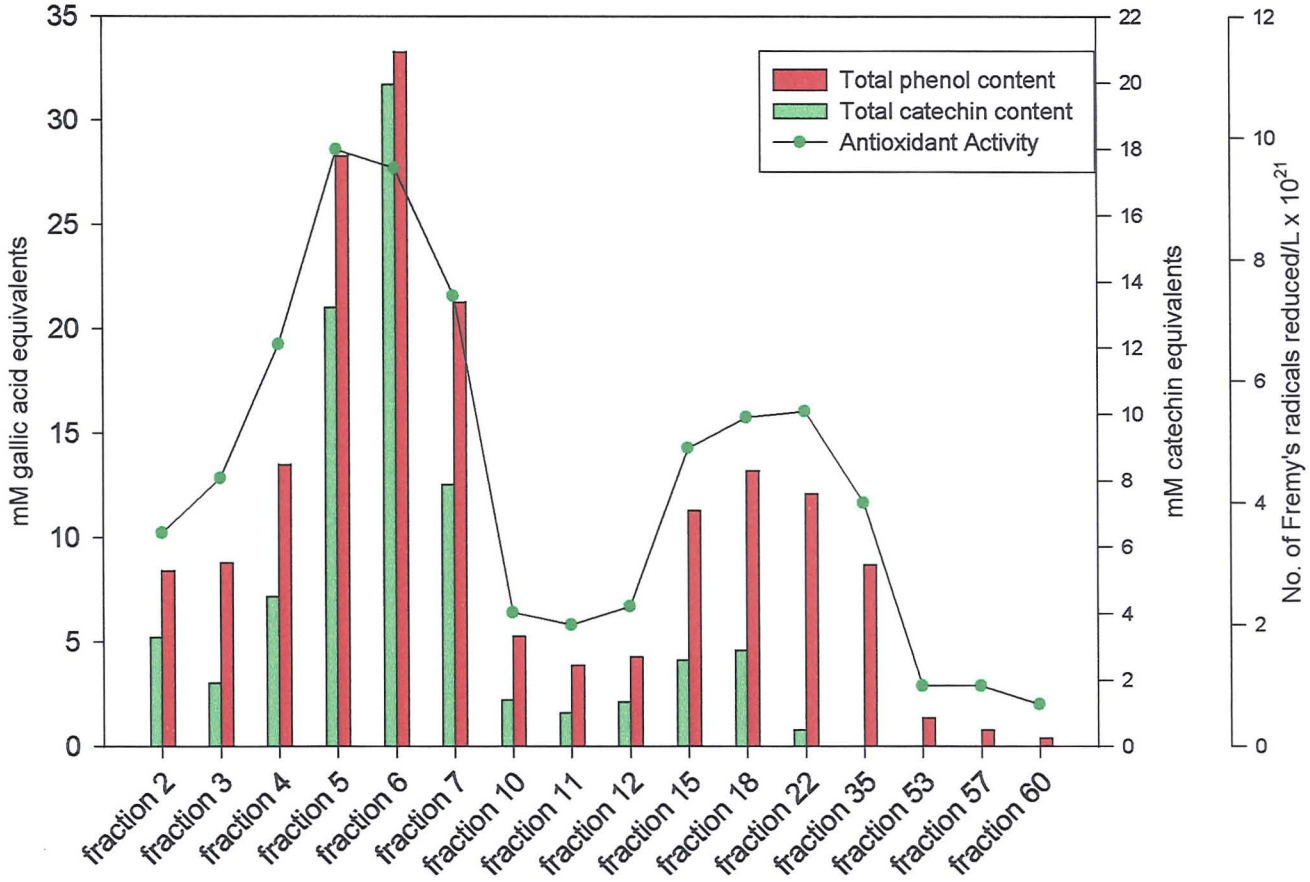


Figure 6.3. Total phenol content, total catechin content and antioxidant activity of selected fractions

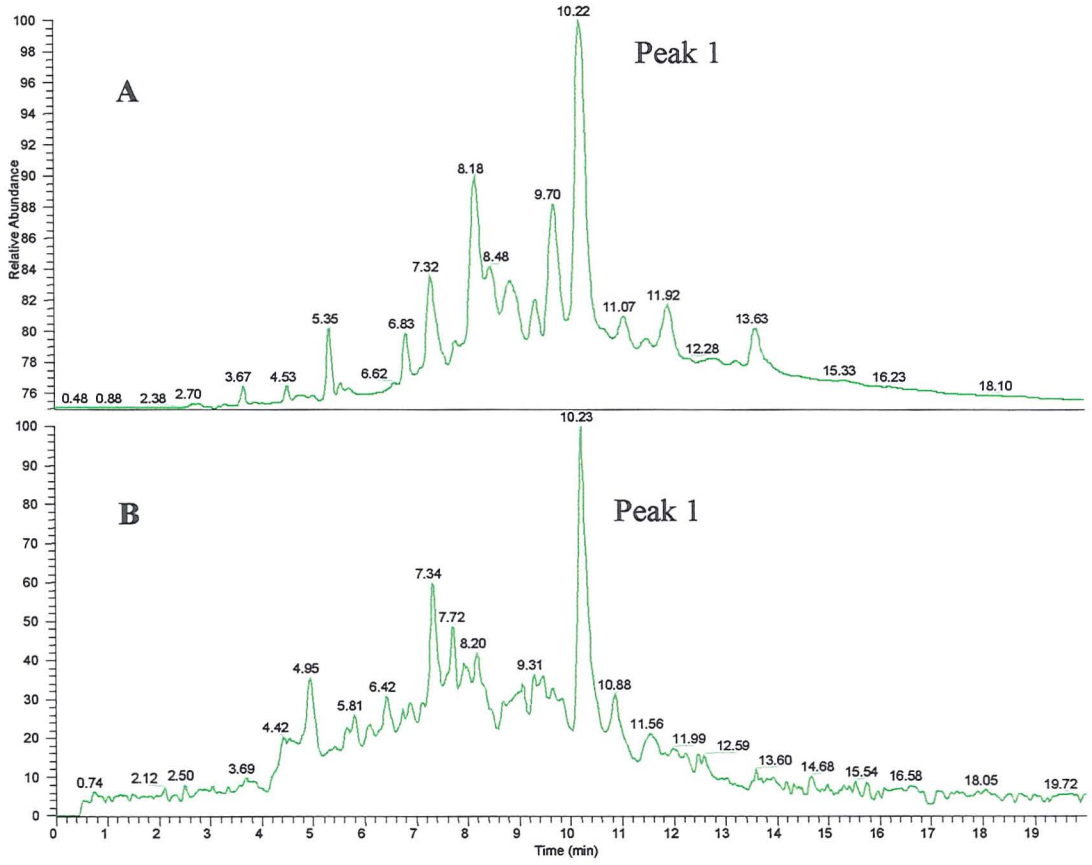


Figure 6.4. LC-MS trace of fraction 5

Analysis of unknown compounds in 20 μL volumes of fraction 5. Column; 250 x 4.6 mm i.d. C_{18} Max RP (Phenomenex). Mobile phase; 3% ACN in 1% aqueous formic acid. Flow rate; 1 mL/min. Detection; (A) total PDA scan (190 – 600 nm) and (B) total ion current (100 – 1500 a.m.u.).

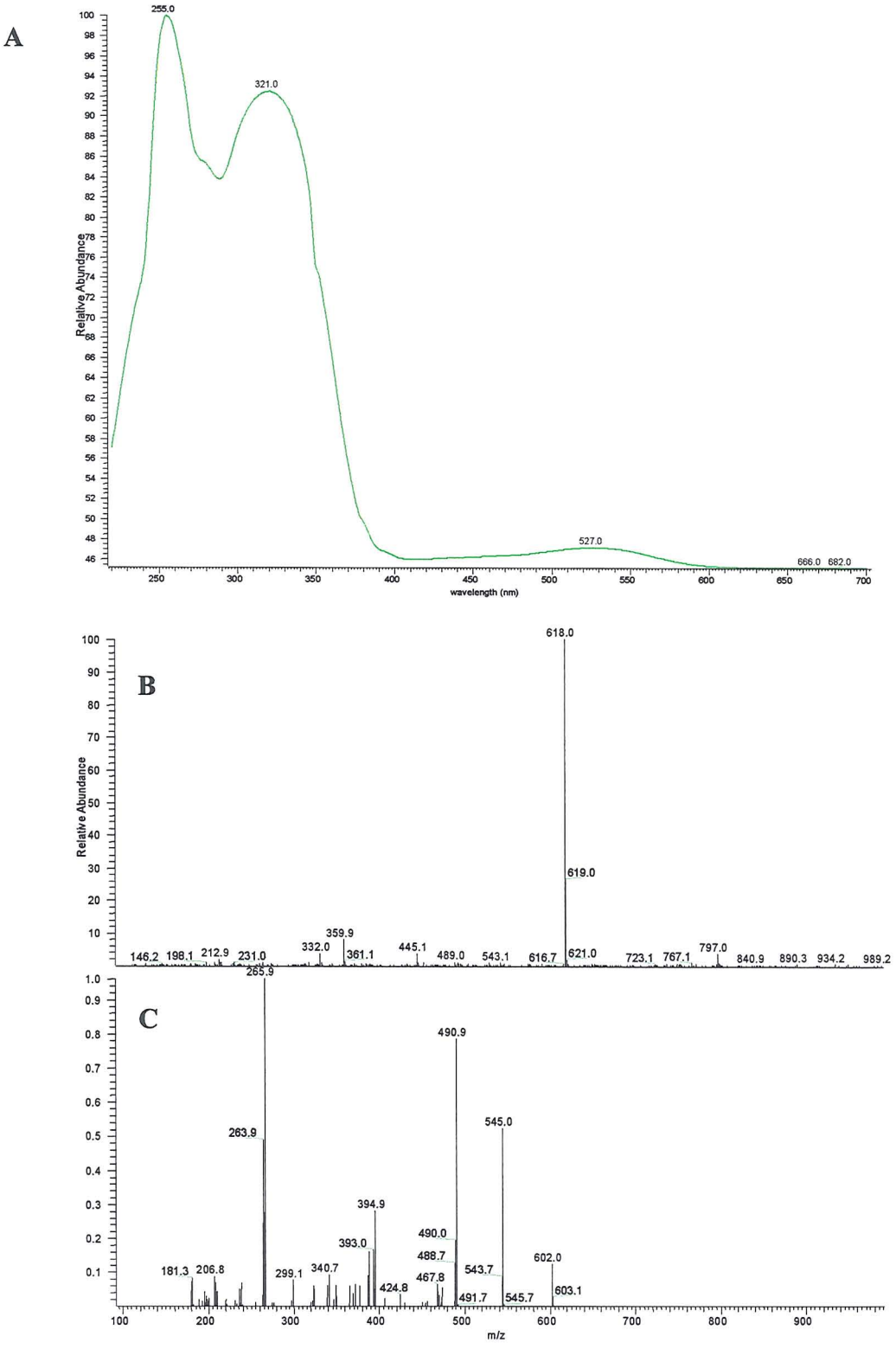


Figure 6.5. Absorbance spectra and mass spectra of peak 1 in Fraction 5

(A) Absorption spectra scan between 190 – 600 nm. (B) Positive ion MS analysis between 100 – 1500 a.m.u) with electrospray interface. (C) MS-MS analysis of 618 a.m.u. ion in (B).

6.8.1.2 Fraction 6

Fraction 6 contains two major peak (peak 2 and peak 3) with retention times of 10.20 and 13.52 min (Fig 6.6 [A]). Peak 2 in Fraction 6 has the same retention time as peak 1 in fraction 5. Comparison of their absorbance spectra and mass spectra confirm that they are identical compounds. This carry-over between fraction 5 and fraction 6 may be due to over-loading of the preparative HPLC column. This theory is also suggested by the asymmetrical peak shape observed for peak 3.

Peak 3 has a very strong absorption spectrum with a maximum at 278 nm (Fig. 6.8 [A]). This peak shape is characteristic of catechin-like compounds. The shoulder observed at around 330 nm is likely to be due to underlying peaks or tailing absorbance from surrounding compounds. The molecular ion of 579 a.m.u. suggests that the peak is likely to be a catechin dimer (Fig. 6.8 [B]). This is confirmed by the presence of the (+)-catechin and (-)-epicatechin molecular ion (290 a.m.u.) after MS-MS analysis (Fig. 6.8 [C]).

6.9 Conclusion

Wine contains four major catechin dimers, Procyanidin B_1, B_2, B_3, and B_4. They contain the basic structural units of (+)-catechin and (-)-epicatechin which are indistinguishable from their absorbance spectra and mass spectra. Pure standards of the procyanidins can not be obtained commercially and have to be extracted from grape seeds and purified. Due to the abundance of Peak 3 it is likely to be the major procyanidin found in wine, namely Procyanidin B_3 or B_4 (Carando et al., 1999).

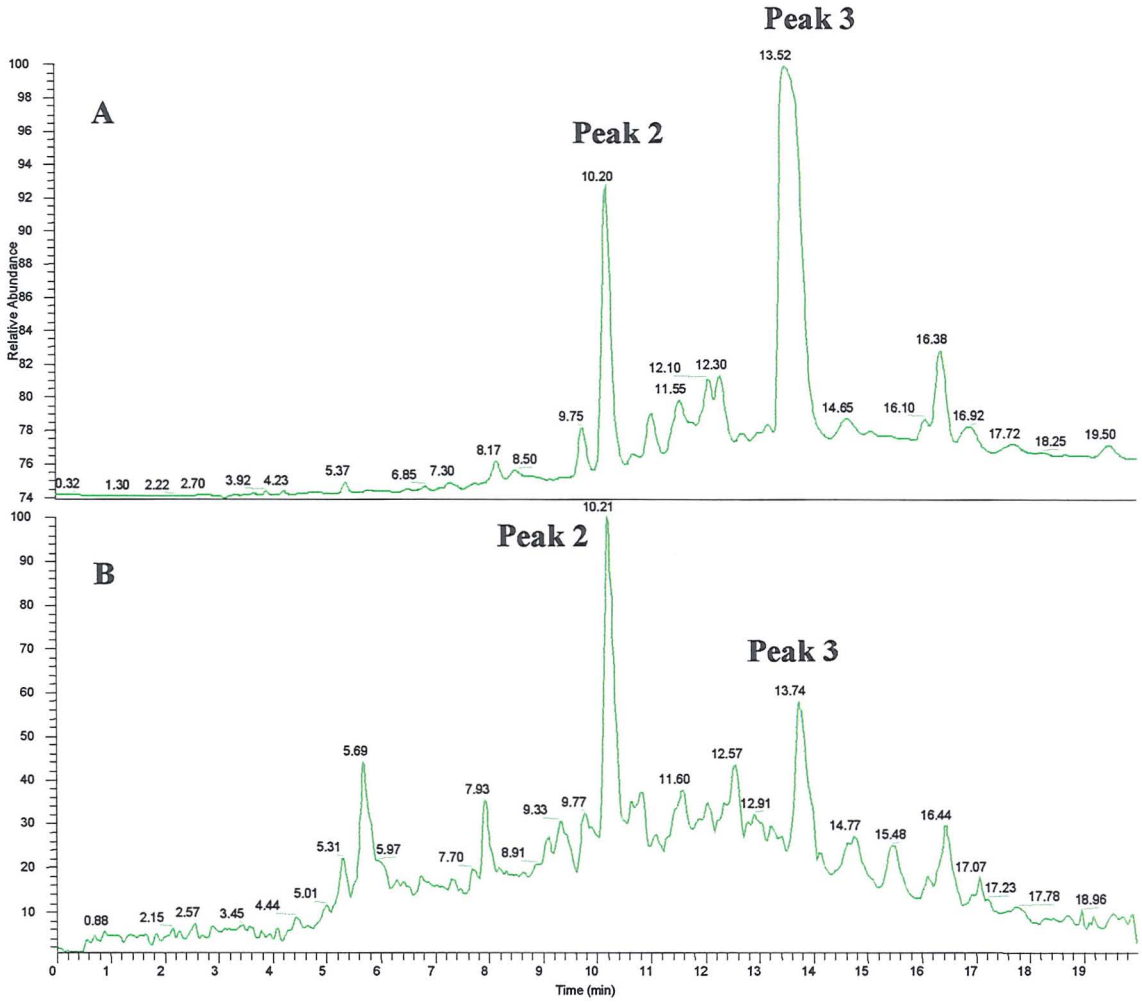


Figure 6.6. LC-MS trace of fraction 6

Analysis of unknown compounds in 20 μL volumes of fraction 6. Column; 250 x 4.6 mm i.d. C_{18} Max RP (Phenomenex). Mobile phase; 3% ACN in 1% aqueous formic acid. Flow rate; 1 mL/min. Detection; (A) total PDA scan (190 – 600 nm) and (B) total ion current (100 – 1500 a.m.u.).

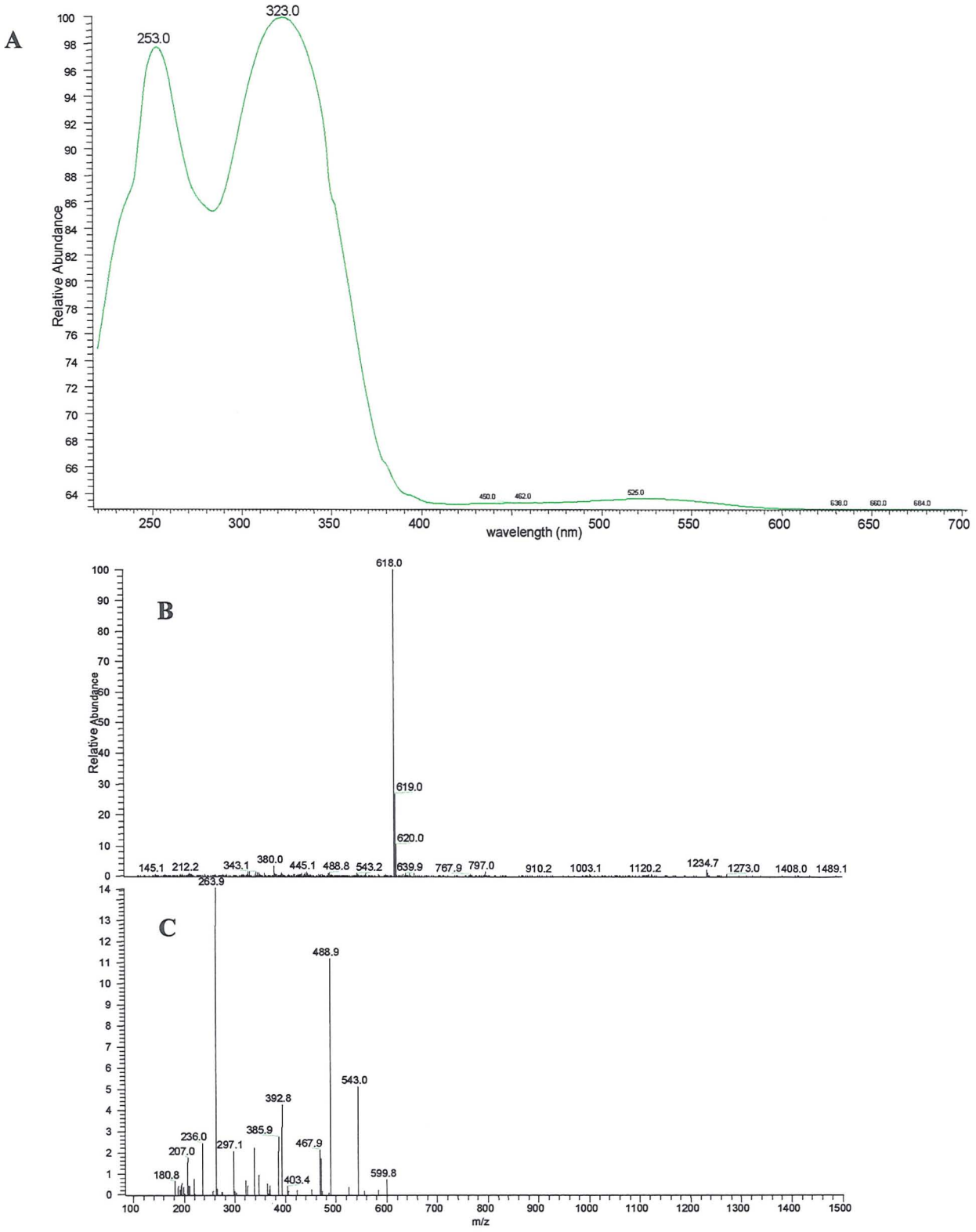


Figure 6.7. Absorbance spectra and mass spectra of peak 2 in Fraction 6

(A) Absorption spectra scan between 190 – 600 nm. (B) Positive ion MS analysis between 100 – 1500 a.m.u) with electrospray interface. (C) MS-MS analysis of 578 a.m.u. ion in (B).

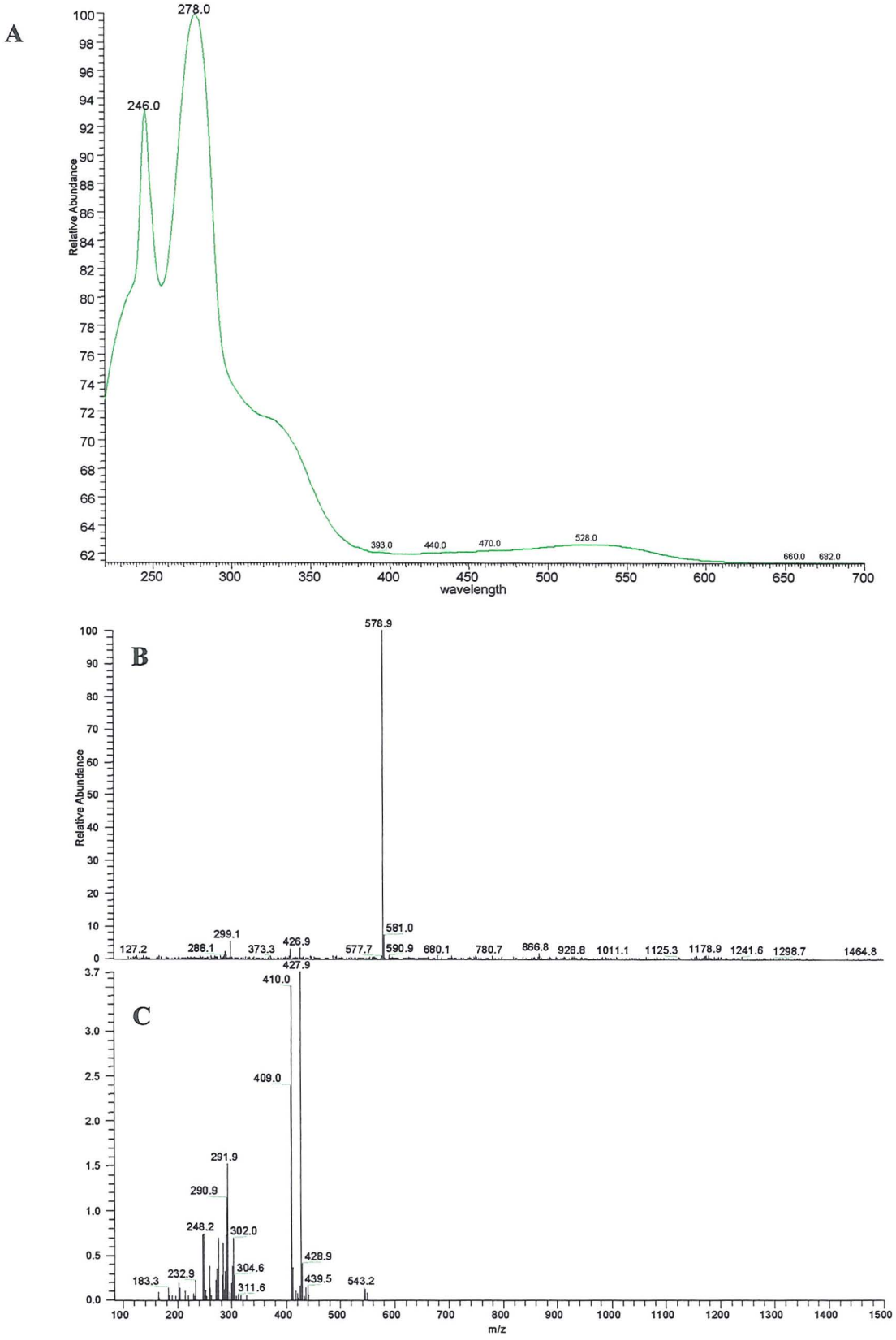


Figure 6.8. Absorbance spectra and mass spectra of peak 3 in Fraction 6

(A) Absorption spectra scan between 190 – 600 nm. (B) Positive ion MS analysis between 100 – 1500 a.m.u) with electrospray interface. (C) MS-MS analysis of 618 a.m.u. ion in (B).

Chapter 7 General Discussion

For many thousands of years man has enjoyed both making and drinking wine. It has featured heavily in the culture of many peoples from biblical times right through to the modern day. In addition to being an integral part of the diet, wine has also been used for medicinal purposes. Indeed for many hundreds of years it was safer to drink alcoholic beverages, such as wine, rather than water.

Wine consumption, particularly that of red wine, has increased over the past few decades, due in part to a growing awareness of its beneficial effects on human health. A moderate consumption of red wine has been correlated with a protection against the development of coronary heart disease (Klatsky et al., 1997). The active components are reported to be phenolic compounds. The dietary intake of food-derived phenolic compounds has been shown to decrease the incidence of cancer as well as CHD.

Phenolic compounds are well-established antioxidants. They are known to scavenge free radicals, chelate metal ions and regenerate the dietary antioxidants, vitamins C and E. Through these roles, and others unknown at present, phenolic compounds are able to inhibit the oxidation of LDL, prevent platelet aggregation and dilate blood vessels.

Red wine contains grape, wood and yeast derived phenolic compounds. The levels of phenolic compounds range from 800 mg/L for white wine to upwards of 3000 mg/L for red wines. There are two main classes of phenolics – the flavonoids and the non-flavonoids. The major flavonoids found in red wine include the flavonols myricetin, quercetin, kaempferol and isorhamnetin and their sugar conjugates; the flavan-3-ols (-)-epicatechin and (+)-catechin and the anthocyanin malvidin-3-glucoside and its conjugates. The non-flavonoid portion includes the hydroxybenzoates gallic and ellagic acids, the hydroxycinnamates caftaric, caffeic and *p*-coumaric acids and their tartrate esters, and finally the stilbenes *trans*-resveratrol and its glucoside.

This thesis reports on a series of investigations undertaken to examine the relationship between the phenolic content of red wines and musts and their antioxidant activity. By determining the major contributors to the antioxidant potential of a wine it should be possible to produce wines with an enhanced concentration of the active component(s). With increased levels of antioxidants in wine it would be possible to drink less alcohol for the same antioxidant intake. Such a development would be of interest to those people who cannot drink much alcohol for medical or social reasons.

In addition, by identifying the major wine derived antioxidants, other natural sources of these compounds can be isolated. The stilbenes *trans*-resveratrol and its glucoside have been reported to be the active ingredients in red wines responsible for the protection against CHD (Soleas et al., 1997b). Red wines contain low levels of *trans*-resveratrol and its glucoside compared with other phenolics (Burns et al., 2000). However the root of *Polygonum cuspidatum*, used in traditional Japanese and Chinese medicine, is rich in *trans*-resveratrol glucoside (Kimura et al., 1985). It could be expected that ingestion of a tea made from *Polygonum cuspidatum* would provide a significantly greater dose of the stilbenes than the corresponding amount of red wine.

7.1 Methodology for HPLC analysis

Many of the phenolic compounds present in red wines are found in very low levels. Due to their close biosynthetic relationship these compounds are alike – both structurally and chemically. As many of the detection methods used in analytical chemistry rely on exploiting a chemical feature unique to the compound of interest, the analysis of phenolic can be problematical.

The approach taken throughout this project involved the separation of phenolic compounds by HPLC and their subsequent identification and quantification using UV and fluorescence detection. Brief use was also made of mass spectrometry.

Due to their low concentrations and similarity with other phenolics, a conscious decision was taken to employ isocratic elution as opposed to a gradient. By making use of short selective isocratic runs the compound of interest can be well separated from interfering peaks, and is less likely to be lost in a congested baseline. In addition it is possible to monitor the eluent at the λ max of the compound of interest rather than at a compromise wavelength for a group of compounds. This problem can be over come, however, with the use of a PDA as opposed to a fixed wavelength detector.

The major disadvantage with the short, selective isocratic approach is the time requirements. Rather than analyse a number of different phenolics in one long gradient run, several shorter runs are necessary. However, although the use of short isocratic runs is time-consuming, this is compensated by increased selectivity and sensitivity.

It has been proposed that this approach is particularly suited to the initial analyses undertaken with a new sample (Bremner et al., 2000). As knowledge of the sample increases it may be possible to develop a suitable gradient elution method.

7.2 Phenolic content of wines

Wine is a complex fluid. It contains water, sugar, acids, alcohol and a wide range of phenolic compounds. The phenolics can be derived from grapes and wood, or be metabolites from yeasts. The phenolic profile of wine is not generic. It varies with many factors including grape variety, climate, geographical location and vinification (Soleas et al., 1997c; McDonald et al., 1998).

In an effort to understand the nature of the factors influencing levels of phenolic compounds in wine, a wide range of bottled wines were analysed. All finished wines were supplied by Safeways Stores plc and have been available for purchase. The wines were analysed in two batches. While the

first sourced primarily Old World wines, the second batch consisted of mainly New World wines.

Levels of the major individual phenolic compounds were quantified by HPLC while the total phenolic content of the wines was determined by two methods. The total of the individual phenolic compounds was calculated (HPLC-derived phenolic content) and Folin-Ciocalteu total phenolic content was estimated. In general the Folin-Ciocalteu total phenolics was ca. 10-fold greater than the HPLC derived value. This over estimation is attributed to the nature of the reagent. The Folin-Ciocalteu reagent will give a positive response to all easily oxidizable substances e.g. phenolics. The results are expressed as gallic acid equivalents which adds to the inaccuracy. It is to be expected that gallic acid will have a different response than some of the larger, more complex phenolics. However the Folin-Ciocalteu assay is widely used throughout the literature and the results obtained from both the total HPLC derived phenolics and the Folin-Ciocalteu total phenolics are very closely correlated (Chapter 4).

While intake of red wine has been reported to be beneficial to human health, the same does not appear to be true for white wine. The major difference between red and white wines is the presence of grape-skin derived phenolics in red wine. These include the flavonols, the flavan-3-ols and most notably the anthocyanins. The major phenolic present in the wines analysed differs dramatically between batch I and II. Although the wines in batches I and II represent quite different vinification methods and growing conditions, their total phenolic contents are overlapping. While the Folin-Ciocalteu total phenolics range from 6.5 mM to 18.6 mM GAE, batch II wines range from 9.3 to 17.1 mM GAE.

Batch I wines are primarily form European vineyards and included the vintages 1992 to 1997, although the majority of wines were from 1995 and 1996. It was noted that the major skin-derived phenolics in thirteen of the sixteen batch I wines were the flavan-3-ols, (+)-catechin and (-)-epicatechin. Although the compounds are found in grape skins they are also present in stem

and seed tissue. In the three remaining wines the major phenolics were the anthocyanins. In general these two families accounted for over 80% of the total skin derived phenolics (Fig. 4.4).

Examination of the second batch of wines produced quite different results. Batch II wines included wine from the New World, particularly the 1997 vintage. In this instance the major phenolics present in seventeen of the twenty-two wines were anthocyanins, with the flavan-3-ols highest in the remainder.

This striking disparity between the two batches of wines is attributed to differences in climate and vinification processed. As opposed to the climate in many of the marginal European vineyards, the Southern Hemisphere grape growing regions enjoy consistent, warm, sunny conditions. Grapes can be left to fully ripen on vines rather than being picked as soon as possible. In recent decades winemaking has become an important industry in the Southern Hemisphere. Novel vinification approaches have been implemented which have encouraged the thorough extraction of phenolics early on in the vinification process.

7.3 Evolution of phenolics during vinification

The observation that wines from the Old World had lower levels of anthocyanins compared with New World wines prompted investigations into the other influences on the eventual phenolic content of wines. Four wines were followed for the first seven to ten days of vinification, with samples also taken of the initial grapes. Two different grape varieties, Merlot and Cabernet Sauvignon, were used, comprising three grades of quality. The fieldwork for this project was undertaken in a vineyard in the Curicó region of Chile. The wines followed were all eventually commercially available either in Chile or the UK. Rather than working with small-scale experimental vinifications it was possible to sample wines that would be bought and consumed.

The phenolic content of the wine samples appeared to vary with vinification approach and grape quality and variety. Two major approaches to vinification were investigated, traditional and thermovinification. Compared with the traditional approach the thermovinified wine was heated to over 60 °C for 1 h and did not immediately undergo alcoholic fermentation. At increased temperatures phenolics are more efficiently extracted into wine from grapes (Ramey et al., 1986). Both *trans*-resveratrol and its glucoside were present in the thermovinified wines at a higher proportion than in traditionally vinified wines. The absence of alcohol in the thermovinified wine also affected the extraction of phenolics. The flavonol myricetin was not well extracted from grapes into thermovinified wine. In the other wines analysed myricetin was the major flavonol present, compared with quercetin in the thermovinified wine.

Grape quality is also an important determinant of the eventual phenolic content of a wine. With increasing quality grapes become smaller and more concentrated in terms of flavour and phenolic content. A high quality grape has a higher ratio of skin to volume than a lower quality grape. Due to the small size of the high quality grapes a much greater volume of these grapes is required in order to make the same volume of wine as would be produced from lower quality fleshier grapes. Wines made from high quality grapes have a higher content of skin derived phenolics than those made from the more diluted lower quality grapes.

It is well-established that different varieties of fruits and vegetables have varying phenolic profiles (Crozier et al., 1997b) and grapes are no exception. A comparison of both Merlot and Cabernet Sauvignon wines found that, irrespective of quality, the anthocyanins found in the two wines were different. Anthocyanin profiles are used in the authentication of wines and other anthocyanin containing products (Mazza, 1995). Likewise Pinot Noir grapes are known to be constituitively higher in catechins than other varieties (Goldberg et al., 1998a).

It is likely that given the extensive selective breeding/cloning of grape varieties it should be possible to isolate a variety that is naturally higher in a particular phenolic that has been identified as an important antioxidant. Levels of such a phenolic compound could also be manipulated through the viticultural and vinification approaches discussed above.

With the recent controversy and public fear over genetic modification of foods such approaches could yield the same eventual result but without the public hysteria.

7.4 Antioxidant activity of red wines

Phenolic compounds have been shown to be potent antioxidants in a range of assays (see Chapter 1.6). The same techniques have also been applied to determine the antioxidant activity of wines. Throughout this study use has been made Electron Spin Resonance Spectrosopy (ESR) to assess the antioxidant activity of samples.

7.4.1 Finished wines

The ESR-derived activity of the finished wines examined from batch I and II are very similar to previously published results (Gardner et al., 1999). Their activity was closely correlated with the total phenol content, derived by both the Folin-Ciocalteu assay and by the summation of individual HPLC analyses. This observation is in keeping with the established literature where the phenolic content of a sample is indicative of its antioxidant activity (Section 1.6.4).

In batch I the wine with the highest antioxidant activity was a Bulgarian Young Vatted Cabernet Sauvignon (wine 3). This had undergone extensive skin extraction prior to the initiation of fermentation. In contrast the lowest antioxidant activity was recorded in Beaujolais from France. This wine is

traditionally extracted lightly to ensure it remains fruity and can be drunk when young.

Spearman rank statistics were used to investigate the significance of the relationship between the levels of individual phenolics in a wine and its antioxidant activity. With the batch I wines the antioxidant activity was significantly correlated with gallic acid ($p = 0.024$), total stilbenes ($p = 0.013$) and total flavan-3-ols ($p = 0.014$), while batch II wines were highly significantly correlated with gallic acid ($p = 0.002$) and total flavan-3-ols ($p = 0.003$) and significantly correlated with polymeric pigments ($p = 0.014$). Gallic acid and the flavan-3-ols are the basic structural units of the hydrolysable and condensed tannin respectively. These compounds are formed and enlarged during the ageing of a wine.

7.4.2 Wines during vinification

In order to further investigate the major contributors to the antioxidant activity of a wine, the relationship between the extraction of phenolic compounds and the increasing antioxidant activity during vinification was followed. Of the four wines investigated, three had undergone traditional fermentation. There was not obvious pattern between the antioxidant activity and phenolic content. Each of the wines had a unique series of significant relationships. However in all of the traditional wines gallic acid, total flavan-3-ols, total flavonols and total hydroxycinnamates were closely correlated with the antioxidant activity of the wine.

By the final sampling day the Folin-Ciocalteu total phenol content of the musts was comparable to a finished wine (8.2 ± 0.1 mM GAE for day 9 wine A). However it was noted that the corresponding antioxidant activity was around 4-fold less than a finished wine. Although a number of the individual phenolics were correlated with antioxidant activity it is apparent that this is not the whole picture. The large difference in the antioxidant activity of the musts and the finished wine suggests that some of the condensation products formed

during the ageing of wine are important contributors to the antioxidant activity of a wine. A study of Spanish red wines found that the older wines had a greater antioxidant activity than their younger counterparts (Larrauriet et al., 1999). They attributed this change to an increase in the tannin content of a wine as it ages.

7.4.3 Fractionation of wine

As the majority of wines consumed by the wine-drinking population are likely to be moderately aged (between 1-3) years, further investigations into the nature of the antioxidant activity of wine were undertaken with a high phenolic containing finished wine in this age range. A Chilean Cabernet Sauvignon (1999) was separated into sixty fractions using a preparative HPLC system. This approach differed from those in the literature. In general wine fractionation has involved liquid-liquid extraction, or SPE techniques to isolate the major families of phenolics, e.g. the anthocyanins, flavan-3-ols or phenolic acids. In this case however wines were separated purely on the basis of their polarity.

The phenolic content of each of the fractions was investigated, and the antioxidant activity of the fraction determined. The first ten fractions to elute were observed to have the greatest antioxidant activity, with two fractions in particular (5 and 6) being particularly potent. LC-MS analysis of these fractions confirmed the presence of two major compounds in these two fractions. While the major peak in fraction 5 was unknown, the second peak in fraction 6 was identified as a procyanidin. The peak was putatively identified as one of the major procyanidins in wines i.e. B_3 or B_4. Further chemical and structural analysis is required to identify the unknown compound in fraction 5 and to confirm the identity of the procyanidin.

7.5 Conclusions

The consumption of red wine is associated with protection against the development of CHD. This protection may be mediated by the high phenolic content of red wines. A range of selective and sensitive isocratic HPLC methods was developed for the separation, identification and quantification of phenolic compounds in red wine. Using this information the relationship between the phenolic profile of a wine and its antioxidant activity was investigated in order to determine the major contributors to the antioxidant activity of red wine.

Analysis of finished wines, musts and fractionated wines have all suggested that the wine tannins have a major role to play in the antioxidant activity of a wine. In the finished bottled wines, gallic acid and the total flavan-3-ols (the basic structural units of the hydrolysable and condensed tannins) were consistently correlated with the provision of antioxidant activity. Likewise, the absence of aged tannin complexes in the musts may account for the 4-fold decrease in antioxidant activity compared with a finished wine. Finally it was noted that the major antioxidant fractions of a wine contained a flavan-3-ol dimer, procyanidin.

7.6 Future work

There has been an explosion of interest in the beneficial effects on human health over the past years. Indeed the publications emanating from this field appear to be increasing exponentially. It is a fascinating field to be working in and the possibilities for continuing research are innumerable.

7.6.1 Methodology

The technology and software associated with the analytical equipment used throughout this study is continually under development. New chromatography columns come onto the market as knowledge about the chemistry of

column/compound interaction increases. The recent addition of an ion-trap LC-MS-MS to the lab will undoubtedly provide a considerable amount of information about some of the phenolic compounds only found in trace levels, or the nature of conjugation of some compounds.

Wine contains a huge number of compounds, phenolics or otherwise and the identity of many of them remains unknown. A considerable amount of work is required to understand the structure of these unknowns, particularly the large complex tannins.

7.6.2 Investigation of the extraction of phenolics during vinification

Investigations into the extraction of phenolics during vinification were undertaken in a vineyard in Chile. The usual practice for such studies involves the use of small-scale experimental vinification vats rather than full-scale commercial vats. During this study, however, the wines analysed were produced on a large-scale for sale within Chile and for export abroad. This allowed us to examine wines that would be available to the wine-consuming population.

Working on such a large scale had its disadvantages. It was impossible to exert any control over the vinification procedure and the science naturally came second to any commercial demands. If any future studies were to be undertaken in this field the use of experimental vinifications would be an improvement. This would allow full control to be taken over conditions such as the timing of pumping over, initiation of fermentation and racking.

7.6.3 Fractionation of wine

Wine was separated into sixty fractions during the course of this study. The first ten fractions were noted to contain the majority of the antioxidant activity. By collecting and pooling these fractions they could be re-

fractionated. Once again a large number of fractions could be collected with the aim of each fraction containing only one compound. Examination of the antioxidant activity and the phenolic profile of each fraction should clarify the major antioxidants in wine.

7.6.4 Absorption and bioavailability

Extensive trials with human subjects are required to deterime the nature and extent of red wine-derived phenolic absorption, bioavailability and metabolism. The limited work carried out in this field to date relies heavily on animal models and the use of pure compounds or plant extracts rather than whole red wine.

Evidence suggests that phenolic compounds are absorbed from the diet and subsequently metabolised. After the consumption of red wine, significant levels of (+)-catechin and (+)-methylcatechin and their sulphate and glucuronide metabolites were identified in plasma (Donovan et al, 1999). Maximum levels were recorded by 1h. Similar results were observed with fruit-derived anthocyanin extracts (Tsuda et al., 1999). Cyanidin-3-glucoside and its methylated derivative were detected in both kidney and liver tissue.

A longterm program of research is required to investigate the bioavailability of red wine phenolics. This multi-stage process would involve animal studies to investigate the major metabolites and sites of reaction. A large range of organs could be collected which would never be feasible in human trials. Knowledge gained from this stage could be applied to feeding trials with human subjects. Trials with human sujects would initially involve acute dosing studies, followed by investigations into the effect of chronic intake.

These trials should enable the major phenolic compounds that are absorbed, and their active metabolites, to be isolated. The ultimate application for this project is then the production of wines enriched with these active compounds.

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Appendix

Appendix Table 1. Summary of the flavonol content of wine A.

sample	Free M	conj M	total M	free Q	conj Q	total Q	free K	conj K	total K	free I	conj I	Total I	total flavonol	% free
grape*	16.3 ± 1.4	13.3 ± 1.8	29.6 ± 3.1	2.9 ± 0.3	43.6 ± 0.3	46.5 ± 0.3	0.8 ± 0.1	6.9 ± 0.2	7.8 ± 0.1	0.8 ± 0.1	n.d.	0.8 ± 0.1	84.6 ± 3.2	22.9
juice/day 0	n.d.	n.d.	n.d.	0.2 ± 0.0	5.8 ± 0.2	6.0 ± 0.2	0.3 ± 0.0	n.d.	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	6.5 ± 0.2	9.2
day 1	n.d.	n.d.	n.d.	0.2 ± 0.0	11.5 ± 0.2	11.8 ± 0.2	0.3 ± 0.0	1.0 ± 0.1	1.3 ± 0.1	0.1 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	13.9 ± 0.3	4.3
day 2	n.d.	2.8 ± 0.2	2.8 ± 0.2	2.3 ± 0.0	16.0 ± 0.6	18.3 ± 0.6	1.1 ± 0.0	1.2 ± 0.1	2.3 ± 0.1	0.3 ± 0.0	1.4 ± 0.1	1.7 ± 0.0	25.1 ± 0.4	14.7
day 3	1.2 ± 0.0	9.8 ± 0.0	11.0 ± 0.1	3.1 ± 0.1	18.0 ± 0.4	21.1 ± 0.6	0.7 ± 0.0	2.9 ± 1.1	3.5 ± 0.1	0.4 ± 0.0	1.9 ± 0.1	2.3 ± 0.1	37.9 ± 0.8	14.2
day 4	1.7 ± 0.2	24.0 ± 1.0	25.7 ± 1.0	5.9 ± 0.3	29.7 ± 1.6	35.6 ± 1.6	1.3 ± 0.0	6.3 ± 0.5	7.6 ± 0.5	0.6 ± 0.0	4.3 ± 0.4	4.9 ± 0.4	73.9 ± 3.4	7.8
day 5	2.0 ± 0.1	35.2 ± 3.1	37.3 ± 3.0	5.9 ± 0.1	38.1 ± 0.5	44.0 ± 4.9	1.5 ± 0.1	8.1 ± 1.3	9.6 ± 1.2	0.7 ± 0.0	6.5 ± 1.0	7.2 ± 1.0	98.0 ± 10.2	10.3
day 6	4.0 ± 0.1	33.1 ± 1.4	37.0 ± 1.3	10.1 ± 0.2	28.1 ± 0.5	38.2 ± 0.3	2.2 ± 0.0	5.6 ± 0.2	7.8 ± 0.2	0.9 ± 0.1	4.6 ± 0.1	5.5 ± 0.1	88.5 ± 1.5	19.4
day 7	4.2 ± 0.1	35.5 ± 3.9	39.8 ± 3.9	6.8 ± 0.2	28.3 ± 2.6	35.1 ± 2.5	0.8 ± 0.0	3.8 ± 0.4	4.6 ± 0.3	0.7 ± 0.0	4.6 ± 0.1	4.2 ± 0.3	83.7 ± 7.0	14.9
day 8	3.8 ± 0.4	34.9 ± 19	38.7 ± 2.2	8.0 ± 0.5	30.0 ± 1.6	38.0 ± 1.3	1.0 ± 0.1	4.0 ± 0.2	5.0 ± 0.2	0.7 ± 0.0	4.3 ± 0.6	5.0 ± 0.7	86.70 ± 3.1	15.5
day 9	7.6 ± 0.2	46.8 ± 0.9	54.8 ± 1.0	9.4 ± 0.0	20.3 ± 0.6	29.7 ± 0.6	1.5 ± 0.2	2.6 ± 0.0	4.2 ± 0.1	1.1 ± 0.1	3.2 ± 0.2	3.2 ± 1.2	91.9 ± 2.2	21.3

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected. M, myricetin; Q, quercetin; K, kaempferol; I, isorhamnetin; conj, conjugated; % free, free flavonols as a % of total.

Appendix Table 2. Summary of the flavonol content of wine B.

sample	free M	conj M	total M	free Q	conj Q	total Q	free K	conj K	total K	free I	conj I	total I	total flavonol	% free
grape*	11.1 ± 0.6	n.d.	11.1 ± 0.6	5.1 ± 0.2	61.9 ± 2.7	66.9 ± 2.8	1.3 ± 0.0	10.1 ± 0.6	11.3 ± 0.6	1.1 ± 0.0	6.1 ± 0.3	7.2 ± 0.4	93.3 ± 3.5	16.5
juice/day 0	4.6 ± 0.2	n.d.	4.6 ± 0.2	5.1± 0.1	15.2 ± 0.7	20.3 ± 0.7	1.3 ± 0.0	1.0 ± 0.1	2.3 ± 0.1	1.1 ± 0.0	n.d.	1.1 ± 0.1	28.2 ± 0.9	42.9
day 1	7.8 ± 0.2	n.d.	7.8 ± 0.2	6.2 ± 0.1	18.4 ± 1.5	24.6 ± 1.5	1.4 ± 0.0	1.7 ± 0.3	3.1 ± 0.3	1.4 ± 0.0	0.4 ± 0.2	1.8 ± 0.2	37.2 ± 1.8	45.2
day 2	6.9 ± 0.2	n.d.	6.9 ± 0.2	6.0 ± 0.1	17.1 ± 0.1	23.0 ± 0.0	1.3 ± 0.1	1.6 ± 0.1	1.3 ± 0.0	1.2 ± 0.0	0.5 ± 0.0	1.7 ± 0.0	32.9 ± 0.2	46.8
day 3	6.0 ± 0.5	n.d.	6.0 ± 0.2	5.7 ± 0.2	18.1 ± 0.4	23.8 ± 0.2	1.3 ± 0.0	1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	n.d.	1.7 ± 0.1	33.5 ± 0.3	44.2
day 4	8.7 ± 0.5	n.d.	8.7 ± 0.5	12.6 ± 0.2	11.7 ± 0.9	24.3 ± 1.0	3.7 ± 0.2	n.d.	3.4 ± 0.2	1.8 ± 0.2	0.1 ± 0.1	1.9 ± 0.1	38.2 ± 1.6	70.2
day 5	5.2 ± 0.3	n.d.	5.2 ± 0.3	4.8 ± 0.0	21.0 ± 4.0	16.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.5	2.4 ± 0.0	1.8 ± 0.0	n.d.	1.6 ± 0.0	25.3 ± 0.3	50.6
day 6	7.5 ± 0.9	n.d.	7.5 ± 0.9	5.9 ± 0.7	19.8 ± 0.5	25.0 ± 0.5	1.1 ± 0.2	2.2 ± 0.0	3.1 ± 0.0	2.2 ± 0.2	0.1 ± 0.0	2.1 ± 0.1	36.8 ± 0.6	51.5
day 7	6.1 ± 0.1	n.d.	6.1 ± 0.1	5.2 ± 0.0	17.0 ± 0.9	22.1 ± 0.9	1.1 ± 0.0	1.4 ± 0.1	2.5 ± 0.2	1.2 ± 0.0	0.5 ± 0.1	1.6 ± 0.1	32.4 ± 1.2	42.0
day 8	4.7 ± 0.2	13.7 ± 0.5	18.4 ± 0.4	2.8 ± 0.1	43.1 ± 1.0	36.6 ± 0.9	0.5 ± 0.1	5.1 ± 0.3	5.6 ± 0.2	2.0 ± 0.1	2.2 ± 0.1	4.2 ± 0.1	65.1 ± 1.3	15.4
day 9	4.4 ± 0.1	14.9 ± 3.1	19.3 ± 3.2	3.9 ± 0.1	36.2 ± 1.9	40.1 ± 1.8	0.6 ± 0.0	5.1 ± 0.4	5. 7 ± 0.4	2.3 ± 0.0	2.1 ± 0.3	4.4 ± 0.3	69.4 ± 5.4	16.1

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected. M, myricetin; Q, quercetin; K, kaempferol; I, isorhamnetin; conj, conjugated; % free, free flavonols as a % of total.

Appendix Table 4. Summary of the flavonol content of wine D.

sample	free M	conj M	total M	free Q	conj Q	total Q	free K	conj K	total K	free I	conj I	total I	total flavonol	% free
grape*	29.1 ± 3.3	59.9 ± 3.7	89.0 ± 2.6	9.6 ± 0.1	151.4 ± 4.5	161.0 ± 4.6	3.4 ± 0.0	40.1 ± 0.9	43.4 ± 0.9	1.5 ± 0.1	33.0 ± 0.8	34.4 ± 0.8	327.9 ± 6.7	13.4
juice/day 0	n.d.	n.d.	n.d.	2.0 ± 0.0	11.2 ± 0.4	13.2 ± 0.4	1.3 ± 0.0	0.3 ± 0.0	1.7 ± 0.0	0.5 ± 0.0	1.1 ± 0.0	1.6 ± 0.0	16.5 ± 0.4	23.0
day 1	1.7 ± 0.1	16.2 ± 1.5	18.0 ± 1.5	2.0 ± 0.0	21.1 ± 0.1	23.0 ± 0.2	0.4 ± 0.0	3.0 ± 0.0	3.4 ± 0.0	0.4 ± 0.0	2.6 ± 0.1	3.0 ± 0.1	47.4 ± 1.8	9.5
day 2	2.5 ± 0.0	36.8 ± 2.6	39.3 ± 2.6	3.5 ± 0.0	32.2 ± 2.2	35.7 ± 2.2	0.6 ± 0.0	5.3 ± 0.3	5.9 ± 0.3	0.5 ± 0.0	4.5 ± 0.5	5.0 ± 0.5	85.9 ± 5.5	8.3
day 3	7.4 ± 0.2	64.4 ± 3.8	71.8 ± 4.0	6.4 ± 0.1	43.0 ± 2.5	49.4 ± 2.7	1.3 ± 0.0	7.6 ± 0.7	8.9 ± 0.7	0.8 ± 0.0	6.9 ± 0.9	7.7 ± 0.9	137.8 ± 8.3	11.5
day 4	6.7 ± 0.5	111.8 ± 1.5	118.5 ± 1.8	8.9 ± 0.3	61.8 ± 0.9	70.7 ± 1.2	1.9 ± 0.1	11.0 ± 0.3	12.9 ± 0.3	1.0 ± 0.0	11.1 ± 0.2	12.1 ± 0.2	214.2 ± 3.5	8.6
day 5	14.5 ± 0.3	81.6 ± 1.8	96.1 ± 1.6	10.7 ± 0.2	43.0 ± 0.9	53.7 ± 0.8	1.8 ± 0.0	7.2 ± 0.2	8.9 ± 0.2	1.1 ± 0.0	8.2 ± 0.44	9.4 ± 0.4	166.1 ± 2.8	16.9
day 6	7.3 ± 0.2	98.3 ± 3.5	105.6 ± 3.5	9.0 ± 0.1	52.9 ± 2.3	61.9 ± 2.3	1.6 ± 0.0	8.3 ± 0.7	9.9 ± 0.6	1.3 ± 0.0	12.6 ± 0.8	13.9 ± 0.8	191.3 ± 7.2	10.0
day 7	6.7 ± 0.2	83.5 ± 4.0	93.5 ± 2.4	9.3 ± 0.0	48.6 ± 2.5	57.9 ± 2.5	2.0 ± 0.0	8.1 ± 0.4	10.1 ± 0.4	1.3 ± 0.0	9.2 ± 0.6	10.6 ± 0.6	171.9 ± 2.0	11.2

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected. M, myricetin; Q, quercetin; K, kaempferol; I, isorhamnetin; conj, conjugated; % free, free flavonols as a % of total.

Appendix Table 3. Summary of the flavonol content of wine C.

sample	free M	conj M	total M	free Q	conj Q	total Q	free K	conj K	total K	free I	conj I	total I	total flavonols	% free
grape*	14.6 ± 0.3	41.4 ± 2.8	61.0 ± 5.3	3.8 ± 0.1	51.3 ± 0.4	56.6 ± 1.5	1.3 ± 0.0	11.2 ± 0.3	12.9 ± 0.4	1.0 ± 0.1	12.1 ± 0.0	13.4 ± 0.4	143.9 ± 7.4	14.2
juice/day 0	n.d.	n.d.	n.d.	0.6 ± 0.1	4.0 ± 0.0	4.6 ± 0.2	0.2 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	5.7 ± 0.1	15.8
day 1	n.d.	n.d.	22.8 ± 1.1	1.4 ± 0.1	20.3 ± 0.2	21.7 ± 0.4	0.4 ± 0.0	4.1 ± 0.2	4.5 ± 0.2	0.3 ± 0.0	3.8 ± 0.1	4.0 ± 0.1	53.3 ± 1.2	3.9
day 2	1.8 ± 0.1	21.4 ± 1.0	23.2 ± 1.0	3.0 ± 0.1	18.2 ± 0.6	21.2 ± 0.5	1.0 ± 0.0	3.5 ± 0.1	4.5 ± 0.1	0.5 ± 0.0	3.6 ± 0.0	4.1 ± 0.0	53.0 ± 0.6	11.9
day 3	8.0 ± 1.1	53.7 ± 2.6	61.7 ± 2.0	5.5 ± 0.5	28.6 ± 2.4	34.0 ± 2.2	1.4 ± 0.1	6.2 ± 0.7	7.6 ± 0.7	0.8 ± 0.1	6.1 ± 0.8	6.8 ± 0.8	110.1 ± 5.8	14.3
day 4	11.6 ± 0.6	66.9 ± 6.9	78.5 ± 7.5	7.7 ± 0.3	30.8 ± 2.2	38.5 ± 2.3	1.8 ± 0.1	6.7 ± 0.5	8.6 ± 0.6	1.3 ± 0.0	6.0 ± 0.4	7.3 ± 0.4	132.8 ± 10.5	16.9
day 5	11.1 ± 0.2	87.5 ± 6.9	98.6 ± 7.0	8.0 ± 0.0	38.6 ± 3.3	46.6 ± 3.3	2.0 ± 0.0	8.4 ± 0.8	10.4 ± 0.8	1.2 ± 0.0	7.9 ± 0.4	9.1 ± 0.4	164.7 ± 11.4	13.5
day 6	8.9 ± 0.7	114.4 ± 7.3	123.3 ± 8.0	6.9 ± 0.1	51.8 ± 3.4	58.8 ± 3.3	1.7 ± 0.1	10.8 ± 0.6	12.4 ± 0.7	1.3 ± 0.0	9.7 ± 0.5	11.0 ± 0.5	205.5 ± 12.3	9.1
day 7	10.0 ± 0.2	108.0 ± 5.6	118.2 ± 5.3	7.3 ± 0.1	46.8 ± 3.2	54.1 ± 3.2	1.6 ± 0.0	9.7 ± 0.8	11.3 ± 0.8	1.1 ± 0.0	8.6 ± 0.8	9.8 ± 0.8	193.3 ± 10.1	10.3
day 8	16.7 ± 0.4	117.6 ± 0.4	134.4 ± 0.2	10.1 ± 0.2	47.3 ± 0.2	57.4 ± 0.1	2.1 ± 0.0	9.4 ± 0.1	11.5 ± 0.1	1.4 ± 0.1	8.7 ± 0.1	10.1 ± 0.2	213.3 ± 0.5	14.2

- data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected. M, myricetin; Q, quercetin; K, kaempferol; I, isorhamnetin; conj, conjugated; % free, free flavonols as a % of total.

Appendix Table 5. Content of (+)-catechin and (-)-epicatechin in wine A.

sample	(+)-catechin	(-)-epicatechin	total	ratio (-)-epi:(+)-cat
grape*	0.5 ± 0.0	0.5 ± 0.0	0.9 ± 0.0	1.1
juice/day 0	5.1 ± 0.8	n.d.	5.1 ± 0.8	n.d.
day 1	4.9 ± 0.2	n.d.	4.9 ± 0.3	n.d.
day 2	12.6 ± 0.3	3.7 ± 0.3	16.3 ± 0.6	3.4
day 3	34.2 ± 1.0	14.3 ± 0.5	48.4 ± 1.4	2.4
day 4	40.0 ± 0.6	16.9 ± 0.3	56.8 ± 0.8	2.4
day 5	51.0 ± 2.7	21.8 ± 1.2	73.1 ± 3.9	2.4
day 6	56.5 ± 0.4	24.1 ± 0.8	80.6 ± 0.8	2.3
day 7	66.9 ± 0.2	35.7 ± 0.5	102.6 ± 0.5	2.0
day 8	64.2 ± 1.0	33.6 ± 0.46	97.8 ± 1.2	1.9
day 9	8.3 ± 1.2	28.5 ± 0.9	86.7 ± 2.1	2.0

* data expressed as μmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected.

Appendix Table 6. Content of (+)-catechin and (-)-epicatechin in wine B.

sample	(+)-catechin	(-)-epicatechin	total	ratio (-)-epi:(+)-cat
grape*	0.4 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	1.0
juice/day 0	45.7 ± 0.1	17.7 ± 0.6	63.5 ± 0.7	2.6
day 1	55.2± 0.8	24.0 ± 0.3	79.2 ± 1.1	2.3
day 2	55.2 ± 1.0	25.4 ± 0.4	80.6 ± 1.3	2.2
day 3	54.6 ± 1.7	23.8 ± 0.4	78.4 ± 1.9	2.3
day 4	44.9 ± 1.1	17.3 ± 0.8	62.2 ± 1.9	2.6
day 5	63.1 ± 1.0	33.0 ± 0.3	96.1 ± 1.2	1.9
day 6	59.5 ± 1.0	28.0 ± 0.7	87.5 ± 1.6	2.1
day 7	66.2 ± 0.6	35.9 ± 0.5	102.1 ± 0.3	1.8
day 8	65.5 ± 0.4	36.4 ± 2.5	99.5 ± 0.6	1.8
day 9	60.9 ± 0.7	32.1 ± 0.7	93.0 ± 1.3	1.9

* data expressed as μmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected.

Appendix Table 7. Content of (+)-catechin and (-)-epicatechin in wine C.

sample	(+)-catechin	(-)-epicatechin	total	ratio (-)-epi:(+)-cat
grape*	0.3 ± 000	0.3 ± 0.0	0.6 ± 0.0	1.1
juice/day 0	3.8 ± 0.0	n.d.	3.8 ± 0.0	n.d.
day 1	7.9 ± 0.2	1.0 ± 0.1	9.0 ± 0.2	7.7
day 2	11.1 ± 0.2	3.0 ± 0.2	14.1 ± 0.2	3.7
day 3	19.4 ± 0.1	5.4 ± 0.3	24.9 ± 0.2	3.6
day 4	32.8 ± 0.2	11.1 ± 0.3	43.9 ± 0.2	2.9
day 5	35.6 ± 0.1	14.1 ± 0.3	49.6 ± 0.2	2.5
day 6	40.4 ± 0.4	17.9 ± 0.3	58.2 ± 0.4	2.3
day 7	48.6 ± 0.2	33.9 ± 0.5	82.5 ± 0.5	1.4
day 8	69.1 ± 0.6	26.2 ± 0.5	95.3 ± 0.4	2.6

* data expressed as μmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected.

Appendix Table 8. Content of (+)-catechin and (-)-epicatechin in wine D.

sample	(+)-catechin	(-)-epicatechin	total	ratio (-)-epi:(+)-cat
grape*	0.6 ± 0.0	0.4 ± 0.0	1.1 ± 0.0	1.4
juice/day 0	7.7 ± 0.3	n.d.	7.7 ± 0.3	n.d.
day 1	27.0 ± 0.4	7.4 ± 0.7	34.3 ± 0.8	3.7
day 2	38.1 ± 0.4	13.6 ± 0.4	51.6 ± 0.7	2.8
day 3	31.9 ± 0.5	8.8 ± 0.2	40.7 ± 0.6	3.6
day 4	53.7 ± 0.4	15.0 ± 0.1	68.7 ± 0.4	3.6
day 5	64.1 ± 0.2	18.5 ± 0.4	82.6 ± 0.5	3.5
day 6	72.1 ± 0.6	20.6 ± 0.5	92.7 ± 0.9	3.5
day 7	64.9 ± 0.4	17.7 ± 0.5	83.6 ± 0.5	3.7

* data expressed as μmol/g grape tissue ± SEM, n=3; wine data expressed as μM ± SEM, n=3; n.d., not detected.

Appendix Table 9. Total anthocyanin content of grapes.

grape	peak 1	peak 2	peak 3	peak 4	peak 5	peak 6	peak 7	Total anthocyanins
A	0.21 ± 0.01	0.02 ± 0.00	0.13 ± 0.01	n.d.	1.20 ± 0.05	0.65 ± 0.01	0.27 ± 0.00	2.5 ± 0.1
B	0.10 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.12 ± 0.00	0.38 ± 0.00	0.14 ± 0.01	0.12 ± 0.00	1.0 ± 0.0
C	0.28 ± 0.01	n.d.	0.20 ± 0.00	n.d.	1.67 ± 0.03	0.83 ± 0.01	0.32 ± 0.00	3.3 ± 0.1
D	0.30 ± 0.00	0.12 ± 0.00	0.21 ± 0.00	0.30 ± 0.00	0.81 ± 0.01	0.23 ± 0.00	0.23 ± 0.00	2.2 ± 0.0

Data expressed as μmol anthocyanin/g grape fresh weight. Peak 1, delphinidin-3-glucoside; peak 2, cyanidin-3-glucoside; peak 3, petunidin-3-glucoside; peak 4, peonidin-3-glucoside; peak 5, malvidin-3-glucoside; peak 6, malvidin-3-acetylglucoside; peak 7, malvidin-3-p-coumaroyl glucoside. n.d., not detected.

Appendix Table 10. Content of free anthocyanins in wine A.

sample	malvidin-3-glucoside	malvidin-3-acetyl glucoside	malvidin-3-*p*-coumaroyl glucoside	total
juice/day 0	n.d.	n.d.	n.d.	n.d.
day 1	3.1 ± 0.0	1.4 ± 0.1	n.d.	4.5 ± 0.1
day 2	20.4 ± 0.5	6.8 ± 0.1	0.9 ± 0.1	28.2 ± 0.2
day 3	19.7 ± 0.1	6.5 ± 0.1	0.6 ± 0.0	26.8 ± 0.2
day 4	20.6 ± 1.5	7.8 ± 0.8	1.2 ± 0.2	29.7 ± 2.5
day 5	23.8 ± 0.5	9.1 ± 0.4	3.2 ± 0.1	36.0 ± 0.8
day 6	19.8 ± 0.3	6.3 ± 0.2	1.4 ± 0.1	27.5 ± 0.2
day 7	21.0 ± 0.2	6.8 ± 0.3	0.9 ± 0.2	28.8 ± 0.3
day 8	26.5 ± 1.0	7.9 ± 0.3	1.4 ± 0.2	35.8 ± 1.5
day 9	50.0 ± 0.4	18.1 ± 0.3	5.6 ± 0.2	73.7 ± 0.9

Results are expressed as μM ± SEM, n=3; n.d., not detected.

Appendix Table 11. Content of free anthocyanins in wine B.

sample	malvidin-3-glucoside	malvidin-3-acetyl glucoside	malvidin-3-*p*-coumaroyl glucoside	total
juice/day 0	n.d.	n.d.	n.d.	n.d.
day 1	n.d.	n.d.	n.d.	n.d.
day 2	0.6 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	1.2 ± 0.1
day 3	n.d.	n.d.	n.d.	n.d.
day 4	5.0 ± 0.5	2.2 ± 0.2	2.1 ± 0.5	9.2 ± 1.1
day 5	3.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.0	5.2 ± 0.0
day 6	5.2 ± 0.3	1.8 ± 0.1	1.5 ± 0.3	8.5 ± 0.6
day 7	4.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.2	7.3 ± 0.4
day 8	4.9 ± 0.2	1.8 ± 0.1	1.7 ± 0.0	8.4 ± 0.3
day 9	12.4 ± 0.2	4.8 ± 0.1	3.4 ± 0.1	20.6 ± 0.4

Results are expressed as μM ± SEM, n=3; n.d., not detected.

Appendix Table 12. Content of free anthocyanins in wine C.

sample	malvidin-3-glucoside	malvidin-3-acetyl glucoside	malvidin-3-*p*-coumaroyl glucoside	total
juice/day 0	2.7 ± 0.1	1.9 ± 0.0	0.3 ± 0.0	4.9 ± 0.0
day 1	19.6 ± 0.2	8.7 ± 0.1	1.2 ± 0.0	29.6 ± 0.2
day 2	15.7 ± 0.2	6.9 ± 0.2	1.0 ± 0.0	23.6 ± 0.1
day 3	42.8 ± 2.4	15.6 ± 1.1	3.9 ± 0.6	62.3 ± 4.1
day 4	57.0 ± 2.3	22.7 ± 1.3	5.5 ± 1.4	85.2 ± 4.9
day 5	39.5 ± 2.0	15.2 ± 0.8	4.1 ± 0.3	58.7 ± 3.1
day 6	132.5 ± 6.6	58.3 ± 3.6	19.1 ± 1.7	209.9 ± 11.7
day 7	91.8 ± 1.5	39.9 ± 0.6	13.3 ± 0.4	145.0 ± 2.1
day 8	149.9 ± 14.2	64.2 ± 7.6	24.9 ± 4.0	239.1 ± 25.7

Results are expressed as μM ± SEM, n=3.

Appendix Table 13. Content of free anthocyanins in wine D.

sample	malvidin-3-glucoside	malvidin-3-acetyl glucoside	malvidin-3-*p*-coumaroyl glucoside	total
juice/day 0	0.5 ± 0.1	0.4 ± 0.0	n.d.	0.8 ± 0.1
day 1	23.8 ± 1.3	11.5 ± 1.2	2.1 ± 0.5	37.4 ± 2.9
day 2	35.3 ± 5.7	16.8 ± 3.5	5.1 ± 2.0	57.2 ± 11.1
day 3	29.6 ± 2.8	9.9 ± 0.9	3.1 ± 0.7	42.7 ± 4.4
day 4	17.6 ± 1.2	6.9 ± 0.6	2.8 ± 0.2	27.3 ± 1.9
day 5	n.d.	n.d.	n.d.	n.d.
day 6	76.6 ± 5.4	7.7 ± 0.3	2.7 ± 0.3	30.9 ± 1.9
day 7	20.5 ± 1.5	7.8 ± 1.1	3.0 ± 0.5	30.0 ± 4.0

Results are expressed as μM ± SEM,n=3; n.d., not detected.

Appendix Table 14. Summary of the hydroxycinnamate content of wine A.

sample	free caffeic acid	caftaric acid	total caffeic acid	free *p*-coumaric acid	conj. *p*-coumaric acid	total *p*-coumaric acid	total hydroxy-cinnamates
grape*	n.d.	195.8 ± 0.10	195.8 ± 0.10	n.d.	149.3 ± 2.3	149.3 ± 2.3	345.2 ± 2.9
juice/day 0	n.d.	18.2 ± 0.3	21.6 ± 0.2	0.7 ± 0.1	10.7 ± 0.3	11.4 ± 0.2	33.0 ± 0.1
day 1	1.0 ± 0.0	10.1 ± 0.0	19.2 ± 0.2	2.0 ± 0.1	30.0 ± 0.7	32.0 ± 0.6	51.2 ± 0.4
day 2	2.3 ± 0.0	18.7 ± 0.1	49.2 ± 0.2	3.4 ± 0.2	77.1 ± 0.7	80.5 ± 0.7	129.7 ± 0.8
day 3	8.3 ± 0.2	61.5 ± 0.5	87.7 ± 0.1	5.2 ± 0.1	87.9 ± 0.3	93.1 ± 0.3	180.8 ± 0.4
day 4	12.1 ± 0.2	131.9 ± 0.7	150.9 ± 2.3	4.1 ± 0.1	181.7 ± 1.1	185.9 ± 1.0	336.9 ± 3.3
day 5	9.9 ± 0.1	112.1 ± 0.6	143.2 ± 0.5	4.0 ± 0.0	188.8 ± 1.2	192.8 ± 1.2	335.7 ± 0.9
day 6	13.0 ± 0.1	141.7 ± 0.4	160.1 ± 1.5	4.5 ± 0.1	236.6 ± 3.4	241.1 ± 3.3	401.2 ± 4.2
day 7	11.6 ± 0.2	114.0 ± 0.9	101.3 ± 0.2	3.5 ± 0.0	160.9 ± 0.7	164.4 ± 0.6	265.7 ± 0.8
day 8	11.7 ± 0.0	107.6 ± 0.1	113.2 ± 0.3	5.1 ± 0.5	164.1 ± 1.2	169.2 ± 1.7	282.4 ± 1.4
day 9	10.8 ± 0.1	104.4 ± 0.6	108.2 ± 0.8	3.6 ± 0.1	159.4 ± 0.8	163.3 ± 0.8	271.4 ± 0.8

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM caffeic, caftaric or *p*-coumaric acids ± SEM, n=3; conj., conjugated. n.d., not detected.

Appendix Table 15. Summary of the hydroxycinnamate content of wine B.

sample	free caffeic acid	caftaric acid	total caffeic acid	free *p*-coumaric acid	conj. *p*-coumaric acid	total *p*-coumaric acid	total hydroxy-cinnamates
grape*	n.d.	79.8 ± 33.0	79.8 ± 33.0	n.d.	74.9 ± 1.3	74.8 ± 1.3	154.6 ± 4.2
juice/day 0	n.d.	49.8 ± 0.3	95.0 ± 0.6	1.8 ± 0.0	187.6 ± 1.1	189.4 ± 1.1	284.4 ± 1.7
day 1	n.d.	73.0 ± 0.1	142 0 ± 0.6	2.4 ± 0.1	268.1 ± 2.6	270.5 ± 2.6	412.5 ± 2.6
day 2	n.d.	59.4 ± 0.3	119 1 ± 0.4	1.5 ± 0.0	214.3 ± 1.1	215.8 ± 1.1	334.8 ± 1.1
day 3	n.d.	55.3 ± 0.4	105.9 ± 0.4	1.8 ± 0.0	206.3 ± 1.2	208.0 ± 1.2	313.9 ± 1.1
day 4	n.d.	47.9 ± 0.2	80.7 ± 0.3	4.7 ± 0.0	248.1 ± 1.8	253.1 ± 1.6	333.6 ± 1.5
day 5	n.d.	58.4 ± 0.1	107.2 ± 0.5	1.1 ± 0.0	172.0 ± 0.8	173.1 ± 0.8	280.2 ± 0.8
day 6	n.d.	62.6 ± 0.2	123 6 ± 1.2	1.3 ± 0.0	209.7 ± 1.6	211.0 ± 1.6	334.5 ± 2.2
day 7	n.d.	54.2 ± 0.1	110.3 ± 0.3	0.9 ± 0.0	192.5 ± 2.1	193.4 ± 2.2	303.6 ± 1.9
day 8	n.d.	57.4 ± 0.1	105.5 ± 0.4	1.6 ± 0.1	183.6 ± 1.4	185.9 ± 1.0	290.7 ± 1.0
day 9	n.d.	61.3 ± 0.1	127.8 ± 0.1	1.1 ± 0.0	228.8 ± 2.1	229.8 ± 2.1	357.6 ± 2.0

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM caffeic, caftaric or *p*-coumaric acids ± SEM, n=3; conj., conjugated. n.d., not detected.

Appendix Table 16. Summary of the hydroxycinnamate content of wine C.

sample	free caffeic acid	caftaric acid	total caffeic acid	free *p*-coumaric acid	conj. *p*-coumaric acid	total *p*-coumaric acid	total hydroxy-cinnamates
grape*	n.d.	189.0 ± 21.5	189.0 ± 21.5	80.5 ± 21.6	100.1 ± 22.5	179.4 ± 20.7	368.3 ± 42.2
juice/day 0	2.0 ± 0.1	31.0 ± 0.2	76.9 ± 0.4	2.0 ± 0.0	23.7 ± 0.5	25.6 ± 0.5	102.5 ± 0.2
day 1	n.d.	4.5 ± 0.1	20.6 ± 0.2	1.1 ± 0.1	52.7 ± 0.4	53.8 ± 0.3	74.4 ± 0.4
day 2	n.d.	31.7 ± 0.1	80.4 ± 0.3	1.9 ± 0.1	145.4 ± 0.8	147.4 ± 0.8	227.8 ± 0.8
day 3	1.2 ± 0.2	52.4 ± 0.0	115.0 ± 0.6	2.0 ± 0.0	209.8 ± 3.7	211.7 ± 3.7	326.7 ± 4.2
day 4	1.5 ± 0.1	77.9 ± 0.4	104.4 ± 0.4	2.6 ± 0.0	195.6 ± 1.6	198.1 ± 1.6	302.6 ± 1.4
day 5	5.4 ± 0.1	92.2 ± 0.3	138.1 ± 0.1	3.5 ± 0.0	268.1 ± 2.1	271.6 ± 2.1	409.7 ± 2.0
day 6	4.8 ± 0.2	82.1 ± 2.2	125.1 ± 0.3	3.0 ± 0.1	258.0 ± 2.8	261.0 ± 2.1	386.1 ± 2.5
day 7	4.3 ± 0.2	81.5 ± 0.9	129.9 ± 0.3	2.7 ± 0.0	292.6 ± 0.1	295.4 ± 0.1	425.2 ± 0.4
day 8	3.5 ± 0.3	81.5 ± 1.6	111.0 ± 1.4	2.7 ± 0.0	272.9 ± 3.5	275.7 ± 3.5	386.7 ± 4.9

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM caffeic, caftaric or *p*-coumaric acids ± SEM, n=3; conj., conjugated. n.d., not detected.

Appendix Table 17. Summary of the hydroxycinnamate content of wine D.

sample	free caffeic acid	caftaric acid	total caffeic acid	free *p*-coumaric acid	conj. *p*-coumaric acid	total *p*-coumaric acid	total hydroxy-cinnamates
grape*	n.d.	171.9 ± 4.1	171.9 ± 4.1	n.d.	151.1 ± 2.0	151.1 ± 2.0	323.0 ± 5.6
juice/day 0	n.d.	12.9 ± 0.1	41.5 ± 0.3	2.4 ± 0.1	55.5 ± 0.3	57.8 ± 0.2	99.4 ± 0.4
day 1	n.d.	24.2 ± 0.0	60.2 ± 0.7	4.0 ± 0.2	87.1 ± 0.2	91.1 ± 0.2	151.3 ± 0.9
day 2	1.3 ± 0.1	35.5 ± 1.7	79.6 ± 0.2	3.6 ± 0.1	137.7 ± 0.6	141.2 ± 0.7	220.8 ± 0.8
day 3	1.7 ± 0.2	42.0 ± 2.2	81.2 ± 0.4	5.9 ± 0.1	177.3 ± 1.2	183.2 ± 1.1	262.3 ± 1.5
day 4	3.4 ± 0.5	46.7 ± 1.0	97.2 ± 0.4	5.9 ± 0.0	262.4 ± 0.9	268.3 ± 0.9	365.4 ± 1.3
day 5	4.1 ± 0.1	45.0 ± 0.3	83.8 ± 0.3	8.0 ± 0.1	233.4 ± 0.2	241.4 ± 0.1	325.3 ± 0.3
day 6	5.0 ± 0.3	44.0 ± 0.2	77.7 ± 0.5	6.0 ± 0.1	213.1 ± 1.3	219.1 ± 1.3	296.8 ± 1.8
day 7	5.8 ± 0.6	51.7 ± 4.0	67.9 ± 0.2	5.1 ± 0.2	185.5 ± 0.5	190.5 ± 0.5	258.4 ± 0.7

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM caffeic, caftaric or *p*-coumaric acids ± SEM, n=3; conj., conjugated. n.d., not detected.

Appendix Table 18. Content of *trans*-resveratrol and *trans*-resveratrol glucoside in grapes and wines A-D.

	wine A		wine B		wine C		wine D	
sample	***trans*-resveratrol**	***trans*-resverarol glucoside**	***trans*-resveratrol**	***trans*-resverarol glucoside**	***trans*-resveratrol**	***trans*-resverarol glucoside**	***trans*-resveratrol**	***trans*-resverarol glucoside**
grape*	2.2 ± 0.2	9.7 ± 0.5	2.4 ± 0.1	31.8 ± 1.5	n.d.	6.8 ± 0.3	n.d.	24.0 ± 1.2
juice	0.1 ± 0.0	0.4 ± 0.1	2.8 ± 0.1	35.5 ± 1.7	n.d.	2.0 ± 0.1	0.0 ± 0.0	1.6 ± 0.0
day 1	0.3 ± 0.0	2.0 ± 0.0	4.2 ± 0.1	73.8 ± 2.7	n.d.	2.1 ± 0.0	0.8 ± 0.1	2.8 ± 0.1
day 2	0.8 ± 0.1	4.7 ± 0.1	4.4 ± 0.1	54.2 ± 2.6	0.2 ± 0.0	4.2 ± 0.1	1.3 ± 0.2	4.4 ± 0.2
day 3	0.6 ± 0.1	4.2 ± 0.1	6.6 ± 0.1	87.6 ± 1.7	0.4 ± 0.1	5.3 ± 0.2	0.2 ± 0.0	3.8 ± 0.1
day 4	0.9 ± 0.0	6.1 ± 0.0	3.6 ± 0.3	60.7 ± 0.9	0.9 ± 0.2	8.2 ± 0.5	0.3 ± 0.0	6.3 ± 0.2
day 5	1.5 ± 0.3	6.5 ± 0.3	4.2 ± 0.2	54.8 ± 0.5	0.6 ± 0.1	8.8 ± 0.5	0.7 ± 0.0	4.4 ± 0.4
day 6	1.8 ± 0.1	8.2 ± 0.2	4.7 ± 0.2	67.1 ± 0.2	1.5 ± 0.4	10.2 ± 0.0	0.3 ± 0.0	4.9 ± 0.2
day 7	1.4 ± 0.2	4.0 ± 0.3	5.0 ± 0.4	73.1 ± 0.3	0.7 ± 0.0	5.9 ± 0.4	0.7 ± 0.1	5.8 ± 0.5
day 8	1.4 ± 0.3	6.1 ± 0.6	5.8 ± 0.2	65.3 ± 0.6	2.1 ± 0.2	11.7 ± 0.3	-	-
day 9	1.1 ± 0.1	5.6 ± 0.3	6.4 ± 0.1	112.0 ± 0.9	-	-	-	-

* data expressed as nmol/g grape tissue ± SEM, n=3; wine data expressed as μM *trans*-resveratrol ± SEM, n=3; n.d., not detected

Publications

(1) Burns,J., Gardner,P.T., O'Neil,J., Crawford,S., Morecroft,I., McPhail,D.B., Lister,C., Matthews,D., MacLean,M.R., Lean,M.E.J., Duthie,G.G., Crozier,A. (2000) Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines. *Journal of Agricultural and Food Chemistry*, **48**, 220-230

(2) Crozier,A., Burns,J., Aziz,A.A., Stewart,A.J., Rabiasz,H.S., Jenkins,G.I., Edwards,C.A., Lean,M.E.J. (2000) Antioxidant flavonols from fruits, vegetables and beverages: measurement and bioavailability. *Biological Research*, **33**, 78-88.

Relationship among Antioxidant Activity, Vasodilation Capacity, and Phenolic Content of Red Wines

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The relationship among antioxidant activity, based on the electron-spin resonance determination of the reduction of Fremy's radical, vasodilation activity, and phenolic content was investigated in 16 red wines. The wines were selected to provide a range of origins, grape varieties, and vinification methods. Sensitive and selective HPLC methods were used for the analysis of the major phenolics in red wine: free and conjugated myricetin, quercetin, kaempferol, and isorhamnetin; (+)-catechin, (−)-epicatechin, gallic acid, *p*-coumaric acid, caffeic acid, caftaric acid, *trans*-resveratrol, *cis*-resveratrol, and *trans*-resveratrol glucoside. Total anthocyanins were measured using a colorimetric assay. The total phenolic content of the wines was determined according to the Folin−Ciocalteu colorimetric assay and also by the cumulative measurements obtained by HPLC. The 16 wines exhibited a wide range in the values of all parameters investigated. However, the total phenol contents, measured both by HPLC and colorimetrically, correlated very strongly with the antioxidant activity and vasodilation activity. In addition, the antioxidant activity was associated with gallic acid, total resveratrol, and total catechin. In contrast, only the total anthocyanins were correlated with vasodilation activity. The results demonstrate that the different phenolic profiles of wines can produce varying antioxidant and vasodilatant activities, which opens up the possibility that some red wines may provide enhanced health benefits for the consumer.

Keywords: *Polyphenols; red wine; HPLC; vasodilation; antioxidant activity; electron spin resonance spectroscopy; Fremy's salt radical*

INTRODUCTION

The ever-increasing human and economic cost of coronary heart disease (CHD) has prompted extensive investigations into the associated risk factors, including alcohol consumption. A number of large-scale epidemiology studies have demonstrated that a moderate consumption of alcohol is associated with reduced mortality and CHD (Klatsky, 1997; Goldberg et al., 1995a). There is evidence that red wine can offer a greater protection than white wine, beer, or spirits (St Leger et al., 1979; Rimm et al., 1996), an association popularized as the "French paradox" (Renaud and de Lorgeril, 1992).

Red wine contains wood- and yeast-derived phenolics in addition to large amounts of phenolic components that originate from grapes, particularly the skins, which are removed during the vinification of white wine (Singleton, 1982). Although structurally diverse, phenolics are classified into two groups—the flavonoids and the nonflavonoids. The flavonoid family includes the flavonols myricetin (**I**; Chart 1), quercetin (**II**), kaempferol (**III**), and isorhamnetin (**IV**), which exist both as aglycons and sugar conjugates; the flavan-3-ols, (+)-catechin (**VI**) and (−)-epicatechin (**VII**); and the anthocyanins such as malvidin-3-glucoside (**VIII**). The nonflavonoids encompass gallic acid (**IX**); hydroxycinnamates, including *p*-coumaric acid (**X**), caffeic acid (**XI**), and caftaric acid (**XII**); and the stilbenes, *trans*-resveratrol (**XIII**), *cis*-resveratrol (**XIV**), and *trans*-resveratrol-*O*-*β*-glucoside (**XV**). A significant proportion of the phenolic content of wine originates from the tannins, which are subdivided into condensed and hydrolyzable tannins. The condensed tannins, known as procyanidins, are oligomers and polymers of (+)-catechin and (−)-epicatechin subunits, whereas the hydrolyzable tannins are based on gallic acid and its derivatives. Red wines

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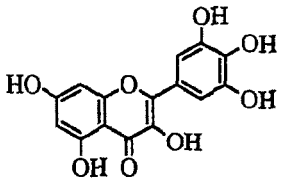
Chart 1. Structures of Myricetin, Quercetin, Kaempferol, Isorhamnetin, Morin, (+)-Catechin, (−)-Epicatechin, Malvidin 3-Glucoside, Gallic Acid, *p*-Coumaric Acid, Caffeic Acid, Caftaric Acid, *trans*-Resveratrol, *cis*-Resveratrol, and *trans*-Resveratrol *O*-β-Glucoside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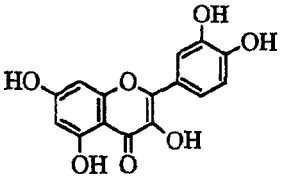
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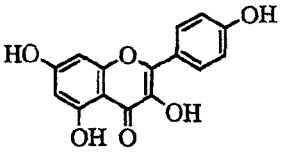
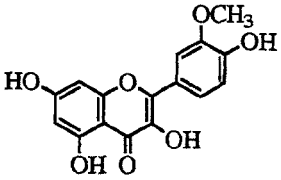

Kaempferol (III)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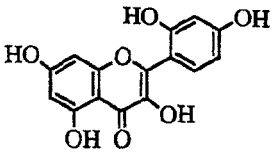
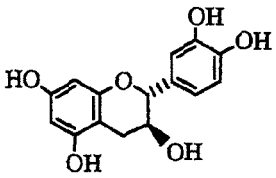
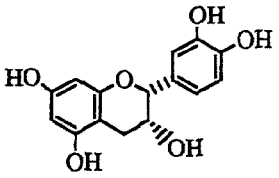
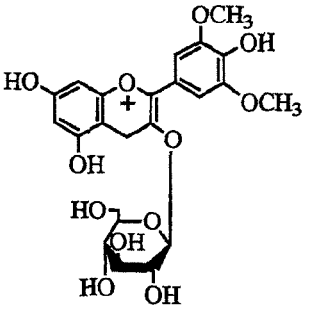

Morin (V)


(+)-Catechin (VI)


(-)-Epicatechin (VII)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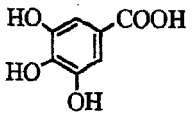
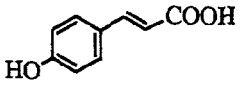

Gallic acid (IX)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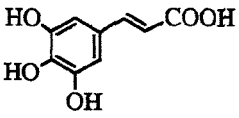
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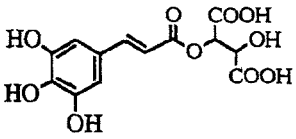
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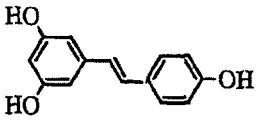
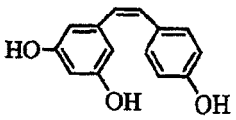
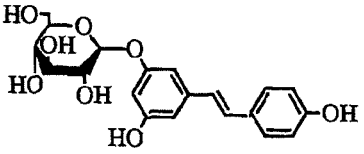

trans-Resveratrol (XIII)


cis-Resveratrol (XIV)


trans-Resveratrol-*O*-glucoside(XV)

contain, in total, 1500−2500 mg/L phenolics (Frankel et al., 1993a), although their presence and structures are affected by a number of factors including grape variety, sun exposure, vinification techniques, and aging (Price et al., 1995; McDonald et al., 1998).

Phenolics have a number of important roles to play in viticulture and enology including UV protection, disease resistance, pollination, color, and defense against predation in plants (Harborne, 1992), as well as haze formation, hue, and taste in wines (Singleton, 1982). Red wines do not contain significant amounts of vitamins or selenium, and their protective effects have been ascribed to phenolic components. It has been proposed that they act as antioxidants. The antioxidant capacity of phenolic compounds is essentially due to the ease with which a hydrogen atom from an aromatic hydroxyl group can be donated to a free radical and the ability of the phenolic moiety to support an unpaired electron due to delocalization around the π-electron system (Kanner et al., 1994). Such activity could reduce free radical-mediated oxidation of low-density lipoprotein (LDL) and so decrease artherogenicity (Frankel et al., 1993b, 1995; Fuhrman et al., 1995; Nigdikar et al., 1998). Other mechanisms have been proposed to explain the beneficial effects of red wine in the prevention of CHD including inhibition of platelet aggregation (Gryglewski et al., 1987; Pace-Asiak et al., 1995) and endothelium-dependent relaxation of blood vessels, mediated by the NO-cGMP pathway (Fitzpatrick et al., 1993).

Flavonols, such as quercetin, have been credited with being a major contributor to the antioxidant potential of red wines (Maxwell, 1997). Although members of this family of compounds do exhibit strong antioxidant activity in a variety of systems (Frankel et al., 1993a; Rice-Evans et al., 1995), they are probably not present in sufficient quantities to be considered major determinants of the total antioxidant capacity of red wines (Gardner et al., 1999). It has also been proposed that the stilbene resveratrol plays a key role in the beneficial effects of red wine (Soleas et al., 1997a), and it is known that resveratrol prevents platelet aggregation (Kimura et al., 1985; Pace-Asiak et al., 1995), inhibits the oxidation of LDL (Frankel et al., 1993b), and lessens the risk of cancer (Jang et al., 1997). However, gallic acid, (+)-catechin, (−)-epicatechin, and flavonols (Frankel et al., 1995; Lamuela-Raventós et al., 1993; Goldberg et al., 1995b; McDonald et al., 1998) are all present in red wines in far higher concentrations than resveratrol and exhibit similar, if not greater, antioxidant and antiplatelet aggregation activities than resveratrol

Table 1. Details of Red Wines Analyzed for Their Phenolic Content

	wine	principal grapes	origin	year	price
1	Chilean Cabernet Sauvignon	Cabernet Sauvignon	Lontué, Chile	1997	£3.99
2	Californian oak-aged Cabernet Sauvignon	Cabernet Sauvignon	California	1995	£4.99
3	Young Vatted Cabernet Sauvignon	Cabernet Sauvignon	Sliven, Bulgaria	1996	£3.29
4	Bulgarian matured Cabernet Sauvignon	Cabernet Sauvignon	Svischtov, Bulgaria	1992	£3.29
5	Cono Sur Pinot Noir, 20 barrels	Pinot Noir	Rapel Valley, Chile	1995	£8.99
6	Fetzer Santa Barbara Pinot Noir	Pinot Noir	California	1994	£7.29
7	Domaine Rossignol Trapet, Gevrey Chambertin	Pinot Noir	Burgundy, France	1995	£13.99
8	Villa Montes oak-aged Merlot	Merlot	Curicó, Chile	1994	£4.99
9	Merlot	Merlot	Languedoc, France	1996	£3.49
10	Cosme Palacio y Hermanos Rioja	Tempranillo	Rioja, Spain	1995	£5.99
11	Viña Albali Tempranillo	Tempranillo	Valdepeñas, Spain	1996	£2.99
12	Fetzer Vineyards Zinfandel	Zinfandel	California	1995	£5.99
13	Beaujolais	Gamy	Beaujolais, France	1996	£3.99
14	Domaine Roche Vue, Minervois	Carignon	Aude, France	1995	£3.99
15	Valpolicella	Corvina, Molinara	Veneto, Italy	1996	£3.49
16	Chianti Classico	Sangiovese, Trebbiano	Tuscany, Italy	1995	£5.75

(Frankel et al., 1993a,b, 1995; Vinson et al., 1995; Teissedre et al., 1996).

This paper reports a study with 16 red wines in which antioxidant capacity was measured by electron spin resonance (ESR) spectroscopy and parallel assessments of vasodilation capacity were also carried out. In addition, a variety of high-performance liquid chromatography (HPLC) procedures were used to identify and quantify the phenolic components present in the individual wines, and anthocyanins were determined by using a spectrophotometric method.

MATERIALS AND METHODS

Chemicals. The selected wines (Table 1) were supplied by Safeway Stores plc. Kaempferol, myricetin, quercetin, *trans*-resveratrol, (+)-catechin, (−)-epicatechin, caffeic acid, *p*-coumaric acid, morin (**V**), and gallic acid were obtained from Sigma (Poole, Dorset, U.K.), and isorhamnetin and *trans*-resveratrol-*O*-*β*-glucoside were purchased from Apin (Abingdon, Oxford, U.K.). *cis*-Resveratrol was obtained by isomerization of *trans*-resveratrol in methanol during a 12 h exposure to high white light. Dr. Creina Stockley of the Australian Wine Research Institute generously provided a sample of caftaric acid, and Dr. Rosa Lamuela-Raventós, Universitat de Barcelona, kindly supplied a sample of *Polygonum cuspidatum* root containing a high concentration of *trans*-resveratrol-*O*-*β*-glucoside. Acetylcholine chloride and phenylephrine hydrochloride were both purchased from Sigma. Ethanol (Analar grade) was obtained from BDH (Poole, Dorset, U.K.), methanol and acetonitrile were from Rathburn Chemicals (Walkerburn, U.K.), and all other chemicals and reagents were obtained from Sigma-Aldrich (Poole, Dorset, U.K.).

Measurement of Antioxidant Potential. The ability of red wines to reduce Fremy's salt (potassium nitrosodisulfonate) was measured as described by Gardner et al. (1998). The wines were diluted to 5% (v/v) with ethanol/water (12:88, v/v). Three milliliter aliquots were reacted with an equal volume of 1 mM Fremy radical in ethanol/water (12:88, v/v). The ESR spectra of the low-field resonance of the Fremy's radical were obtained after 20 min, by which time the reaction was complete. Signal intensity was obtained by double integration and concentration calculated by comparison with a control reaction using ethanol/water (12:88, v/v) without red wine. Spectra were obtained at 21 °C on a Bruker ECS 106 spectrometer equipped with a cylindrical (TM110 mode) cavity and operating at ~9.5 GHz (X-band frequency). The microwave power and modulation amplitude were set at 2 mW and 0.01 mT, respectively.

Assay of Vasodilation Capacity. New Zealand white adult rabbits (~2.5 kg) were studied. They were killed by sodium pentobarbitone (200 mg/kg), and the thoracic aortas were removed and cleaned of adhering fat and connective tissue. Each aorta was cut into rings (4−5 mm long), suspended from force displacement transducers in 10 mL organ baths, and bathed in Krebs buffer solution (pH 7.4) [composition (mM): NaCl, 118.4; $NaHCO_3$, 25; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 0.6; $CaCl_2$, 2.5; glucose, 11.0; and EDTA, 23.0] at 37 °C. The buffer was bubbled continuously with 16% O_2/5% CO_2 balanced with N_2 to give values similar to those found in vivo. Tension (2 g) was then applied to all rings. Following a 1 h equilibration period the response to 50 mM KCl was determined, followed by wash-out and further equilibration. All tissues were contracted submaximally with phenylephrine (PE; 0.1 μM), and once a stable plateau had been reached, cumulative concentration-dependent response curves (CCRCs) to the wine extracts (1−5000 μg/mL) using various dilutions of these extracts were constructed. Only one wine extract was used for each ring. All drugs and solutions were prepared in distilled water. Extracts were prepared by removing water and alcohol from the wine first by vacuum and then under nitrogen to ensure they were dry. The samples were then diluted to give an initial solution of 500 mg/mL, from which further dilutions were made. Fresh wine extract dilutions were made up daily and used within 24 h. As an index of potency, the pIC_{50} (the concentration in micrograms per milliliter at which each extract caused 50% of maximum vasodilation) for each wine extract was determined using graphical interpolation for each CCRC constructed and expressed as mean ± standard error (SE). Graphically the potency is displayed as $1/pIC_{50} \times 10^3$.

Analysis of Phenolics by HPLC. Red wines were analyzed using a Shimadzu (Kyoto, Japan) LC-10Avp series automated liquid chromatograph comprising an SCL-10Avp system controller, two LC-10ATvp pumps, an SIL-10ADvp autoinjector with sample cooler, a CTO-10Avp column oven operating at 40 °C and linked to a Waters 996 photodiode array (PDA) detector (Waters, Milford, MA), and an RF-10A fluorometer (Shimadzu). Data from both detectors were collected and processed via a Millennium Chromatography Manager (Waters). Sample treatment, HPLC column, solvent conditions, and detector systems used for the different phenolics are summarized below. To optimize resolution, mobile phase conditions for isocratic analyses were designed to provide K' values of ~4−5 for the compounds of interest. In addition, for each group of compounds, different reverse-phase columns with varying selectivities and polarities were evaluated with red wines to ensure that impurities did not impinge on the homogeneity of quantified peaks.

Flavonols. Free and conjugated myricetin, quercetin, kaempferol, and isorhamnetin were analyzed in samples with a morin internal standard, before and after acid hydrolysis (McDonald et al., 1998) using a 150 × 3.0 mm i.d., 4 μm C_{18} Genesis column (Jones Chromatography, Mid-Glamorgan, U.K.) eluted at a flow rate of 0.5 mL/min with a 20 min gradient of 20−40% acetonitrile in water adjusted to pH 2.5 with trifluoroacetic acid (TFA). After passing through the PDA detector operating at 365 nm, the column eluate was mixed with methanolic aluminum nitrate in 7.5% acetic acid pumped at a flow rate of 0.5 mL/min and fluorescent flavonol complexes were detected with a fluorometer (excitation = 425 nm, emission = 480 nm) as described by Aziz et al. (1998). Sample volumes analyzed were equivalent to 4.6 μL of wine.

Stilbenes. trans- and *cis*-resveratrol in 10 μL volumes of red wine were analyzed on a 250 × 4.6 mm i.d., 5 μm ODS-Hypersil (Shandon, Astmoor, U.K.) column, packed in-house and eluted at a flow rate of 1 mL/min with 25% acetonitrile in water adjusted to pH 1.5 with TFA using a PDA detector at 307 nm. *trans*-Resveratrol-*O*-β-glucoside was analyzed under similar conditions except that the mobile phase was 17% acetonitrile in water adjusted to pH 1.5 with TFA.

Gallic Acid. The gallic acid contents of 5 μL volumes of red wines were analyzed on a 150 × 3.0 mm i.d., 4 μm C_{18} Genesis column (Jones Chromatography) eluted at a flow rate of 1.0 mL/min with 2% methanol in water adjusted to pH 1.5 with TFA using a PDA detector at 280 nm.

Hydroxycinnamates. Five microliter volumes of samples were analyzed before and after alkaline hydrolysis, which was used to cleave conjugated caffeic acid and *p*-coumaric acid. Five microliter volumes of hydrolysate are equivalent to 1.67 μL of wine. This was achieved by mixing 1 mL of red wine and 1 mL of 4 N NaOH in a 3 mL glass V-vial, which was incubated in darkness at room temperature for 2 h before being acidified with 1 mL of 6 M HCl. The method was adapted and optimized from that of Rapisarda et al. (1998). Samples were analyzed on a 150 × 3.0 mm i.d., 5 μm C_{18} Nemesis column (Phenomenex, Macclesfield, U.K.) eluted at a flow rate of 1 mL/min with either 5 or 9% acetonitrile in water adjusted to pH 1.5 with TFA and a PDA detector operating at 313 nm. Caffeic acid and caftaric acid were analyzed with 5% acetonitrile, whereas a 9% acetonitrile mobile phase was used for free *p*-coumaric acid as well as caffeic acid and *p*-coumaric acid released by alkaline hydrolysis. Using a 9% acetonitrile mobile phase, ferulic and sinapic acids could also be separated but were not found in detectable levels in the wines under study.

(+)-Catechin and (−)-Epicatechin. A 150 × 4.6 mm i.d., 5 μm C_{18} Luna column (Phenomenex, Macclesfield, U.K.) eluted at a flow rate of 1 mL/min with 10% acetonitrile in water adjusted to pH 1.5 with TFA was used to analyze the (+)-catechin and (−)-epicatechin contents of 5 μL volumes of red wines. (+)-Catechin and (−)-epicatechin were detected with a fluorometer operating at excitation = 280 nm and emission = 310 nm (Arts and Hollman, 1998) and by absorbance at 280 nm. The method also allowed the separation of epigallocatechin, epigallocatechin gallate, and epicatechin gallate. These compounds could be detected only by using absorbance at 280 nm and were not found in detectable levels in the wines analyzed.

Colorimetric Analysis of Anthocyanins. The anthocyanin content of red wines was estimated using a pH shift method adapted from Ribéreau-Gayon and Stonestreet (1965). Two test tubes were set up, each containing 1 mL of wine and 1 mL of 0.1% concentrated HCl in 95% ethanol. Ten milliliters of 2% concentrated HCl (pH 0.6) was added to one tube and 10 mL of pH 3.5 buffer (300 mL of 0.2 M Na_2HPO_4 and 700 mL of 0.1 M 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 correction of the 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 coefficient $\epsilon = 28000$. At pH <1 anthocyanins are found entirely in their red flavylium form, allowing the determination of the total anthocyanins. However, at pH 3.5 the flavylium form of the anthocyanin is primarily in equilibrium with the colorless 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.

Determination of Total Phenol Content. The total phenol contents of the wines were determined using the Folin−Ciocalteu method of Singleton and Rossi (1965).

Statistics. Data are presented as mean values ± standard error (SEM) ($n = 3$). A matrix plot was used to graphically represent the data obtained. Some relationships between results were apparently nonlinear; therefore, nonparametric Spearman rank correlations were used to assess the strength of the association between them using Minitab software, version 12 (Minitab Inc., Addison-Wesley Publishing Co., Reading, MA).

Table 2. Antioxidant Activity, Vasodilation Activity, and Phenolic Content of Red Wines

wine	ESR-based antioxidant activity[a]	vasodilation activity[b]	Folin−Ciocalteu total phenolics[c]	HPLC total phenolics[d]
1	6.99 ± 0.17	39 ± 8	10.48 ± 0.20	1.01 ± 0.02
2	5.99 ± 0.18	188 ± 64	10.16 ± 0.06	1.06 ± 0.02
3	9.29 ± 0.27	9 ± 3	18.6 ± 0.10	1.66 ± 0.01
4	6.16 ± 0.23	127 ± 27	11.1 ± 0.12	0.95 ± 0.00
5	7.27 ± 0.27	46 ± 6	13.28 ± 0.09	1.62 ± 0.02
6	6.60 ± 0.13	239 ± 51	11.78 ± 0.03	1.12 ± 0.00
7	8.03 ± 0.06	28 ± 7	15.74 ± 0.06	1.33 ± 0.10
8	5.98 ± 0.08	46 ± 6	10.39 ± 0.06	0.87 ± 0.02
9	7.98 ± 0.23	28 ± 6	14.55 ± 0.06	1.37 ± 0.01
10	7.49 ± 0.25	37 ± 5	14.24 ± 0.03	1.23 ± 0.01
11	6.60 ± 0.15	50 ± 10	12.06 ± 0.03	1.20 ± 0.01
12	6.37 ± 0.08	54 ± 10	11.55 ± 0.03	1.17 ± 0.0
13	4.13 ± 0.13	256 ± 123	6.47 ± 0.03	0.92 ± 0.01
14	8.29 ± 0.13	21 ± 4	17.39 ± 0.09	2.03 ± 0.02
15	4.45 ± 0.02	380 ± 142	7.72 ± 0.09	0.84 ± 0.00
16	7.05 ± 0.09	27 ± 6	13.50 ± 0.06	1.23 ± 0.01

[a] Antioxidant capacity of red wines, measured by ESR spectroscopy, presented as the number of Fremy's radicals reduced by 1 L of wine × 10^{21}. [b] Vasodilation capacity expressed as concentration of wine extract required to give 50% maximal contraction of aortic rings, pIC_{50}. [c,d] Total phenol content of red wine determined by using the Folin−Ciocalteu method (mM gallic acid equivalents GAE) and from HPLC analysis of individual phenolics (mM). All data expressed as mean values ± SE.

RESULTS

Antioxidant Activity. The ability of the 16 wines to reduce the Fremy's salt free radical in the ESR-based antioxidant assay was assessed, and values ranging from 4.13 × 10^{21} to 9.29 × 10^{21} radicals reduced/L were obtained (Table 2). This compares with a range of 6.59 × 10^{21} to 8.55 × 10^{21} radicals/L for 7 red wines studied previously (Gardner et al., 1999). In this chemical model system, Beaujolais (wine 13) and Valpolicella (wine 15) showed the lowest activities, whereas Bulgarian Young Vatted Cabernet Sauvignon (wine 3) and Minervois (wine 14) were ranked first and second, respectively.

Vasodilation Activity. The vasodilation activity of the wines was also assessed, and the pIC_{50} values were determined (Table 2). Although all showed activity, a varying response was observed across the range of wines. The young vatted Bulgarian Cabernet Sauvignon (wine 3) and the Minervois (wine 14) were once again found to be the most active, whereas Beaujolais (wine 13) and Valpolicella (wine 15) exhibited the lowest activity.

Analysis of Individual Phenolic Compounds. To investigate the phenolic content of the red wines in detail, samples were analyzed using a number of HPLC systems custom designed for the different categories of phenolic components. Anthocyanins were measured using a spectrophotometric method. The data obtained were as follows.

Flavonols. The flavonols were analyzed by gradient RP-HPLC with detection at A_{365nm}, as used in previous studies with fruits, vegetables (Crozier et al., 1997), and wines (McDonald et al., 1998). However, in this instance an additional, on-line postcolumn derivatization step was used to provide sensitive and selective detection of flavonols (Aziz et al., 1998). The value of this procedure can be seen by comparing the absorbance and fluorescence traces obtained with an aliquot of an acid-

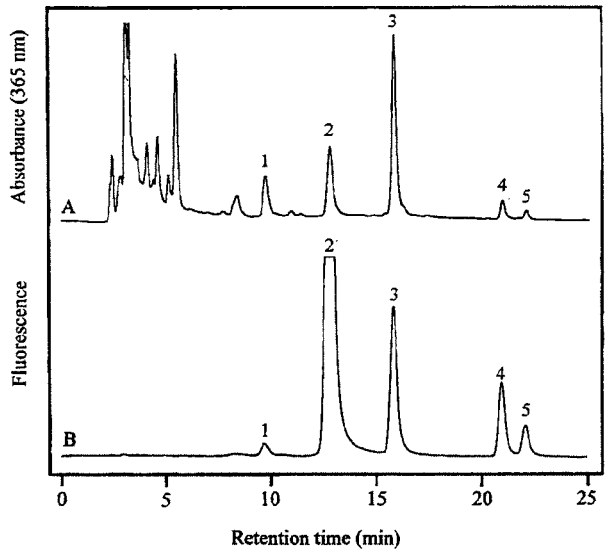


Figure 1. Gradient reversed-phase HPLC analysis of free and conjugated flavonols in wine 14, a 1995 French Minervois: column, 150 × 3.0 mm i.d., 4 μm Genesis C_{18} at 40 °C; mobile phase, 20 min gradient of 20–40% acetonitrile in water adjusted to pH 2.5 with TFA; flow rate, 0.5 mL/min; samples, extract aliquot equivalent to 4.6 μL of wine after acid hydrolysis; detection, (A) absorbance at 365 nm and (B) fluorescence (excitation = 425 nm, emission = 480 nm) after postcolumn derivatization with methanolic aluminum nitrate. Peaks: (1) myricetin, (2) morin (internal standard), (3) quercetin, (4) kaempferol, and (5) isorhamnetin.

hydrolyzed sample of wine 14, the 1995 Minervois, which contains myricetin, quercetin, kaempferol, and isorhamnetin, as well as morin, which was added as an internal standard (Figure 1).

The flavonol contents of the 16 red wines are presented in Table 3. All of the wines contained free and conjugated myricetin, quercetin, kaempferol, and isorhamnetin in various concentrations and with different aglycon/conjugate ratios. As in our previous study with red wines (McDonald et al., 1998), there are >10-fold differences in total flavonol contents, with values ranging from 17.6 μM in the 1992 Bulgarian Cabernet Sauvignon (wine 4) to >187 μM in the Chilean Cabernet Sauvignon (wine 1) and Pinot Noir (wine 5) and Minervois (wine 14). The slightly higher flavonol levels observed in the present study are attributable to the additional detection of isorhamnetin and kaempferol with postcolumn derivatization and fluorescence detection.

Resveratrol. Information on the levels of *cis*- and *trans*-resveratrol and *trans*-resveratrol-*O*-β-glucoside in the wines under study is presented in Table 4. *cis*-Resveratrol-*O*-β-glucoside was not detected in any of the wines analyzed. Typical HPLC traces obtained in the analysis of the stilbenes in wine 14 are illustrated in Figure 2. Most wines contained much higher levels of the aglycons than the conjugate, although high concentrations of the glucoside were present in the French Gevrey Chambertin (wine 7) and Minervois (wine 14). Total resveratrol content ranged >20-fold from 4.3 μM in wine 2, a Californian Cabernet Sauvignon, to 87.9 μM in the conjugate-rich, Pinot Noir-based Gevrey Chambertin (wine 7). Wines 5 and 6, Pinot Noirs from California and Chile, also contained high total levels of resveratrol.

Table 3. Free and Conjugated Flavonol Contents of Red Wines[a]

wine	free M	conj M	total M	free Q	conj Q	total Q	free K	conj K	total K	free I	conj I	total I	total flavonols	% free
1	6.0 ± 0.2	51.0 ± 3.3	55.1 ± 4.3	11.9 ± 0.1	87.8 ± 11.2	95.8 ± 14.3	2.3 ± 0.0	7.3 ± 0.3	8.8 ± 0.7	2.3 ± 0.1	25.8 ± 2.8	27.4 ± 3.5	187.1 ± 19.1	12.0
2	12.5 ± 0.5	16.6 ± 1.6	29.1 ± 1.0	26.8 ± 0.8	41.1 ± 3.3	67.9 ± 2.6	3.8 ± 0.1	1.5 ± 0.5	5.4 ± 0.4	6.1 ± 0.2	7.0 ± 1.8	13.1 ± 1.6	115.5 ± 4.6	42.6
3	8.5 ± 0.1	12.4 ± 0.4	20.9 ± 0.3	3.5 ± 0.1	13.2 ± 0.7	16.7 ± 0.8	1.7 ± 0.0	0.7 ± 0.2	2.3 ± 0.2	0.3 ± 0.0	13.4 ± 0.9	13.6 ± 0.9	53.5 ± 1.7	26.2
4	2.3 ± 0.1	3.5 ± 0.2	5.8 ± 0.3	1.8 ± 0.1	3.2 ± 0.1	5.0 ± 0.1	nd	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	6.3 ± 0.1	6.6 ± 0.1	17.6 ± 0.5	25.0
5	18.1 ± 0.1	13.8 ± 1.5	32.5 ± 0.6	36.6 ± 1.6	51.2 ± 0.2	88.6 ± 2.1	2.7 ± 0.1	2.7 ± 0.2	5.4 ± 0.3	6.8 ± 0.1	54.2 ± 3.6	61.1 ± 3.5	187.6 ± 7.9	34.2
6	8.4 ± 0.4	8.8 ± 1.0	17.2 ± 0.6	17.1 ± 1.4	29.9 ± 2.5	47.1 ± 1.6	1.8 ± 0.0	1.6 ± 0.1	3.4 ± 0.2	2.4 ± 0.1	30.5 ± 0.9	33.0 ± 0.9	100.7 ± 2.4	29.5
7	3.2 ± 0.2	9.1 ± 0.6	12.4 ± 0.4	21.2 ± 0.5	20.0 ± 0.2	41.7 ± 0.1	2.1 ± 0.1	0.3 ± 0.0	2.5 ± 0.0	1.8 ± 0.0	16.8 ± 0.1	18.6 ± 0.1	75.2 ± 0.5	37.6
8	10.7 ± 0.7	22.7 ± 4.8	27.6 ± 0.8	11.0 ± 0.6	28.8 ± 5.5	34.6 ± 0.7	1.7 ± 0.1	1.5 ± 1.0	2.4 ± 0.2	2.8 ± 0.1	12.1 ±2.8	13.6 ± 1.6	78.2 ± 3.9	35.5
9	6.4 ± 1.4	12.8 ± 2.9	19.2 ± 3.1	32.8 ± 3.8	29.2 ± 4.9	62.0 ± 5.1	4.0 ± 0.3	0.9 ± 0.3	4.8 ± 0.1	4.4 ± 0.5	16.9 ± 0.9	21.3 ± 1.1	107.3 ± 9.2	44.4
10	20.7 ± 1.1	18.4 ± 5.0	39.1 ± 5.5	3.5 ± 0.1	20.3 ± 3.7	23.9 ± 3.8	1.8 ± 0.0	1.8 ± 0.6	3.6 ± 0.6	0.3 ± 0.0	17.6 ± 3.1	17.9 ± 3.1	84.5 ± 11.9	31.1
11	17.1 ± 0.4	9.0 ± 1.8	26.1 ± 1.4	15.0 ± 0.1	15.3 ± 1.4	30.2 ± 1.5	0.9 ± 0.0	0.7 ± 0.1	1.6 ± 0.1	3.2 ± 0.0	11.4 ± 0.8	14.6 ± 0.8	72.5 ± 3.7	49.9
12	7.6 ± 0.1	4.5 ± 0.7	12.1 ± 0.7	7.4 ± 0.2	13.3 ± 1.1	20.8 ± 1.0	1.6 ± 0.0	0.6 ± 0.1	2.1 ± 0.1	0.7 ± 0.0	5.0 ± 0.4	5.7 ± 0.4	40.7 ± 1.7	42.5
13	3.4 ± 0.1	4.9 ± 0.4	8.3 ± 0.5	6.8 ± 0.2	9.2 ± 1.2	16.0 ± 1.3	0.2 ± 0.0	0.6 ± 0.1	0.7 ± 0.1	1.2 ± 0.0	8.7 ± 0.9	9.9 ± 1.0	34.9 ± 2.7	33.2
14	20.7 ± 4.4	30.9 ± 8.6	51.7 ± 4.3	41.9 ± 4.6	62.8 ± 14.7	104.7 ± 10.5	4.5 ± 0.2	3.2 ± 1.1	7.6 ± 0.9	7.1 ± 0.6	24.3 ± 6.1	31.4 ± 5.6	195.4 ± 21.2	38.0
15	3.1 ± 0.6	8.8 ± 1.2	12.0 ± 0.6	2.3 ± 0.2	16.6 ± 1.7	18.9 ± 1.5	1.1 ± 0.0	1.0 ± 0.1	2.0 ± 0.2	0.2 ± 0.0	16.0 ± 1.6	16.2 ± 1.6	49.1 ± 3.8	13.6
16	4.9 ± 1.3	17.0 ± 1.4	21.9 ± 0.5	20.9 ± 2.5	36.1 ± 2.4	57.0 ± 1.5	1.6 ± 0.1	0.8 ± 0.1	2.4 ± 0.0	0.5 ± 0.0	7.6 ± 0.2	8.1 ± 0.2	89.4 ± 1.9	31.2

[a] Data are expressed as μM ± SE (n = 3). M, myricetin; Q, quercetin; K, kaempferol; I, isorhamnetin; nd, not detected; % free, free flavonols as % of total; conj, conjugated.

Table 4. Resveratrol and Gallic Acid Content of Red Wines[a]

	wine	year	*trans*-resveratrol	*cis*-resveratrol	*trans*-resveratol glucoside	total resveratrol	gallic acid
1	Chilean Cabernet Sauvignon	1997	2.1 ± 0.3	2.5 ± 0.5	2.8 ± 0.3	7.4 ± 0.6	130.8 ± 4.9
2	Californian oak-aged Cabernet Sauvignon	1995	2.3 ± 0.1	2.0 ± 0.1	nd	4.3 ± 0.1	167.1 ± 0.6
3	Young Vatted Cabernet Sauvignon	1996	29.4 ± 0.6	22.8 ± 0.7	8.3 ± 0.2	60.5 ± 1.1	416.6 ± 4.0
4	Bulgarian Matured Cabernet Sauvignon	1992	27.9 ± 0.3	8.2 ± 0.2	3.1 ± 0.0	39.2 ± 0.5	344.7 ± 0.7
5	Cono Sur Pinot Noir, 20 barrels	1995	39.0 ± 2.2	28.1 ± 0.4	7.8 ± 1.6	74.9 ± 2.9	205.2 ± 2.6
6	Fetzer Santa Barbara Pinot Noir	1994	46.3 ± 2.6	32.7 ± 0.7	nd	79.0 ± 3.1	282.9 ± 1.7
7	Domaine Rossignol Trapet, Gevrey Chambertin	1995	30.4 ± 0.8	27.1 ± 0.4	30.4 ± 0.8	87.9 ± 2.1	300.7 ± 4.1
8	Villa Montes oak-aged Merlot	1994	2.1 ± 0.1	6.6 ± 0.2	nd	8.7 ± 0.1	45.9 ± 1.7
9	Merlot	1996	22.8 ± 2.2	22.1 ± 1.1	7.0 ± 0.4	51.9 ± 2.1	300.0 ± 1.8
10	Cosme Palacio y Hermanos Rioja	1995	15.1 ± 0.2	11.3 ± 0.4	2.9 ± 0.2	29.3 ± 0.4	145.1 ± 0.5
11	Viña Albali Tempranillo	1996	9.1 ± 0.3	6.0 ± 0.1	7.1 ± 0.2	22.2 ± 0.5	225.8 ± 1.4
12	Fetzer Vineyards Zinfandel	1995	5.7 ± 0.0	4.6 ± 0.3	2.6 ± 0.1	12.9 ± 0.4	245.8 ± 1.7
13	Beaujolais	1996	11.0 ± 0.5	9.9 ± 0.1	1.3 ± 0.2	22.2 ± 0.5	89.1 ± 0.4
14	Domaine Roche Vue, Minervois	1995	18.6 ± 0.8	7.1 ± 0.8	18.6 ± 0.8	44.3 ± 0.9	274.4 ± 4.1
15	Valpolicella	1996	9.2 ± 0.9	4.4 ± 0.1	9.2 ± 0.9	22.8 ± 1.9	147.4 ± 2.6
16	Chianti Classico	1995	9.1 ± 0.8	4.7 ± 0.7	9.1 ± 0.8	22.9 ± 1.0	335.5 ± 2.4

[a] Data are expressed as μM ± SE ($n = 3$). nd, not detected.

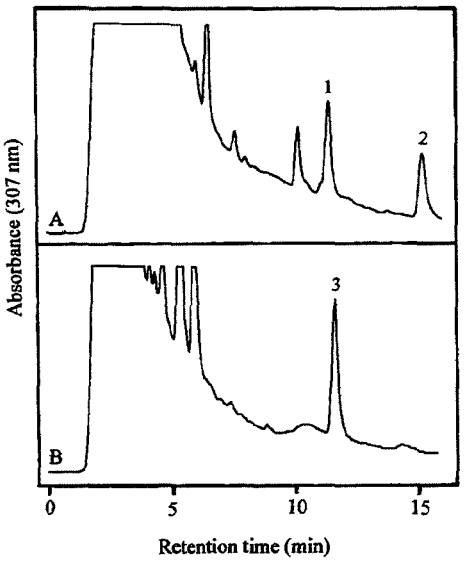


Figure 2. Reversed-phase HPLC analysis of *trans*- and *cis*-resveratrol and *trans*-resveratrol-*O*-β-D-glucoside in 10 μL aliquots of wine 14, a 1995 French Minervois: (A) analysis of *trans*-reseveratrol (1) and *cis*-resveratrol (2) using a 250 × 4.6 mm i.d., 5 μm ODS-Hypersil column eluted with 25% acetonitrile in water adjusted to pH 1.5 with TFA at 1 mL/min and detection at 307 nm; (B) analysis of *trans*-resveratrol-*O*-β-glucoside (3) using a 250 × 4.6 mm i.d., 5 μm ODS-Hypersil column eluted with 17% acetonitrile in water adjusted to pH 1.5 with TFA and detection at 307 nm.

Gallic Acid. A typical trace illustrating HPLC analysis of gallic acid in wine 14 is presented in Figure 3A. The levels of gallic acid varied almost 10-fold from 416.6 μM in wine 3, a 1996 Bulgarian Cabernet Sauvignon, to 45.9 μM in wine 8, a Chilean Merlot (Table 4).

Hydroxycinnamates. Quantitative estimates of caftaric acid, caffeic acid, *p*-coumaric acid, and conjugated *p*-coumaric acid are presented in Table 5, and an HPLC trace illustrating the analysis of the hydroxycinnamates is shown in Figure 3B. Wine 13, a Beaujolais produced by light extraction of Gamay grapes, contained by far the highest concentration of caftaric acid, 331.8 μM. Wine 14, the Minervois, contained the highest levels of both free and conjugated *p*-coumaric acid, 210.6 and 462.2 μM, respectively. As a consequence, the overall concentration of hydroxycinnamates in the Minervois, 903.4 μM, was ~2-fold higher than the levels detected in any of the other wines. With the exception of the Minervois, the total hydroxycinnamate content did not vary greatly in the wines that were investigated (Table 5). Unlike the skin-derived stilbenes and flavonols, the hydroxycinnamates are located primarily in the flesh of the grape and as such are found in comparable levels in both red and white wines.

Catechins. The concentrations of (+)-catechin and (−)-epicatechin are presented in Table 6, and a typical HPLC trace upon which these estimates are based is illustrated in Figure 3C,D. Note that fluorescence detection provided greater sensitivity and selectivity than the more traditional absorbance at 280 nm. Although the method also separates epigallocatechin, epigallocatechin gallate, and epicatechin gallate, only (+)-catechin and (−)-epicatechin were present in the wines in detectable quantities. The highest total catechin concentrations, 645.6 and 637.5 μM, were found in wines 5 and 7, Pinot Noirs from Chile and France, respectively, which also contained high levels of total resveratrol. Wine 10, a Spanish Rioja, contained the lowest amount of total catechins, 172.6 μM, albeit only 3.5-fold less than the highest value in wine 5. (+)-Catechin was invariably present in larger amounts than (−)-epicatechin, with ratios ranging from 2.7 in the Chilean Pinot Noir, wine 5, to 16.0 in wine 14, the Minervois. The catechin levels presented in Table 6 are in keeping with the findings of Goldberg et al. (1998), who also found highest concentrations in wines made from Pinot Noir grapes, which appear to be constitutively higher in (+)-catechin and (−)-epicatechin than other grape varieties.

Anthocyanins. Polymeric anthocyanins were present in all wines in larger amounts than free anthocyanins with ratios varying from 1.4 to 6.1. The highest total anthocyanin concentrations, 325.5 and 308.8 μM malvidin-3-glucoside equivalents, were detected in wine 1, the Chilean Cabernet Sauvignon, and wine 14, the Minervois, respectively. The lowest level, 101.5 μM, was observed in wine 7, the Gevrey Chambertin Pinot Noir (Table 6).

Total Phenolic Content of Red Wines. The total phenolic content of the 16 red wines determined by using the Folin–Ciocalteu colorimetric method are presented in Table 2. There are almost 3-fold differences in the levels present in the different wines, with concentrations ranging from 6.47 to 18.6 mM of gallic acid equivalents (GAE). These figures, corresponding to

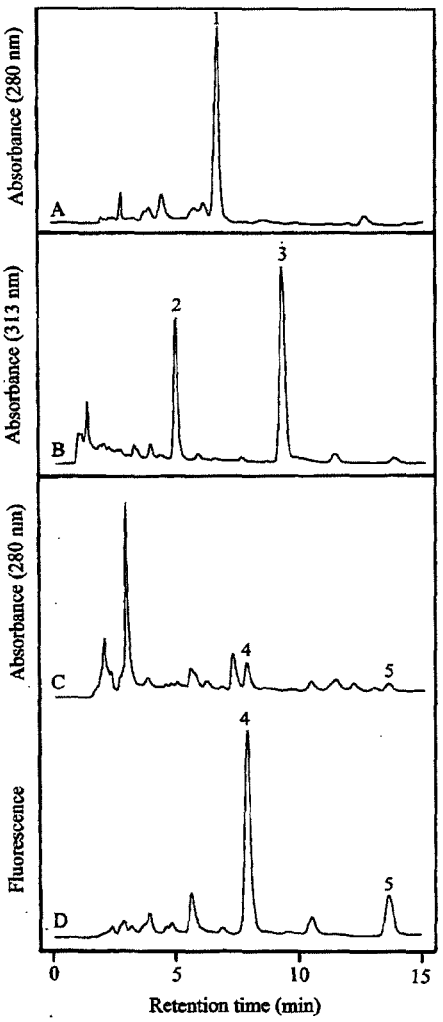


Figure 3. Reversed-phase HPLC analysis of gallic acid, hydroxycinnamates, (+)-catechin, and (−)-epicatechin in wine 14, a 1995 French Minervois: (A) analysis of gallic acid (1) in 5 μL of wine using a 150 × 3.0 mm i.d., 4 μm Genesis C_{18} column eluted at a flow rate of 1.0 mL/min with 2% methanol in water adjusted to pH 1.5 with TFA and detection at 280 nm; (B) analysis of caffeic acid (2) and *p*-coumaric acid (3) after alkaline hydrolysis using a 150 × 3.0 mm C_{18}, 5 μm Nemesis column eluted at a flow rate of 1.0 mL/min with 9% acetonitrile in water adjusted to pH 1.5 with TFA and detection at 313 nm (5 μL injection of hydrolysate is equivalent to 1.67 μL of wine); (C) analysis of (+)-catechin (4) and (−)-epicatechin (5) in 5 μL wine using a 150 × 4.6 mm i.d., 5 μm C_{18} Luna column eluted at a flow rate of 1 mL/min with 10% acetonitrile in water adjusted to pH 1.5 with TFA and detection at 280 nm; (D) same as (C) but with fluorescence detection (excitation = 280 nm, emission = 310 nm).

1100−3165 mg/L GAE, are comparable with values obtained for red wines by other investigators (Frankel et al., 1995; Sato et al., 1996; Ritchey and Waterhouse, 1999). In the current study phenolic-rich wines included the 1996 Young Vatted Bulgarian Cabernet Sauvignon (wine 3), the 1995 Minervois (wine 14), and the 1995 Gevrey Chambertain Pinot Noir (wine 7). Lowest concentrations were detected in wine 13, the 1996 Beaujolais, and the 1996 Valpolicella (wine 15).

A second method was used to assess the total phenolic content of the red wines. This involved combining the figures obtained from the HPLC-based analyses of flavonols, hydroxycinnamates, (+)-catechin, (−)-epicatechin, *cis*-resveratrol, *trans*-resveratrol, *trans*-resveratrol-*O*-β-glucoside, and gallic acid as well as anthocyanin values obtained with the colorimetric assay. The figures based on this method of assessment of the total phenolic content are presented in Table 2. Although showing a similar trend, they are ~10-fold lower than those obtained with the Folin−Ciocalteu assay. There are two likely reasons for this discrepancy. First, the analysis of the individual components in the wines did not include the condensed tannins, the oligomers and polymers of (+)-catechin and (−)-epicatechin, so their contribution to the total phenolic content of the wines was not determined using the HPLC methods. However, according to Soleas et al. (1997b) and Singleton (1982), these components comprise only ~20% of the total phenolics in red wines. Second, and probably the main cause of the difference between figures obtained by the two methods, is the fact that the Folin−Ciocalteu method does not provide a specific assay for phenolics as it reacts positively with many easily oxidizable nonphenolic compounds present in red wines and other matrices (Singleton, 1982). In addition, as different phenolics have widely varying reaction stoichiometries, expressing the Folin−Ciocalteu results as GAE may cause an overestimation in the total phenolic content of the wines.

Relationship among Antioxidant Activity, Vasodilation Activity, and Phenolics. The statistical significance of the relationships among antioxidant activity, vasodilation capacity, and the total phenolic contents of the red wines (see Table 2) was analyzed using nonparametric Spearman rank correlation and Minitab software. The ESR-based antioxidant potentials were found to correlate strongly with the Folin−Ciocalteu estimates of total phenol content ($r_S = 0.96$ and $p < 0.001$). The total phenolic content based on HPLC analyses also correlated with the antioxidant potential ($r_S = 0.94$ and $p < 0.001$). The pIC_{50} values for the wines were closely associated with their total HPLC-derived phenolic content ($r_S = -0.811$, $p < 0.001$) and the Folin−Ciocalteu estimate of phenolic content ($r_s = -0.862$, $p < 0.001$). The vasodilation activity of the wines was found to correlate very strongly with their antioxidant activity as determined by the ESR method ($r_S = -0.883$, $p < 0.001$). Figure 4 demonstrates the relationship between the ESR-based antioxidant activity, the vasodilation activity, and the Folin−Ciocalteu- and HPLC-derived phenolic contents of the wines. This close association between the ESR chemical model system and an ex vivo biological assay suggests that the chemical method has biological relevance.

Attempts to correlate statistically the levels of specific phenolics in the red wines (Tables 3−6) with antioxidant activity and vasodilation capacity were less successful. Although correlations with antioxidant activity were detected with gallic acid ($r_S = 0.56$, $p = 0.024$), total resveratrol ($r_S = 0.61$, $p = 0.013$), and total (+)-catechin and (−)-epicatechin ($r_S = 0.60$, $p = 0.014$), the other individual correlations were lower (total hydroxycinnamates, $r_S = 0.26$, $p = 0.341$; total flavonols, $r_S = 0.45$, $p = 0.08$; total anthocyanins, $r_S = 0.35$, $p = 0.182$). Likewise, although the pIC_{50} values of the wines were closely correlated with their total HPLC-derived phenolic contents ($r_S = -0.811$, $p < 0.001$), this association was not evident with individual phenolics with the sole exception of total anthocyanins ($r_S = -0.52$, $p = 0.038$).

Table 5. Hydroxycinnamate Content of Red Wines[a]

	wine	year	caftaric acid	free caffeic acid	free *p*-coumaric acid	conj *p*-coumaric acid	total hydroxy-cinnamates
1	Chilean Cabernet Sauvignon	1997	111.2 ± 0.7	23.0 ± 0.7	23.9 ± 2.3	25.5 ± 0.8	183.6 ± 4.3
2	Californian oak-aged Cabernet Sauvignon	1995	42.9 ± 1.7	32.3 ± 0.6	131.9 ±0.6	35.9 ± 0.7	243.0 ± 2.5
3	Young Vatted Cabernet Sauvignon	1996	95.7 ± 0.5	13.3 ± 0.4	115.6 ± 0.4	14.8 ± 0.4	239.4 ± 9.6
4	Bulgarian matured Cabernet Sauvignon	1992	82.9 ± 1.6	15.3 ± 0.7	71.6 ± 0.6	17.0 ± 0.8	186.8 ± 0.8
5	Cono Sur Pinot Noir, 20 barrels	1995	188.0 ± 0.5	50.6 ± 0.3	32.7 ± 4.7	56.2 ± 0.3	327.5 ± 4.3
6	Fetzer Santa Barbara Pinot Noir	1994	49.5 ± 2.7	94.2 ± 0.2	33.9 ± 0.3	131.6 ± 1.8	309.2 ± 2.2
7	Domaine Rossignol Trapet, Gevrey Chambertin	1995	89.8 ± 1.9	106.4 ± 7.0	31.7 ± 1.2	25.7 ± 1.4	253.6 ± 7.7
8	Villa Montes oak-aged Merlot	1994	17.4 ± 0.4	74.4 ± 0.5	14.7 ± 0.2	197.6 ± 0.7	304.1 ± 1.1
9	Merlot	1996	110.0 ± 0.4	24.1 ± 0.1	16.1 ± 0.5	188.2 ± 0.5	338.4 ± 1.2
10	Cosme Palacio y Hermanos Rioja	1995	128.5 ± 0.9	47.6 ± 0.8	23.7 ± 0.2	319.2 ± 1.0	519.0 ± 1.9
11	Viña Albali Tempranillo	1996	117.9 ± 0.4	6.6 ± 0.2	33.9 ± 0.3	131.6 ± 1.8	290.0 ± 2.2
12	Fetzer Vineyards Zinfandel	1995	94.4 ± 1.2	101.0 ± 0.3	54.6 ± 0.2	201.5 ± 1.6	451.5 ± 3.0
13	Beaujolais	1996	331.8 ± 9.8	28.9 ± 0.4	26.4 ± 0.2	32.1 ± 0.4	419.2 ± 9.3
14	Domaine Roche Vue, Minervois	1995	189.7 ± 2.2	40.9 ± 0.3	210.6 ± 0.3	462.2 ± 1.8	903.4 ± 5.5
15	Valpolicella	1996	110.5 ± 0.1	11.1 ± 0.2	74.7 ± 0.7	52.8 ± 1.9	249.1 ± 1.6
16	Chianti Classico	1995	121.8 ± 0.9	30.6 ± 0.3	21.8 ± 0.8	142.0 ± 0.8	316.2 ± 1.1

[a] Data are expressed as μM ± SE (n = 3). Caftaric acid quantified as caffeic acid equivalents. Conj, conjugated.

Table 6. Flavan 3-ol and Anthocyanin Contents of Red Wines[a]

	wine	year	(+)-catechin	(−)-epi-catechin	total catechins	(+)-cat./(−)-epi ratio	free antho-cyanins	polymeric pigments	total antho-cyanins
1	Chilean Cabernet Sauvignon	1997	239.8 ± 16.4	23.8 ± 0.7	263.6 ± 16.3	10.1	72.0	253.7	325.7
2	Californian oak-aged Cabernet Sauvignon	1995	270.3 ± 16.6	47.4 ± 2.0	317.7 ± 15.6	5.7	75.0	135.3	210.3
3	Young Vatted Cabernet Sauvignon	1996	468.1 ± 7.8	130.5 ± 2.0	598.6 ± 8.3	3.6	82.7	202.3	285.0
4	Bulgarian matured Cabernet Sauvignon	1992	188.9 ± 0.6	59.4 ± 3.0	248.3 ± 3.7	3.2	15.5	94.7	110.2
5	Cono Sur Pinot Noir, 20 barrels	1995	472.6 ± 4.5	173.0 ± 5.5	645.6 ± 10.1	2.7	72.4	110.6	183.0
6	Fetzer Santa Barbara Pinot Noir	1994	233.2 ± 1.3	91.5 ± 0.8	324.7 ± 0.6	2.5	25.3	83.9	109.2
7	Domaine Rossignol Trapet, Gevrey Chambertin	1995	490.5 ± 8.5	147.0 ± 8.1	637.5 ± 16.5	3.3	26.1	75.4	101.5
8	Villa Montes oak-aged Merlot	1994	186.6 ± 7.0	36.6 ± 1.3	223.2 ± 7.6	5.1	80.2	154.2	234.4
9	Merlot	1996	296.8 ± 1.2	83.8 ± 0.9	380.6 ± 2.1	3.5	56.1	135.0	191.1
10	Cosme Palacio y Hermanos Rioja	1995	151.0 ± 2.0	21.6 ± 1.0	172.6 ± 1.3	7.0	112.7	163.3	276.0
11	Viña Albali Tempranillo	1996	299.3 ± 4.6	69.1 ± 2.2	368.4 ± 5.8	4.3	90.9	135.3	226.2
12	Fetzer Vineyards Zinfandel	1995	202.7 ± 0.9	53.5 ± 1.2	256.2 ± 1.4	3.8	41.2	121.4	162.6
13	Beaujolais	1996	193.8 ± 1.9	30.6 ± 1.1	224.4 ± 3.0	6.5	49.3	84.9	134.2
14	Domaine Roche Vue, Minervois	1995	284.5 ± 1.1	17.8 ± 1.5	302.3 ± 2.6	16.0	87.3	221.2	308.5
15	Valpolicella	1996	198.6 ± 2.2	48.9 ± 1.1	247.5 ± 3.3	4.1	52.7	74.5	127.2
16	Chianti Classico	1995	242.2 ± 2.3	58.4 ± 3.8	300.6 ± 6.0	4.1	46.3	120.8	167.1

[a] Data for (+)-catechin and (−)-epicatechin expressed as μM ± SE (n = 3). (+)-cat./(−)-epi ratio, ratio of (+)-catechin to (−)-epicatechin. Anthocyanin data expressed as μM malvidin 3-glucoside equivalents.

DISCUSSION

The varying capacities of the 16 red wines to act both as ex vivo vasodilators and as in vitro antioxidants appear to be associated with the phenolic content of the wines, whether determined by the Folin–Ciocalteu assay or by the summation of the levels of individual phenolics analyzed primarily by HPLC. These relationships become more evident in Figure 5 in which the vasodilation pIC$_{50}$ figures are plotted as inverse values $\times 10^3$ and the wines are ranked to visualize the concomitant reductions in vasodilation and antioxidant activity that are paralleled by decreasing phenolic content. Although wines all showed activity, there is a large spread in the antioxidant capacity of the individual wines and likewise in their ability to relax precontracted aorta. The phenolic-rich Bulgarian Young Vatted Cabernet Sauvignon (wines 3) has high antioxidant and vasodilation capacities, whereas at the other end of the scale the low phenolic content of the Beaujolais (wine 13) is characterized by markedly lower vasodilation and antioxidant activities. The Bulgarian Young Vatted Cabernet Sauvignon (wine 3) underwent extensive skin extraction using a rotary extractor during vinification, facilitating the release of more phenolics than would have otherwise been possible using more traditional methods. In contrast, the Beaujolais (wine 13) was produced conventionally from thin-skinned Gamy grapes that undergo carbonic maceration and are, therefore, only lightly extracted.

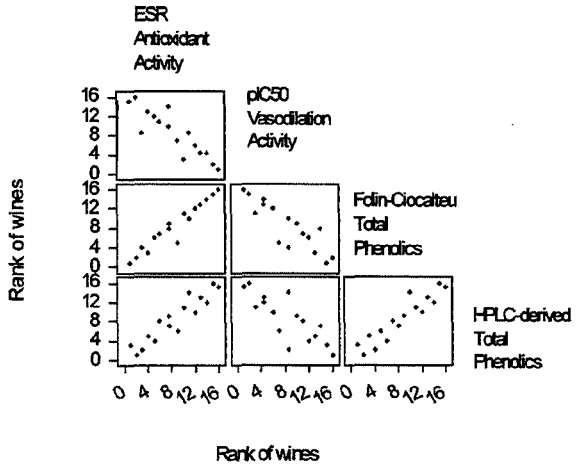


Figure 4. Matrix plot derived from Spearman rank correlations, highlighting the relationships between ESR-based antioxidant activity, vasodilation activity, and total phenolics. Antioxidant activity was determined as the number of Fremy's radicals reduced per liter of wine × 10^{21}; vasodilation activity was expressed as the concentration in μg/mL at which each extract caused 50% maximal vasodilation, pIC$_{50}$; total phenolic content was determined by Folin–Ciocalteu colorimetric assay and HPLC.

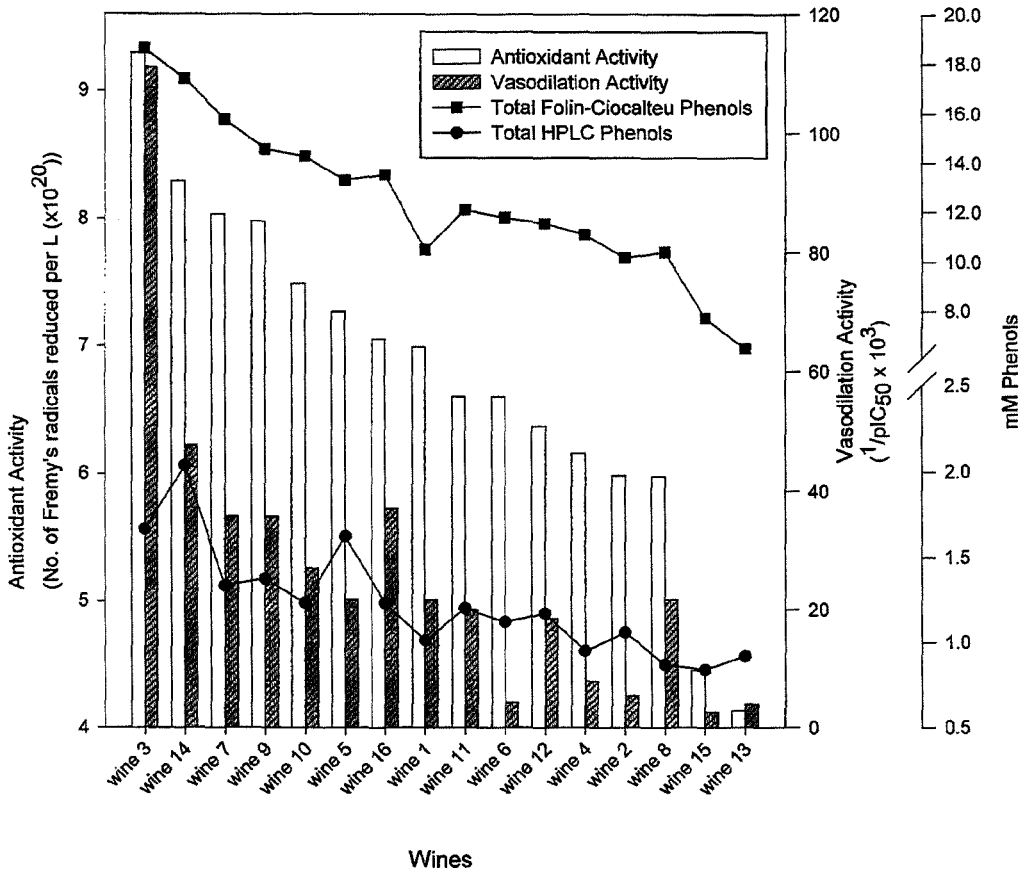


Figure 5. Relationship between antioxidant activity, vasodilation activity, and phenolic content of red wines. Antioxidant activity was determined as the number of Fremy's radicals reduced per liter of wine × 10^{21}; vasodilation activity was expressed as $1/pIC_{50}$ × 10^{21}, where pIC_{50} is the concentration in μg/mL of wine extract at which there is 50% maximal contraction of aortic rings. Total phenolic content was determined by two methods, the Folin–Ciocalteu colorimetric assay [results expressed as mM gallic acid equivalents (GAE)] and HPLC (results expressed as mM of individual phenolics as presented in Tables 2–6). All data are expressed as mean values ± SE.

A number of studies have previously investigated the vasodilation activity of a range of plant extracts, including red wine, grape juice, and grape skin extract (Fitzpatrick et al., 1993, 1995; Andriambeloson et al., 1998). There is clear evidence that the most active compounds are skin-derived, supported by the low vasodilation activity of white wine as opposed to red wine, for which there is extensive grape skin extraction. Recent studies have attempted to identify the vasoactive agent. Investigations using the flavonols apigenin, isorhamnetin, kaempferol, luteolin, myricetin, quercetin, and rutin showed that apart from rutin they all induced significant relaxation of rat pulmonary arteries (MacLean et al., 1997). In contrast, Andriambeloson et al. (1998) found that only the anthocyanin and oligomeric condensed tannin containing fractions of red wine showed the same activity as the original red wine polyphenolic fraction. The present study found that although there is a strong correlation between vasodilation activity and total phenolics quantified either by the Folin–Ciocalteu assay or by HPLC, the only correlation with a phenolic family was with the total anthocyanins.

Although a substantial amount of work has been carried out into the action of phenolics using in vitro systems, until recently there has been a dearth of information on their absorption. However, there is now a significant body of evidence supporting the theory that the flavonol conjugates are preferentially absorbed and that the nature of the conjugation may be important (Hollman et al., 1995). In studies on the absorption and excretion of flavonols after onion consumption, it has been shown that quercetin-4′-glucoside and isorhamnetin-4′-glucoside accumulate in human plasma and are excreted in urine to a much greater extent than their aglycons (Aziz et al., 1998; Aziz, 2000). Conjugated quercetin can be similarly detected in human plasma after the consumption of red wine (Crozier et al., 1999). A recent study has demonstrated the presence of catechin metabolites in human plasma after red wine consumption. Although free (+)-catechin and its metabolite, 3′-*O*-methylcatechin, were detected, their levels were low compared to those of (+)-catechin sulfate and/or glucuronide conjugates (Donovan et al., 1999). Anthocyanins also appear to be absorbed as anthocyanin-like compounds have been detected in human urine after consumption of red wine (Lapidot et al., 1998). In addition to flavonoids, the bioavailability of other wine phenolics is now coming under investigation, and it has been demonstrated with a model rat system that resveratrol accumulates in plasma after ingestion of the stilbene (Bertelli et al., 1996; Juan et al., 1999). As with other dietary compounds, extensive postconsumption metabolism of phenolics is possible, and it may be, in some instances, that metabolites rather than the parent

compound are responsible for the advantageous effects of red wine.

It seems likely that the beneficial effects of red wine are due to the cumulative effects of several phenolics rather than one individual compound. Identification of the bioavailable antioxidant and/or vasodilatant agents will open up the possibility of identifying wines that are rich in these components and, which, as a consequence, may provide enhanced protection against CHD. This could also be achieved by making wines from grapes that have been genetically modified to produce high levels of the active phenolics in both the flesh and the skin.

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