An assessment of the anti-oxidant and pro-oxidant profiles of red wine and selected phenolic components

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DECLARATION

I hereby declare that this submission is my own work and has been carried out at Kingston University, UK.

The contents of this thesis have not been submitted for any other degree at this or any other university.

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ABSTRACT

INTRODUCTION: Red wine contains a rich matrix of phenolic compounds which have been found to possess anti-oxidant properties. These phenolic anti-oxidants have considerable potential in preventing inflammation and oxidative stress. However, many of these dietary components can also exhibit pro-oxidant activity under certain experimental conditions, such as in the presence of redox-active transition metal ions. A great deal of research has focussed on the anti-oxidant potential of red wine. However, studies on the potential pro-oxidant effects are limited.

AIMS: This research aimed to contribute to existing knowledge by delineating both the anti-oxidant and pro-oxidant profiles of red wine and selected individual phenolic compounds in the presence of various oxidant systems.

METHODS: A new functional-based TLC approach was used to screen the anti-oxidant profile of red wine in the presence of hydrogen peroxide, Fe^{3+} and Cu^{2+} metal ions. A quantitative approach using a reversed-phase HPLC method was developed to further assess the relative anti-oxidant activities of wine and its phenolic compounds. Pro-oxidant effects of wine were investigated using the hydroxyl radical-mediated deoxyribose degradation assay and inhibition of linoleic acid peroxidation assay. The effect of Fe^{3+} and Cu^{2+} on the anti-oxidant activities of other grape-based products was also carried out using modified forms of the $ABTS^{\bullet+}$ and DPPH assays.

RESULTS: Functional TLC revealed that quercetin and caffeic acid were found to be the most potent anti-oxidants, with overall ranking of the five anti-oxidants in the order: quercetin > caffeic acid > gallic acid > *p*-coumaric acid > chlorogenic acid. RP-HPLC showed similar results, with quercetin and caffeic acid exhibiting the highest anti-

oxidant efficacies. Gallic acid and *p*-coumaric acid, however, showed lower activities. The Fenton systems were shown to have a greater oxidising power relative to the oxidants added alone. In the hydroxyl radical-mediated deoxyribose degradation assay, red wine exhibited decreased anti-oxidant potential as the concentration increased, and was pro-oxidant at 640 mg/L. However, it was an efficient inhibitor of linoleic acid peroxidation, with inhibition ranging from 73.93-82.59 %. All phenolic standards showed pro-oxidant activities, with gallic acid the highest (-62.12 %), and kaempferol the lowest (-19.70 %). The modified DPPH and $ABTS^{\bullet+}$ assays revealed a reduction in anti-oxidant capacity for wines and grape juices in the presence of metal ions, with Cu^{2+} showing a greater reduction in activity than Fe^{3+} in the $ABTS^{\bullet+}$ assay. For red wine, anti-oxidant activity ranged from 4556.72-4782.09 mg TE/L in the presence of Fe^{3+}, whereas this decreased to between 3444.78 and 3600.00 mg TE/L when Cu^{2+} was added.

CONCLUSIONS: The study demonstrated the first application of functional TLC and HPLC to delineate the anti-oxidant profile of red wine in the presence of different oxidant systems involving H_2O_2 and redox-active metal ions. The results suggested that red wine and its phenolic compounds can exhibit pro-oxidant potential under certain experimental conditions. The reduced anti-oxidant capacity of other grape-based products in the presence of metal ions also suggested potential pro-oxidant effects. Overall, a more detailed examination of metal ion-phenolic anti-oxidant interactions must be fully explored in order to determine potential pro-oxidant effects in biological systems.

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LIST OF ABBREVIATIONS

AA, Ascorbic Acid

AAPH, 2,2'-diazobis-(2-amidinopropane) dihydrochloride

ABTS, 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt

BHT, Butylated Hydroxytoluene

BODIPY, 4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene

1-BuOH, 1-Butanol

C, Catechin

CA, Caffeic Acid

CAD, Coronary Artery Disease

CCl_4, Carbon tetrachloride

CE, Capillary Electrophoresis

ChA, Chlorogenic Acid

Cu^{2+}, Cupric ion

Cu^{+}, Cuprous ion

CUPRAC, Cupric Reducing Anti-oxidant Capacity

CuZnSOD, Copper-Zinc Superoxide Dismutase

DCFH, 2'7'-Dichlorodihydrofluorescin

$DMPD^{\cdot+}$, N,N-Dimethyl-l,4-phenylenediamine

DPPD, N,N'-Diphenyl-p-phenylenediamine

DPPH, 2,2-Diphenyl-1-picrylhydrazyl

EC, Epicatechin

EDTA, Ethylenediaminetetraacetic Acid

EGC, Epigallocatechin

EGCG, Epigallocatechin-3-Gallate

EGF, Epidermal Growth Factor

eNOS, Endothelial Nitrc Oxide Synthase

ESR, Electron Spin Resonance

EtOAc, Ethyl Acetate

EtOH, Ethanol

FC, Folin-Ciocalteau

Fe^{3+}, Ferric ion

Fe^{2+}, Ferrous ion

FMD, Flow Mediated Dilation

FRAP, Ferric Reducing Anti-oxidant Power

FTIR, Fourier Transform Infrared Spectroscopy

F-TLC, Functional Thin-Layer Chromatography

GA, Gallic Acid

GAE, Gallic Acid Equivalents

GC, Gas Chromatography

GPx, Glutathione Peroxidase

GSH, Glutathione

GSPE, Grape Seed Proanthocyanidin Extract

GSSG, Glutathione Disulphide

HA, Hexane

HCl, Hydrochloric Acid

HCOOH, Formic Acid

HCOR, Aldehyde

HDL, High Density Lipoprotein

H_2O_2, Hydrogen Peroxide

$HO^{\cdot}_2$, Hydroperoxyl Radical

HOAc, Acetic Acid

HOCL, Hypochlorous Acid

HPLTC, High Performance Thin-Layer Chromatography

HRSA, Hydroxyl Radical Scavenging Assay

ICAM-1, Intercellular Adhesion Molecule-1

ICP-OES, Inductively Coupled Plasma Optical Emission Spectrometry

iNOS, Inducible Nitric Oxide Synthase

KAEM, Kaempferol

LDL, Low Density Lipoprotein

LMWA, Low Molecular Weight Anti-oxidant

LOD, Limit of Detection

LOQ, Limit of Quantification

LW UV, Long Wave Ultraviolet

MCP-1, Monocyte Chemoattractant Protein-1

MeOH, Methanol

mM, Millimolar

MnSOD, Manganese Superoxide Dismutase

MRP, Maillard Reaction Product

MS, Mass Spectrometry

MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MYR, Myricetin

NADH, Nicotinamide Adenine Dinucleotide

NHEK, Normal Human Epidermal Keratinocyte

NMR, Nuclear Magnetic Resonance

NOS, Nitric Oxide Synthase

$O_2^{\cdot -}$, Superoxide

$^1O_2$, Singlet Oxygen

O_3, Ozone

·OH, Hydroxyl Radical

8OHdG, 8-Hydroxy-2'-Deoxyguanosine

$ONOO^-$, Peroxynitrite

ORAC, Oxygen Radical Absorbance Capacity

PCA, Protocatechuic Acid

***p*-CA**, *p*-Coumaric Acid

PE, Phycoerythrin

PG COX, Prostaglandin Cyclooxygenase

Pgp, P-glycoprotein

PLE, Pressurised Liquid Extraction

PMN, Polymorphonuclear Leukocytes

QUE, Quercetin

RES, Resveratrol

R_f, Retention Factor

RGJ, Red Grape Juice

RNS, Reactive Nitrogen Species

RO·, Alkoxyl Radical

ROO·, Peroxyl Radical

ROS, Reactive Oxygen Species

RP-HPLC, Reversed-Phase High Performance Liquid Chromatography

RSD, Relative Standard Deviation

RW, Red Wine

SEM, Standard Error of the Mean

SFE, Supercritical Fluid Extraction

SPE, Solid Phase Extraction

SOD, Superoxide Dismutase

SRSA, Superoxide Radical Scavenging Assay

SW UV, Short Wave Ultraviolet

SYR, Syringic Acid

TBARS, Thiobarbituric Acid Reactive Subtances

TE, Trolox Equivalents

TEAC, Trolox Equivalent Anti-oxidant Capacity

TGF-β, Transforming Growth Factor-Beta

TOL, Toluene

TRAP, Total Radical Trapping Anti-oxidant Parameter

TROLOX, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UPLC, Ultra-Performance Liquid Chromatography

VCAM-1, Vascular Cell Adhesion Molecule-1

v/v, volume/volume

WGJ, White Grape Juice

w/v, weight/volume

WW, White Wine

w/w, weight/weight

Chapter 1

INTRODUCTION

1.1. The role of reactive oxygen and nitrogen species in oxidative stress

The use of natural products obtained from plant compounds (bioflavonoids) is an area of increasing interest due to the many reported benefits to human health. These naturally occurring plant secondary metabolites, predominantly found in fruits, vegetables, nuts, tea, medicinal herbs and red wine, have been shown to be associated with a reduced risk of developing chronic diseases associated with oxidative stress, including cardiovascular disease, neurodegenerative diseases, type II diabetes, cancer, and rheumatoid arthritis (Boudet, 2007; Espín et al., 2007). Consequently, these benefits have fuelled growing interest and demand from consumers for health-promoting products. It has been claimed that the main mechanism by which these compounds exert these effects is by acting as anti-oxidants (Leopoldini et al., 2011), although other mechanisms may be involved.

Reactive oxygen/nitrogen species (ROS/RNS) are known to be involved in promoting oxidative damage to biological molecues such as lipids, proteins, and DNA, which can contribute to various oxidative stress-related diseases and pathologies shown in Figure 1 (Halliwell, 2009).

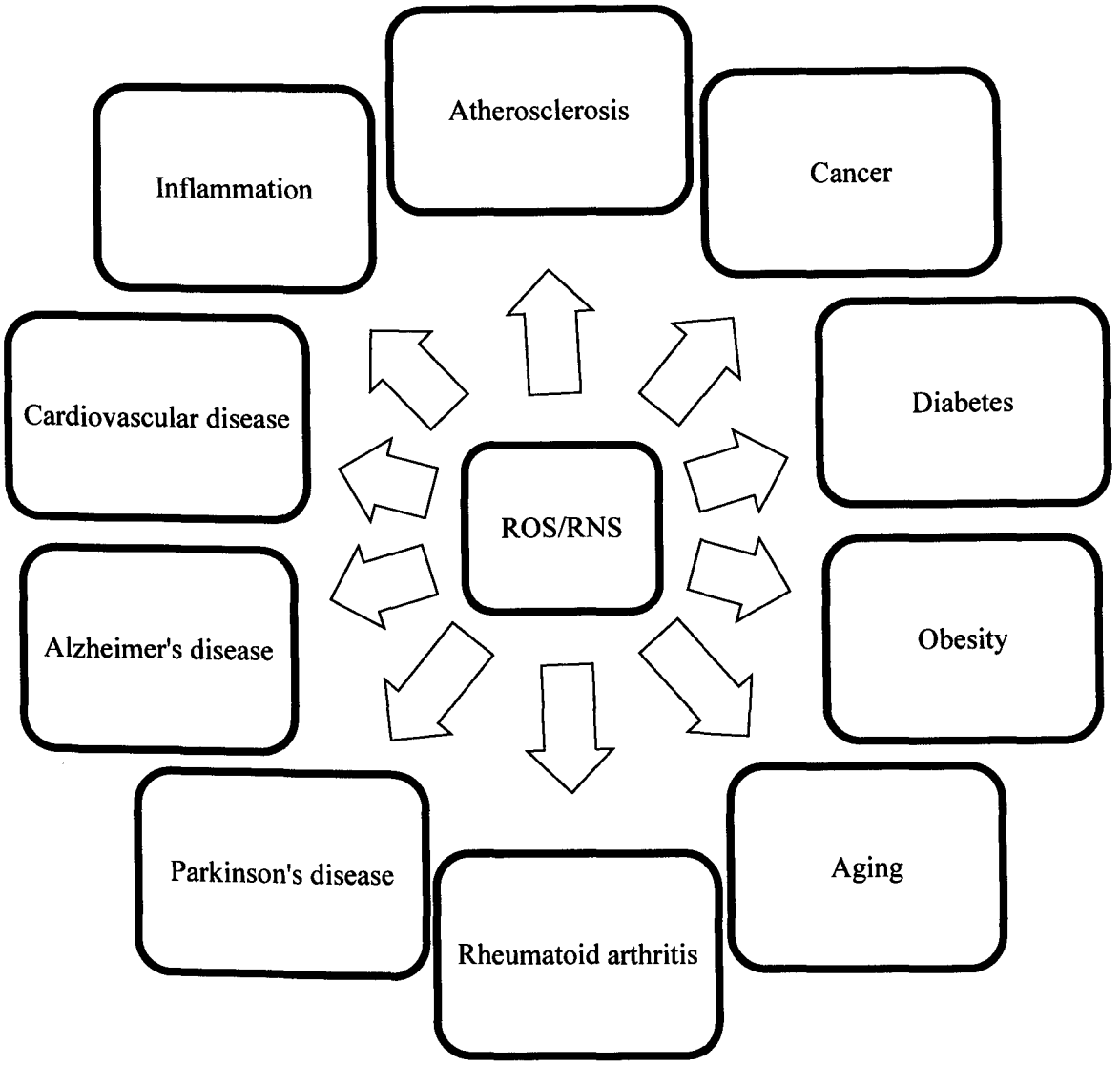

Figure 1: The various disease states and pathologies attributed to reactive oxygen/nitrogen species.

ROS/RNS are derived from exogenous and endogenous sources. Some of the main exogenous sources include: ionising and non-ionising irradiation; pollutants; xenobiotics (pesticides, herbicides); toxins and food oxidation in the gastrointestinal tract (peroxides, aldehydes, oxidised fatty acids, and transition metals) (Limón-Pacheco and Gonsebatt, 2009).

ROS/RNS are also formed as a result of endogenous sources. Mitochondrial respiration is the main site of production of superoxide radicals (Buonocore et al., 2010). Phagocytes (neutrophils, eosinophils, basophils, monocytes, and lymphocytes) produce ROS in response to infection by foreign organisms including bacteria and viruses (Cannizzo et al., 2011). Enzymes such as xanthine oxidase produce ROS indirectly, i.e. as a by-product of their activity. Other enzymes, however, directly produce ROS, and include NO·-and $O_2^{\cdot -}$ -producing enzymes (nitric oxide synthase), and H_2O_2-producing enzymes (peroxidases; peroxisomal oxidases; and monoamine oxidase) (Genestra, 2007). Many diseases associated with impaired metal metabolism, cardiovascular disorders, and inflammation, are also known to be involved in the initiation or production of ROS.

There are two groups of ROS/RNS: radicals and non-radicals. Oxygen radicals include superoxide ion ($O_2^{\cdot -}$), hydroxyl radical (·OH), peroxyl radical (ROO·), alkoxyl radical (RO·), and nitric oxide (NO·). They are characterised by the presence of at least one unpaired electron in the shells surrounding the atomic nucleus. This results in high reactivity due to the ability of the radical to donate or accept another electron (Cannizzo et al., 2011). Non-radical oxygen derivatives include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), aldehydes (HCOR), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$) (Sorg, 2004).

Superoxide exists as either $O_2^{\cdot -}$ or hydroperoxyl ($HO_2^{\cdot}$) depending on the environment and pH. At low pH levels, the hydroperoxyl is the predominant form. The superoxide radical acts as a powerful oxidising agent, reacting with compounds such as tocopherol and ascorbate, which can donate H^+ to the radical. The reaction of the superoxide

radical with another superoxide radical involves the dismutation to H_2O_2 and O_2 as shown below (Kohen and Nyska, 2002):

$$O_2^{\cdot -} \rightarrow O_2 + e^- \qquad k \sim 10^6\ M^{-1}\ s^{-1}$$

$$O_2^{\cdot -} + e^- + 2H^+ \rightarrow H_2O_2$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$$

Hydrogen peroxide is considered to be a relatively stable non-radical. However, it can cause damage to cells at concentrations as low as 10 μM. It is able to oxidise DNA, lipids, -SH groups and keto acids of protein membranes. In addition to this, it plays a key role in the formation of hydroxyl radicals (Kohen and Nyska, 2002).

The hydroxyl radical, ·OH, is a highly reactive damaging radical species which interacts with biological molecules at the site of its production by abstracting H^+ or transferring electrons (Sorg, 2004). It is usually formed by the reaction of H_2O_2 with transition metal ions, mainly Fe and Cu, and is referred to as the Fenton reaction (Buonocore et al., 2010; Valko et al., 2006):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\cdot}OH + OH^-$$

The iron ions are usually chelated to proteins or other molecules in the form of Fe^{3+}. However, a reducing agent can convert ferric ions to ferrous ions. Superoxide can also reduce Fe^{3+} to Fe^{2+} in a process known as the Haber-Weiss reaction (Buonocore et al., 2010; Jomova & Valko, 2011):

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^- \quad (2)$$

Nitric oxide is catalysed by the nitric oxide synthases (NOSs), of which three types of this enzyme occur: neuronal NOS, endothelial NOS (eNOS), and inducible NOS (iNOS) (Dedon & Tannenbaum, 2004). In the reaction, L-arginine is converted to nitric oxide and L-citrulline. The nitric oxide radical ($NO^{\cdot}$) can react with a number of radicals, but when it reacts with superoxide, the highly reactive peroxynitrite ($ONOO^-$) is formed as shown below (Dedon & Tannenbaum, 2004):

$$NO^{\cdot} + O_2^{\cdot-} \rightarrow ONOO^- \qquad k \sim 7.0 \times 10^9\ M^{-1}\ s^{-1}$$

The powerful oxidising agent, ONOOH, which is the protonated form of peroxynitrite, can react with most organic and inorganic biomolecules, causing deleterious effects.

The main biological targets that are susceptible to oxidative damage as a result of ROS are lipids, proteins, and DNA (Genestra, 2007). Damage to lipids, referred to as lipid peroxidation, involves three stages. Initiation is the first stage, and involves the ROS abstracting an H^+ from a methylene group in the lipid membrane. The resulting fatty acid radical retains one electron, which in the presence of oxygen, can react to form $ROO^{\cdot}$. This is referred to as propagation, and is the second stage of lipid peroxidation. These radicals in turn can abstract another H^+ atom from the lipid membrane, and continues until all the unsaturated lipids in the membrane have been oxidised. The final stage in the lipid peroxidation process is termination, which occurs when one $ROO^{\cdot}$ reacts with another radical or an anti-oxidant. However, although fatty acids with one or no double bonds can be oxidised, they cannot undergo the chain lipid peroxidation

process (Laguerre et al., 2007). Protein membranes are another site of attack for various ROS. The highly reactive ·OH and $ONOO^-$ are the key radicals thought to be involved in protein oxidation. Aldehydes, keto compounds, and carbonyls are usually formed as a result of oxidation, fragmentation or degradation of specific amino acid residues of proteins (Cannizzo et al., 2011).

DNA is another key molecule subjected to damage in the presence of ROS. However, although the less reactive ROS such as $O_2^{\cdot -}$ and H_2O_2 do not necessarily cause damage, they can act as sources for the highly reactive radicals (·OH and $ONOO^-$) (Kohen and Nyska, 2002). Interaction of ·OH and $ONOO^-$ with DNA can lead to several types of damage including single- and double-DNA breaks, modification of DNA bases, and damge to the deoyribose sugar (Valko et al., 2007).

When exposed to these radicals, various positions of the DNA molecule are attacked, such as guanine at the C-8 position, giving rise to 8-hydroxydeoxyguanosine (an oxidation product) (Wallace, 2002). Oxidation of adenine, gives rise to 8 (or 4-5-)-hydroxyadenine. Exposure of ·OH and $ONOO^-$ to the pyrimidines, thymine, cytosine, and uracil, can lead to formation of thymine peroxide, thymine glycols, and 5-(hydroxymethyl) uracil, as well as other oxidation products (Wallace, 2002).

1.2. Mechanisms of defence against oxidative stress

Various mechanisms exist to defend against oxidative damage by reactive metabolites. Indirect mechanisms exist such as repair enzymes, metal chelators (transferrin, lactoferrin and caeruloplasmin), and physical defence of biological membranes (Halliwell, 2009; Valko et al., 2007). However, unlike these indirect mechanisms, anti-oxidants directly interact with ROS, ensuring their removal and subsequent protection

of biological sites (Ratnam et al., 2006). When the balance between anti-oxidants and pro-oxidants is disrupted, in favour of the latter, oxidative stress can result (Figure 2).

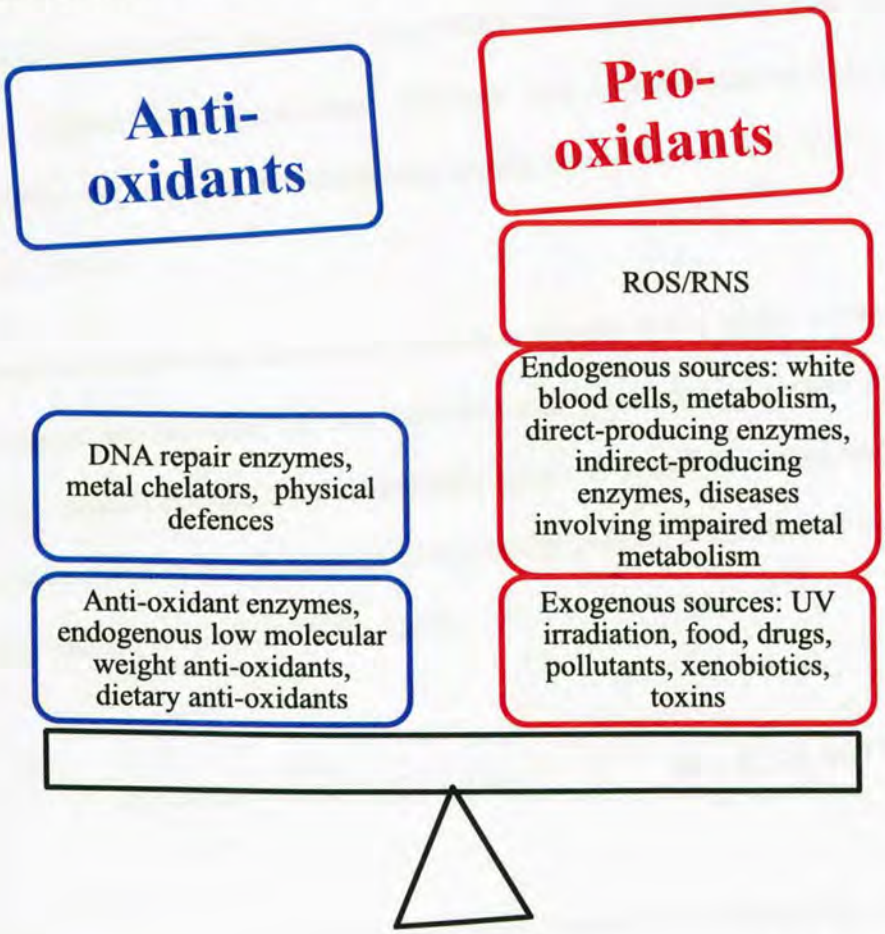

Figure 2: The anti-oxidant/pro-oxidant balance. A disturbance in this balance in favour of pro-oxidants results in oxidative stress.

Anti-oxidants encompass an array of defence mechanisms that help to maintain cellular redox homeostasis. These anti-oxidants comprise: anti-oxidant enzymes, low-molecular weight anti-oxidants (LMWA), and chelating agents (Procházková et al., 2011). Their role is to prevent or inhibit oxidation of substrates and biological molecules by removing pro-oxidants that would otherwise promote oxidative stress.

1.2.1. The enzymatic anti-oxidant network

This network of anti-oxidants includes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Ratnam et al., 2006). Other enzymes also exist, but their role is to support these anti-oxidant enzymes, and include glucose-6-phosphate dehydrogenase and xanthine dehydrogenase among others (Kohen and Nyska, 2002; Valko et al., 2006).

SOD comprises copper-zinc superoxide dismutase (Cu-Zn SOD), which is localised in the cytoplasm and consists of two subunits each possessing an active site, and manganese superoxide dismutase (Mn-SOD) which is found in mitochondria. The enzyme is involved in the dismutation of superoxide radicals to H_2O_2, as shown by the following reaction (Jomova & Valko, 2011):

$$O_2^{\cdot-} + O_2^{\cdot-} \xrightarrow{SOD} H_2O_2 + O_2 \qquad k \sim 2.5 \times 10^9\ M^{-1}\ s^{-1}$$

Catalase, a metalloprotein containing iron haem (porphyrin), catalyses the two-electron dismutation of H_2O_2 to oxygen and water. Following oxidation, the first H_2O_2 molecule produces Fe^{4+} called compound 1. Two H_2O_2 molecules are subsequently converted to oxygen and water as shown in the following reaction (Kohen and Nyska, 2002; Valko et al., 2006):

$$H_2O_2 \xrightarrow{Catalase} (Compound\ 1) \qquad (1)$$

$$(Compound\ 1) + H_2O_2 \rightarrow O_2 + 2H_2O \qquad (2)$$

$$2H_2O_2 \xrightarrow{Catalase} O_2 + 2H_2O \qquad (3)$$

Peroxidases are another group of enzymes involved in removal of H_2O_2. Glutathione peroxidase catalyses the reaction between two molecules of glutathione and one molecule of H_2O_2 to produce oxidised glutathione and water. Unlike catalase, this reaction produces no oxygen (Valko et al., 2006):

$$2GSH + H_2O_2 \xrightarrow{\text{Peroxidase}} GSSG + 2H_2O$$

The supporting enzymes also play an important role in the prevention and removal of ROS. Glucose-6-phosphate dehydrogenase is important in the production of NADPH, which is necessary for regeneration of oxidised anti-oxidants, such as the regeneration of oxidised glutathione (GSSG) to the reduced form (GSH) (Fang et al., 2002).

1.2.2. The non-enzymatic anti-oxidant network

1.2.2.1. Endogenous sources

The array of anti-oxidant enzyme defences allows efficient protection against free radical damage. However, under certain conditions, these defences are not sufficient. The non-enzymatic anti-oxidant network includes low molecular weight compounds (LMWA) which serve to provide further protection against free radical damage. This group is further divided into endogenously or exogenously derived anti-oxidants. Endogenous anti-oxidants are limited, and are either synthesised by the cell or generated as waste products. Those synthesised by the cell include: glutathione, uric acid, lipoic acid, histidine dipeptides, bilirubin, and metal-binding proteins (Fang et al., 2002).

Glutathione (GSH) is another important enzymatic anti-oxidant involved in the removal of H_2O_2. Like catalase it is a tetrameric protein, but each monomer contains selenium at the catalytic site (Valko et al., 2006). One of the functions of this compound is as an electron donor, which involves the removal of H_2O_2 by peroxidase. In addition, it acts as a chelating agent for copper ions. GSH can react with the highly reactive radicals such as ·OH, peroxyl radicals (ROO·), and alkoxyl radicals (RO·) (Fang et al., 2002; Ndhlala et al., 2010).

Histidine dipeptides are comprised of carnosine, homocarnosine, and anserine. These compounds are very efficient scavengers of ROS, as well as effective chelators of transition metal ions. In addition, unlike other anti-oxidants, they have not been found to demonstrate pro-oxidant behaviour (Kohen and Nyska, 2002; Ndhlala et al., 2010). Melatonin is another compound with strong anti-oxidant capacity produced by the pineal gland, and functions in regulating sleep and maintaining circadian rhythm. It also possesses strong anti-oxidant properties by scavenging ROS, possibly by donating a hydrogen atom from the (-NH) group in its structure. Another mechanism of action could be by upregulating the synthesis of anti-oxidant enzymes or enhancing the secretion of other anti-oxidants (Kohen and Nyska, 2002). Uric acid, a cellular waste product of purine metabolism, is a result of the reaction between hypoxanthine and xanthine by the enzyme xanthine oxidase. Urate has been found to be an effective anti-oxidant *in vivo*, able to react with various ROS and chelate transition metal ions (Ndhlala et al., 2010).

The iron proteins, transferrin and ferritin, sequester iron and prevent it from participating in free radical reactions. Ferritin can hold ≈ 2500 ferric ions per molecule, whereas transferrin can bind two ferric ions per molecule. However, in the event of

damage to the protein, the iron can be released from the core of the protein, and thus participate in oxidation reactions (Kohen and Nyska, 2002).

1.2.2.2. Exogenous sources

Although LMWA from endogenous sources contribute to prevention of oxidative stress, LMWA derived exogenously, i.e. from the diet, are the main sources of anti-oxidant intake. These are mainly derived from fruits, vegetables, nuts, and seeds, and include ascorbic acid, tocopherols, carotenoids, and flavonoids. The latter group, flavonoids, is the subject of extensive research due to their many health benefits (Wang et al., 2011).

Ascorbic acid is an efficient water-soluble reducing agent. It can donate two electrons, with donation of one electron resulting in the ascorbyl radical. Although this radical is relatively stable, further oxidation produces dehydroascorbic acid. This compound, dehydroascorbic acid, is not stable however, and oxidises to various decomposition products (Ndhlala et al., 2010). Although ascorbate has been shown to act as a very efficient anti-oxidant *in vitro*, *in vivo* data on its activity is scarce (Traber and Stevens, 2011). Tocopherols comprise a family of lipophilic LMWA, and are collectively known as vitamin E. The vitamin E family consists of eight naturally occurring forms, four tocopherols and four tocotrienols: d-α, d-β, d-γ, and d-δ-tocopherols; and d-α, d-β, d-γ, and d-δ-tocotrienols. However, d-α-tocopherol is the most efficient anti-oxidant of the eight forms (Traber and Stevens, 2011). Oxidation of tocopherol results in tocopherolquinone and tocopherylquinone, which are subsequently regenerated to the reduced form from another reducing agent (Traber and Stevens, 2011).

1.3. Main components of wine

Wine is composed of a complex mixture of organic and inorganic substances (Table 1). Organic substances include volatile and non-volatile compounds. Ethanol is the most prevalent volatile compound (Grindlay et al., 2011). Methanol, esters, and terpenes are other volatile compounds, but these are present at very low concentrations relative to ethanol. Non-volatile compounds include sugars, organic acids, and other substances such as amino acids, polyphenols, and flavonoids (Garrido and Borges, 2011). These latter substances make up less than 1 g/L of wine. The inorganic compounds in wine include salts and metal ions. The most abundant element is potassium, followed by calcium, magnesium, and sodium. Trace metals, such as iron, copper, manganese, and zinc, are present in concentrations ranging from 0.1-10 mg/L (Grindlay et al., 2011).

Table 1: The composition of wine (Grindlay et al., 2011).

Wine composition			**Concentration**
Volatile organics	Ethanol		8-19% (*v/v*)
Non-volatile organics	**Non-volatile alcohols**	Glycerol, butylethylglycol	1-10 g/L
	Sugars	Glucose, fructose, galactose, mannose	1-200 g/L
	Organic acids/salts	Tartaric, malic, citric, acetic	1-8 g/L
	Other substances	Amino acids, flavonoids, polyphenols, etc	< 1 g/L
Inorganic salts	Cl^-, PO_4^{3-}, SO_4^{2-}, SO_3^{2-}		> 10 mg/L
Elements	**Major**	Na, Mg, K, Ca	> 10 mg/L
	Trace	B, Al, Mn, Fe, Cu, Zn, Sr, Rb	0.1-10 mg/L
	Ultratrace	Li, Sc, Ti, V, Cr, Co, Ni, As, Se, Mo, Ag, Cd, Sn, Sb, Ba, rare earth, Hg, Tl, Pb, etc	< 0.1 mg/L

The primary (endogenous) origin of metals in wine comes from natural sources such as soil, grape variety, and climatic conditions during growth. The secondary (exogenous) origin of metals arises from external impurities including environmental pollution, pesticides, fungicides, and fertilisers (Pohl, 2007). Metals also arise during winemaking processes including bottling, aging/storage, process type and equipment, and addition of substances during wine production. These metals may form complexes with organic acids, peptides, proteins, or polyphenols in wines, or exist as free ions (Pohl, 2007; Ibanez et al., 2008).

Consumption of wine has been found to contribute to total daily intake of essential elements, including Fe, Cu, Mn, Co, Ca, and Zn, and others. They also have an important contribution in the winemaking process (Pohl, 2007). The major metals, K, Mg, Ca, and Na, ensure pH and balance in wine by regulating cellular metabolism of yeast. The trace elements Cu, Zn, Fe, and Mn, however, affect taste, flavour, colour, and stability of the wine (Ibanez et al., 2008).

The concentration of total phenolic compounds in red grapes is approximately 1.5 times higher than white grapes (Stockley and Hoj, 2005). Red wine and grape juice contain more than 500 mg/L of flavonoids, whereas white wine and beer comprise less than 60 mg/L (van de Wiel et al., 2001). The main steps involved in the production of red and white wine are shown in Figure 3.

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Figure 3: The main steps involved in the production of red and white wine. Adapted from Pretorius and Høj, (2005).

1.4. Structures of phenolic compounds in red wine

Wine is composed of a complex matrix of phenolic substances. These compounds possess one structural feature, a phenol, which is an aromatic ring possessing at least one hydroxyl substituent (Waterhouse, 2002). They can be further divided into two groups: simple phenols and polyphenols (Figure 4).

The structures of the key phenolic compounds in red wine are shown in Figure 5. Simple phenols are non-flavonoids and include phenolic acids, which possess one carboxylic acid functional group. Hydroxybenzoic and hydroxycinnamic structures are the two classes of simple phenolic acids. Hydroxycinnamic acids include caffeic, chlorogenic, *p*-coumaric, ferulic, and sinapic acids (Waterhouse, 2002). The main hydroxycinnamic acids found in grapes and wines are caftaric acid (caffeoyltartaric acid), *p*-coutaric acid (coumaroyltartaric acid), and fertaric acid (feruloyltartaric acid). The most abundant hydroxybenzoic acids include gallic, protocatechuic, syringic, and vanillic acids (Garrido and Borges, 2011). Stilbenes comprise the other non-flavonoid component of wine, and include piceid, astringin, pterostilbene, and pallidol. However, the most studied stilbene is resveratrol (Garrido and Borges, 2011).

Flavonoids are polyphenolic compounds which comprise a flavane (2-phenyl-benzo-ɣ-pyran) nucleus. This three-ring system consists of two benzene rings (A and B) linked by an oxygen-containing pyran ring (C) (Leopoldini et al., 2011). The major classes of wine flavonoids are flavan-3-ols, flavonols, and anthocyanins. Flavones, isoflavones, and flavanones are other classes found in many foods including fruits and vegetables (Covas et al., 2010; Stockley and Hoj, 2005).

Flavan-3-ols are the most abundant class found in wine and grapes, and are present in both the seeds and skins of grapes. They include two stereoisomers: the *trans* form is

(+)-catechin and the *cis* form is (-)-epicatechin. Unlike other flavonoids in wine, flavan-3-ols are not found as glycosides. Most flavan-3-ols also form polymeric compounds (tannins), i.e. hydrolysable and condensed tannins, and are responsible for the astringent properties of wine. Condensed tannins are the predominant forms in grapes and wine, and occur as polymers of flavonoids linked at positions 4→8 and 4→6 (Waterhouse, 2002). Hydrolysable tannins contain gallic acid or ellagic acid esterified to a carbohydrate such as D-glucose, L-rhamnose, glucorhamnose, galactose, and arabinose (Leopoldini et al., 2011). Flavonols are present in the grape berry skin and also occur in a wide range of other foods. They are found as glycosides, producing glucosides, glucuronides, galactosides, and diglycosides (glucosylarabinoside, glucosylgalactoside, glucosylxyloside, and glucosylrhamnoside). The main flavonols in wine include quercetin, myricetin, and kaempferol (Garrido and Borges, 2011).

Anthocyanins form another major group of compounds in wine and grapes, and other plant-based foods. They are the key compounds responsible for the colour of red wine, and differ from the other flavonoids by the presence of a charged oxygen atom in the C ring (Leopoldini et al., 2011). Anthocyanidins are usually found in their glucosylated form, with glucose, galactose, rhamnose, arabinose, and rutinose being the most common sugar moieties (Garrido and Borges, 2011). There are five main anthocyanins in wine: cyanidin, peonidin, delphinidin, petunidin, and malvidin-the most abundant anthocyanin in red wine (Garrido and Borges, 2011; Waterhouse, 2002).

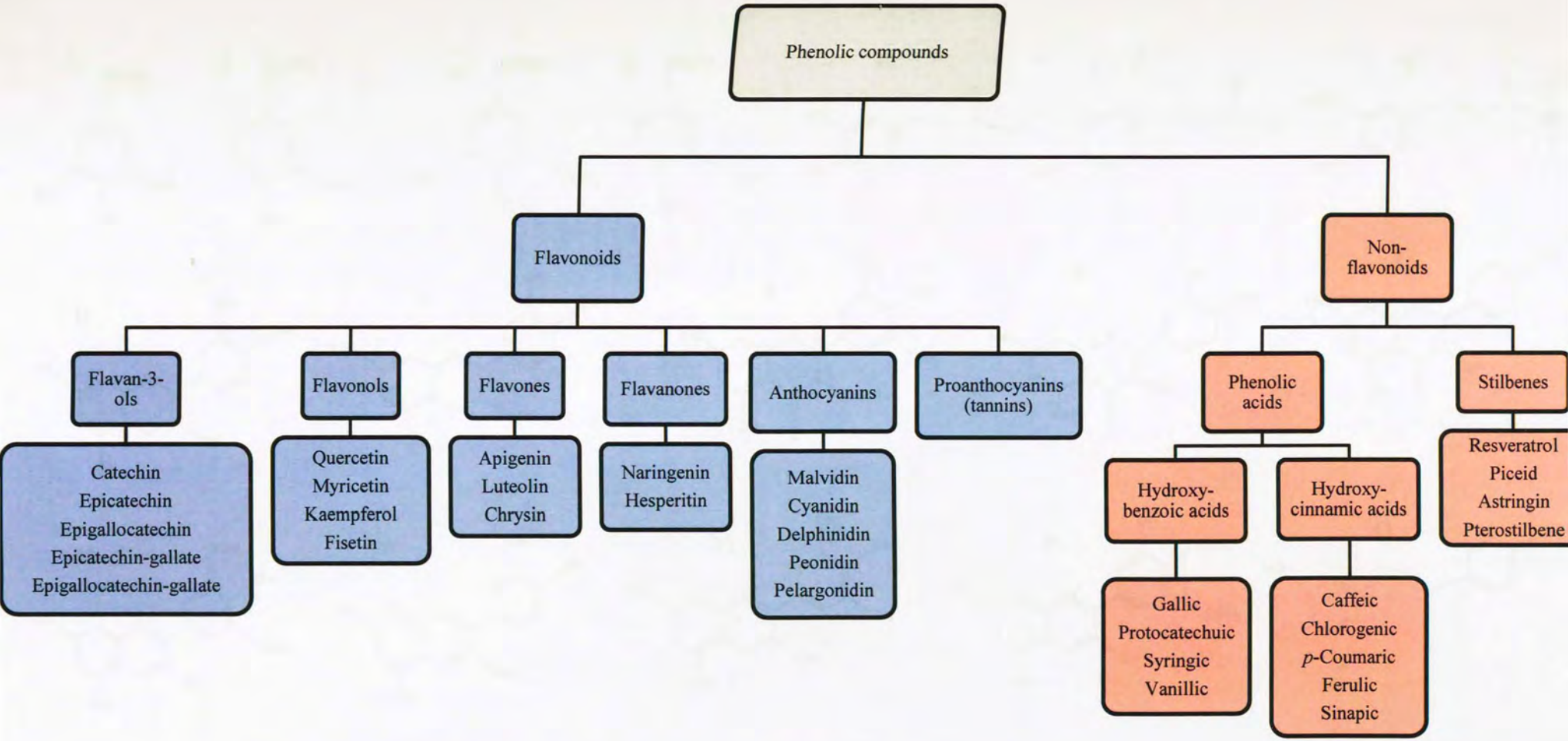


Figure 4: The main phenolic compounds in red wine.

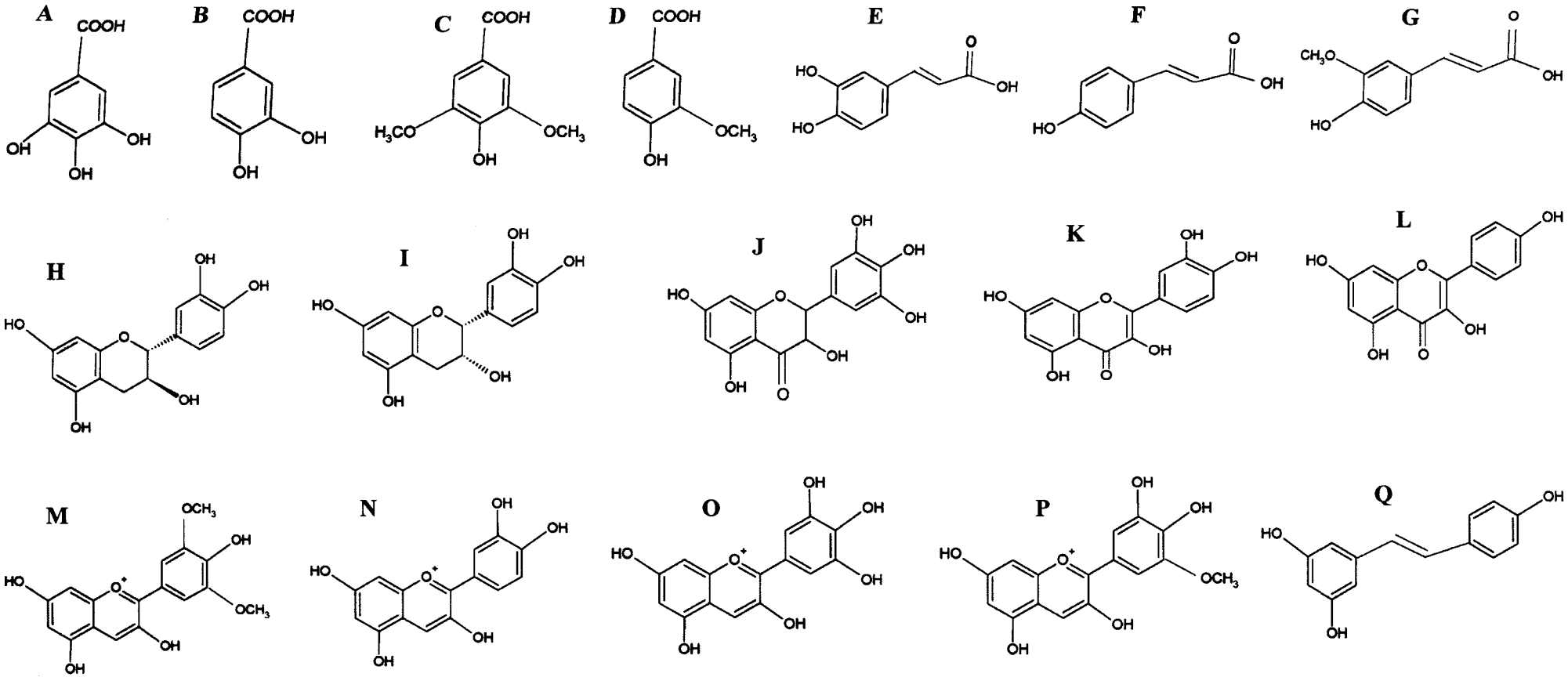


Figure 5: The structures of the key phenolic compounds in red wine: **(A)**=Gallic acid; **(B)**=Protocatechuic acid; **(C)**=Syringic acid; **(D)**=Vanillic acid; **(E)**=Caffeic acid; **(F)**=*p*-Coumaric acid; **(G)**=Chlorogenic acid; **(H)**=Catechin; **(I)**=Epicatechin; **(J)**=Myricetin; **(K)**=Quercetin; **(L)**=Kaempferol; **(M)**=Malvidin; **(N)**=Cyanidin; **(O)**=Delphinidin; **(P)**=Petunidin; **(Q)**=*trans*-Resveratrol.

1.5. Biological effects and mechanisms of phenolic action

Phenolic compounds have been found to exhibit several beneficial health effects as outlined in Figure 6. However, the most studied mechanism is their anti-oxidant action (Halliwell, 2008; Heim et al., 2002; Yoo et al., 2010). The mechanism of action of phenolic compounds can be divided into two groups: general or non-specific mechanisms and specific mechanisms.

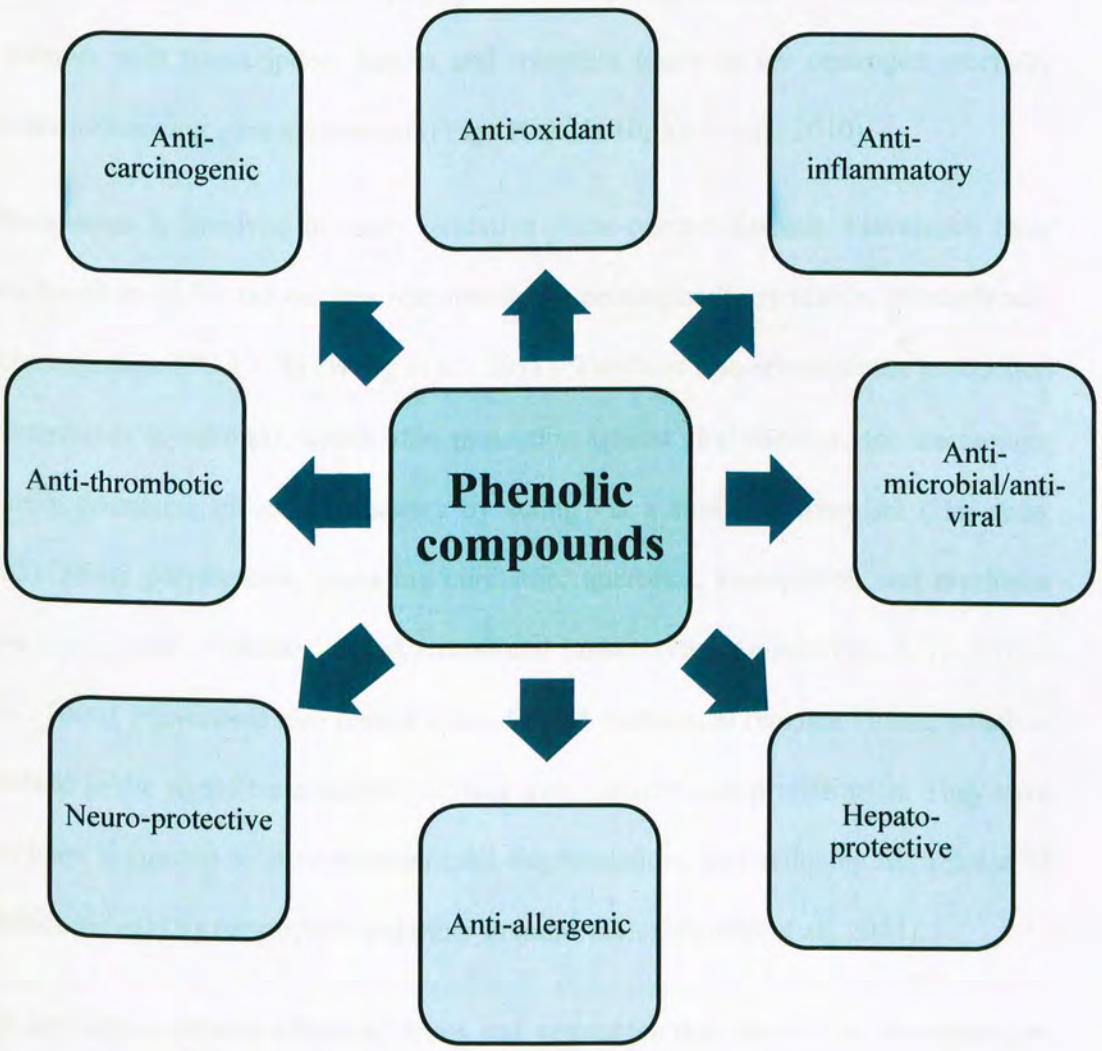


Figure 6: Some of the reported health benefits of phenolic compounds.

1.5.1. Specific mechanisms

Specific mechanisms of phenolic action include interaction with enzymes. The enzymes targeted by phenolics are those with purine as substrates (such as kinases, ATPases, cyclic nucleotide phosphodiesterase, adenylate cyclase, reverse transcriptase, xanthine oxidase, RNA and DNA polymerases, ribonuclease, and human DNA ligase) (Fraga et al., 2010). Other enzymes include those with NADPH as a cofactor (such as aldose reductase, malate dehydrogenase, lactic dehydrogenase, nitric oxide synthase, glutathione reductase, and 11-β-hydroxysteroid dehydrogenase). Phenolics are also able to interact with transcription factors and receptors (such as the oestrogen receptor) thereby influencing gene expression (Fraga et al., 2010; Yoo et al., 2010).

Inflammation is involved in many oxidative stress-related diseases. Flavonoids have been found to inhibit the enzyme responsible for prostaglandin synthesis, prostaglandin cyclooxygenase (PG COX) (Wang et al., 2011). They can also stimulate the production of interferons (cytokines), which offer protection against viral damage, and are thought to have promising effects in diabetes by acting via a similar mechanism (Havsteen, 2002). Many polyphenols, including curcumin, quercetin, kaempferol, and myricetin have been found to inhibit 5-lipoxygenase and 12-lipoxygenase activities *in vitro* (Issa et al., 2006). Flavonoids also reduce cytosolic and membranal tyrosine kinase, which is involved in the signal transduction pathway that regulates cell proliferation. They have also been suggested to prevent neutrophil degranulation, thus reducing the release of arachidonic acid by neutrophils and other immune cells (Nijveldt et al., 2001).

The anti-atherosclerotic effects of fruits and vegetables that are rich in flavonoids are well documented (Mladěnka et al., 2010). They have been found to protect unsaturated fatty acids from peroxidation, and also have an effect on the Na^+/K^+ pump by inhibiting

acidification of lysosomal enzymes. Cholesterol-lowering effects have also been reported, which is thought to be attributed to the inhibition of HMG-CoA reductase, a key enzyme involved in cholesterol biosynthesis (Havsteen, 2002).

Phenolic compounds also play a role in ameliorating hypertension. They appear to inhibit phosphodiesterase, which results in increased flow of water from blood into renal tubular cells, thus lowering blood pressure (Mladěnka et al., 2010). One group of compounds which have been found to have a blood pressure-lowering effect are hydroxyethyl-rutosides (Havsteen, 2002).

Anti-proliferative and chemo-preventive effects of flavonoids in *in vitro* and *in vivo* studies have been reported (Bennett et al., 2012). These compounds are thought to function in a number of ways to inhibit growth of cancer cells. This mainly involves interfering with key regulatory pathways, including growth, apoptosis, cell division, gene repair, transcription, and energy metabolism among others (Issa et al., 2006).

The protein phosphokinases are involved in tyrosine, serine, and threonine phosphorylation. In particular, tyrosine phosphorylation plays a major role in oncogenesis (Reuter et al., 2010). Some flavonoids have been found to prevent tyrosine-specific protein kinases, topoisomerases I and II, and cell division protein kinases, thus reducing growth and cell division (Havsteen, 2002). The energy demand of tumour cells is high, which subsequently leads to failure of the respiratory chain through phosphorylation of the β-chain of the Na^+/K^+-ATPase pump in the membrane. Flavonoids such as quercetin can inhibit this phosphorylation and re-establish normal cell function (Havsteen, 2002; Wang et al., 2011). Phenolic compounds are also able to detoxify carcinogenic environmental toxins, such as aromatic hydrocarbons. They are thought to do this by facilitating the decomposition of these toxins to smaller products,

such as aromatic carbonic acids, which can subsequently be excreted (Wang et al., 2011).

Flavonoids are also effective anti-bacterial agents. They are thought to inhibit bacterial infection by targeting ion channels and inhibiting the enzymes that hydrolyse proteoglycan and the protein meshwork of connective tissues, such as hyaluronidase. Other mechanisms include interference with various bacterial virulence factors, including toxins and signal receptors (Cushnie and Lamb, 2011). The anti-viral properties of flavonoids are also well known. One mechanism of action involves inhibiting the fusion of the viral membrane with that of the lysosome. In addition, they can inhibit the formation of P-glycoprotein (Pgp), which participates in fusion of cell membranes. Some flavonoids, such as quercetin, have also been found to inhibit the reverse transcriptase of RNA viruses (Havsteen, 2002). Recently, polyphenols combined with antibiotics have shown promise as a new strategy to combat microbial resistance (Daglia, 2012).

Flavonoids have also been found to exert neuro-protective effects through a number of mechanisms including interaction with neuronal/glial intracellular signalling pathways and inflammatory mediators, or chelation of redox-active transition metal ions (Spencer et al., 2012). Flavonoids have also been found to play a role in reducing allergy by ameliorating the secretion of histamine and serotonin from mast cells. However, this mechanism is not fully understood (Havsteen, 2002).

1.5.2. Non-specific mechanisms

1.5.2.1. Anti-oxidant action

Non-specific mechanisms refer to the anti-oxidant action of phenolics. Two main strategies are employed: free radical scavenging and metal ion chelation. Anti-oxidants break free radical chain reactions by interfering with initiation or propagation, and subsequent oxidative damage (Procházková et al., 2011). The main structural features responsible for this activity are the phenolic OH groups that remove free radicals by donating an electron, and the aromatic structures that stabilise the resultant aroxyl radicals by resonance (Fraga et al., 2010), as shown below by the following equation:

$$LOO^{\cdot} + AH \rightarrow LOOH + A^{\cdot}$$

Three main structural features of the flavonoid backbone have been identified for efficient radical scavenging (Figure 7A). These include: 1) an *ortho*-dihydroxyl (catechol) structure in the B ring for electron delocalisation, 2) 2,3-double bond in conjugation with a 4-oxo function in the C ring, and 3) hydroxyl groups at positions 3 and 5, which provide hydrogen bonding to the oxo group (Procházková et al., 2011).

Polyphenolics containing a phenol ring have been found to be generally more pro-oxidant than polyphenols containing a catechol ring. The more readily oxidisable flavonoids are the most effective, with catechols possessing a lower redox potential being more readily oxidised than the higher redox potential phenols (Galati et al., 2002).

Another anti-oxidant strategy is chelation of redox-active transition metal ions. When phenolic compounds reduce Fe^{3+} or Cu^{2+}, the reduced forms of the metal ions (Fe^{2+} and Cu^{+}) can form complexes with the phenolic compound rendering these metal ions inert. However, the phenolic compound can regenerate the Fe^{2+}/Cu^{+} from Fe^{3+}/Cu^{2+} by

Fe^{2+}/Fe^{3+} and Cu^{+}/Cu^{2+} recycling. Catechol moieties and combinations of hydroxyl and carbonyl groups are the main centres present in the phenolic structure for which metal ions have high affinity (Malešev and Kuntic, 2007) (Figure 7B). Binding to Fe^{2+}/Cu^{+} will change the redox potential for converting the reduced ion to the oxidised state, thereby reducing oxidative damage (Khokar and Apenten, 2003).

Several studies have measured the anti-oxidant activity of flavonoid-metal ion complexes compared to the free flavonoid. Bukhari et al., (2009), Chen et al., (2009), and Dehghan and Khoshkam, (2012) measured the anti-oxidant activity of quercetin complexed with Cu^{2+}, Cr^{3+}, and Sn^{2+}, respectively. Bukhari et al., (2009) and Chen et al., (2009) found that the anti-oxidant ability of the quercetin complexes were comparatively higher than the free flavonoid. However, Dehghan and Khoshkam, (2012) found that the anti-oxidant activity of quercetin decreased after chelating Sn^{2+}.

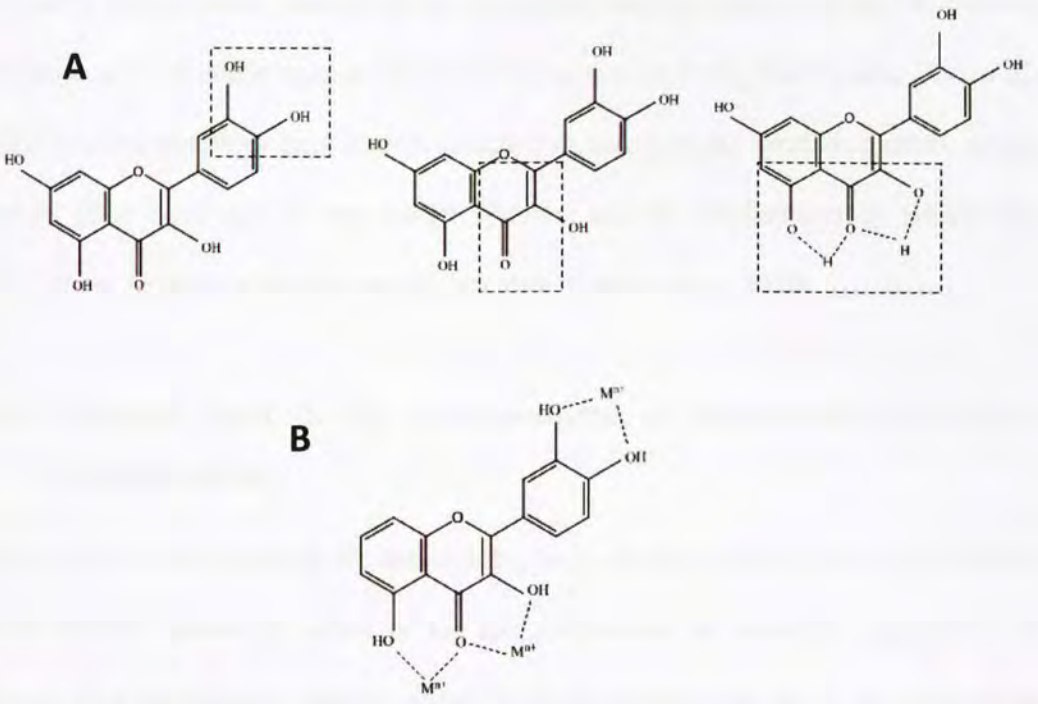


Figure 7: The binding sites on the flavonoid backbone structure involved in (**A**) radical scavenging and (**B**) metal chelation (Procházková et al., 2011).

Catalytic polyphenol-metal complexes have been found to mimic the action of the metal-containing enzymes, SOD and catalase, and have emerged as potential therapeutic agents in attenuating ROS-induced insults (Fraga et al., 2010). They have been found to reduce the deleterious effects of metal ions by different mechanisms, including removal of metal ions, redox-silencing, and dissolution of metal ion deposits (Hague et al., 2006). Many studies have demonstrated the potential SOD mimetic activities of flavonoids such as rutin, taxifolin, epicatechin, and luteolin (Kostyuk et al., 2004) and curcumin (Barik et al., 2007). Catalase-like mimics include metalloporphyrins, salens, and other metal complexes that can dismutate H_2O_2. Glutathione peroxidase-like mimics include mono-selenium and di-selenium mimics, which mimic the action of glutathione peroxidase in H_2O_2 dismutation.

The use of nano-particles as anti-oxidant enzyme mimetics has become a growing area of interest, which unlike natural dietary chelators, display great resistance to extreme conditions and are stable against denaturation, as well as being low in cost (Xie at al., 2012). Dietary chelators have shown promise in ameliorating oxidative stress-related diseases in *in vitro* and *in vivo* model systems, but the mechanisms by which they display these protective actions are still largely unknown (Day, 2009).

1.6. Methods used in the measurement of anti-oxidant and pro-oxidant activity

There are two main methods for determining anti-oxidant activity: direct and indirect. Direct methods assess the effect of the test compounds on oxidative degradation of lipids or lipid membranes, whereas indirect methods measure the ability of anti-oxidants to scavenge ROS/RNS or by transferring hydrogen atoms or electrons (Laguerre et al., 2007). ROS/RNS scavenging assays involve measuring the inhibition of specific

radicals such as SOD, H_2O_2, $^{\cdot}OH$, and $^1O_2$, by addition of anti-oxidant(s) (Antolovich et al., 2002). Hydrogen atom transfer includes oxygen radical absorbance capacity (ORAC), total radical trapping anti-oxidant parameter (TRAP), crocin, and LDL oxidation assays. Electron transfer assays comprise total phenol, ferric reducing anti-oxidant power assays (FRAP), trolox equivalent anti-oxidant capacity (TEAC) or ABTS assay, Cu^{2+} reduction (CUPRAC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays (Antolovich et al., 2002; MacDonald-Wicks et al., 2006; Huang et al., 2005).

Measurement of superoxide anion scavenging ability usually involves the use of xanthine oxidase and xanthine to generate superoxide. The ability of superoxide to reduce nitroblue tetrazolium to formazan is then determined spectrophotometrically at 560nm. Anti-oxidant scavenging of the radical leads to decolourisation, and a decrease in the rate of reaction (MacDonald-Wicks et al., 2006). H_2O_2 scavenging employs peroxide-based assays such as horseradish peroxidase and H_2O_2 to oxidise scopoletin. Chemiluminescence reaction of luminal with hypochlorite is another method to measure H_2O_2 scavenging (Antolovich et al., 2002; Roginsky and Lissi, 2005).

The electron transfer assays are based on the following reaction:

Probe (oxidant) + e (from anti-oxidant) → reduced probe + oxidised anti-oxidant

The TRAP assay measures total anti-oxidant capacity of plasma or serum. Peroxyl radicals are generated from an azo initiator, such as 2,2'-diazobis-(2-amidinopropane) dihydrochloride (AAPH). The oxidation of peroxidisable materials in the plasma or serum is recorded by measuring the oxygen consumed in the reaction. Protein targets are used as probes for assessment of radical scavenging activity in the TRAP assay, and include hydrophilic and lipophilic compounds. Hydrophilic compounds can be

phycoerythrin (PE), fluorescin, DCFH, alizarin red, propylgallate, pyranine, and pyrogallol red. The lipophic compounds include crocin, β-carotene, BODIPY, cis-parinaric acid, and DPPD (Niki, 2010).

The ORAC assay uses phycoerythrin as an oxidisable substrate. When a source of peroxyl radicals is applied, a decrease in the fluorescence of the protein indicates oxidative damage to the protein (MacDonald-Wicks et al., 2006). Prevention of oxidation by an anti-oxidant is monitored against a reference compound, usually trolox (Magalhães et al., 2008). Another method, the FRAP assay, measures the effect of anti-oxidant compounds on the reduction of the Fe^{3+} complex, tripyridyltriazine $Fe(TPTZ)^{3+}$, to the ferrous form $Fe(TPTZ)^{2+}$ (Magalhães et al., 2008).

The TEAC assay measures anti-oxidant activity through the ability of polyphenolic compounds to scavenge the radical cation $ABTS^{\cdot+}$ which is generated through the oxidation of ABTS (2,2'-azinobis(3-ethylbenzothiaziline-6 sulfonate). When a hydrogen atom donor reacts with the radical cation, ABTS is converted from a coloured solution to a non-coloured form of ABTS. The amount of $ABTS^{\cdot+}$ consumed by a phenolic is expressed in trolox equivalents, which is defined as the number of $ABTS^{\cdot+}$ radicals consumed per one molecule of anti-oxidant (MacDonald-Wicks et al., 2006). A similar method, the DPPH assay, assesses anti-oxidant activity by monitoring the decrease in absorbance as the anti-oxidant reduces the DPPH radical from a purple coloured chromophore to colourless (Antolovich et al., 2002).

The total phenol index of a food or beverage sample is usually carried out using Folin-Ciocalteau (FC) reagent due to the ease, reproducibility, and convenience of the test system. Although HPLC analysis is another method that can be used to measure total phenol capacity, the Folin-Ciocalteau is the method that is most commonly used for

measuring total phenolics. The basis of the assay involves measuring the ability of the phenolic compounds in the sample to reduce the FC reagent (MacDonald-Wicks et al., 2006).

Compared to the other hydrogen atom transfer and electron transfer methods, the ORAC assay is more specific, in that it directly measures the capacity of an anti-oxidant to quench free radicals (Prior and Cao, 1999). Although the electron transfer methods are simpler to measure, the $ABTS^{\cdot+}$ and DPPH radicals are not present *in vivo*, with the latter radical being stable, unlike the radicals in living organisms (MacDonald-Wicks et al., 2006). However, the ORAC assay does have limitations. The protein substrate may have an interfering effect in the assay, and although the assay measures the scavenging ability of anti-oxidants against peroxyl radicals which are present *in vivo*, the methods used to generate these radicals are not physiological (Pérez-Jiménez et al., 2008). Although the *in vitro* anti-oxidant capacity assays have many advantages, they may not be reproducible due to the differing experimental conditions and methodologies used to obtain results. Possible interferences include solvents used, and the presence of non anti-oxidant components which may react in the assays and produce an over estimation of anti-oxidant activity (Pérez-Jiménez et al., 2008). In addition, the measured reducing capacity may not reflect the anti-oxidant capacity of the test substance. (Magalhães et al., 2008).

In addition, most of the assays are not suitable for measuring hydrophilic and lipophilic anti-oxidants, except for TEAC and ORAC. It is therefore important to employ a number of different tests to evaluate a test compound/s true anti-oxidant potential (MacDonald-Wicks et al., 2006). Although it is difficult to accurately mimic *in vivo* conditions, these assays do not take into account the bioavailabilty, synergistic effects

of anti-oxidant compounds or storage within tissues (MacDonald-Wicks et al., 2006). Consequently, this has led to results that are incomparable and often conflicting since they are conducted under non-physiological conditions, and may not exert the same effects *in vivo*. (Halliwell, 2008; Halliwell, 2009).

Assays of pro-oxidant action include the deoxyribose assay, bleomycin assay and copper-phenanthroline assay. The deoxyribose assay measures the reduction of Fe^{3+}-EDTA chelates to form OH in the presence of H_2O_2, which subsequently causes deoxyribose degradation. When anti-oxidants such as ascorbate or phenolic compounds are added to the deoxyribose reaction mixture, a reduction of the Fe^{3+}-EDTA complex gives an indication of pro-oxidant action (Aruoma, 2003). Incubation with Fe^{3+}-EDTA, ascorbate, and H_2O_2 has also been found to initiate significant DNA damage. When ascorbate is removed from this system a decrease in DNA base modifications is observed (Aruoma, 2003).

The bleomycin assay employs the anti-tumour antibiotic, bleomycin, to assess pro-oxidant action binding to metal ions such as Fe^{3+}. The assay measures the reduction of the bleomycin-Fe^{3+} complex in the presence of a reducing agent or O_2. The formation of ferric bleomycin peroxide (BLM-Fe^{3+}-O_2H^-) by reaction with hydrogen peroxide results in DNA damage (Aruoma, 2003). The copper-phenanthroline assay, another technique for pro-oxidant activity, measures oxidative damage to DNA bases. Addition of ascorbate or other reducing agents initiates enhanced damage to DNA bases. As with the other systems, the ability of added dietary anti-oxidants to reduce the copper-1, 10-phenanthroline complex gives an indication of pro-oxidant action (Aruoma, 2003).

The use of biomarkers to measure oxidative damage *in vivo* can test for systemic anti-oxidant or pro-oxidant activity. The oxidation products of lipids, proteins, and DNA

which can be measured are many. Potential indicators of lipid peroxidation include: diene conjugates, isoprostanes, malonaldehyde and other aldehydes, 4-hydroxynonenal, ethane and/or pentane in expired air, and thiobarbituric acid-reactive substances (TBARS), which is a common method for analysis of lipid peroxidation (Jackson, 1999). Protein biomarkers include protein carbonyls, hydroperoxide-modified proteins, crosslinked proteins, myeloperoxidases, nitro-,chloro-, bromo-amino acids, and cleavage products (Niki, 2010). The GSSH:GSH content of cells is a common index used to assess cellular oxidative stress. Biomarkers for DNA strand breaks or DNA base oxidation products include comet assays, thymine glycols, 5-hydroxyuracil, 2-8-hydroxyadenine, and 8-hydroxyguanine, among others (Niki, 2010).

1.7. *In vitro* and *in vivo* studies on the anti-oxidant activity of red wine and grape products

Several epidemiological studies have found a decreased risk in developing diseases associated with oxidative stress, particularly cardiovascular disease, and increased consumption of red wine (Avellone et al., 2006; Guarda et al., 2005; Micallef et al., 2007). The French paradox, i.e. a low incidence of coronary heart disease in southern France despite a high fat diet, has been attributed not only to the ethanol component of wine, but also to the phenolic constituents (Gresele et al., 2011). The mechanisms of action appear to be inhibition of platelet aggregation and LDL oxidation, increased HDL, vaso-relaxation, and modulation of endothelial action (Xanthopoulou et al, 2010). Although red wine has been shown to be more beneficial than white wine, there are studies that have found that white wine can provide cardioprotective effects if it is rich in the components hydroxytyrosol and tyrosol (Dudley et al., 2008).

Those who consume wine generally have an approximately 25-35 % lower risk of developing cardiovascular disease than those who consume beers and spirits (Stockley and Hoj, 2005). Moderate consumption is defined as approximately 10 to 20 g alcohol/day. It is unclear however, whether the reductions in cardiovascular disease risk are due to the ethanol component of wine. This is because approximately 90 % of the ethanol is readily absorbed into the bloodstream. In contrast, data suggest that the phenolic component is absorbed much less, and may be 10 to 100-fold less than the quantities measured *in vitro*, to have any biological activity (Stockley and Hoj, 2005).

A study by Karlsen et al., (2007) investigated the effects of daily consumption of 150 mL red wine on biomarkers of anti-oxidant status, oxidative stress and inflammation in men and women. Subjects were randomised to a red wine group or a control group. Those in the red wine group consumed a glass of red wine for three weeks, and biomarkers in blood samples were then analysed. The results showed that daily consumption of red wine did not affect biomarkers of anti-oxidant status, oxidative stress, and inflammation in men. In women, even minor adverse effects were observed in a few biomarkers. However, a study by Noguer et al., (2012) found that intake of alcohol-free red wine increased the activities of endogenous anti-oxidant enzymes in a human intervention study. Subjects followed a low phenolic diet in the first week, and in the second, they drank 300 mL of alcohol-free red wine in addition to the low phenolic diet. The overall results showed that the polyphenolic composition of the wine was responsible for the increase in activities of enzymes rather than the alcohol component.

Epidemiological studies have also found a significant correlation between wine polyphenol consumption and higher levels of omega-3 fatty acids. De Lorgeril et al.,

(2008) found that in a cross-section of 353 male patients with coronary heart disease, moderate wine consumption was related to higher marine ω3 concentrations in plasma.

Resveratrol, a stilbene derivative present in grape skins, has received particular attention due to its protective effects *in vitro* (Pezzuto, 2008). It has been shown to decrease the oxidation of LDL by having a negative effect on the enzymes NADPH oxidase, hypoxanthine/xanthine oxidase, 15-lipoxygenase, and myeloperoxidase, which are involved in LDL oxidation (Gresele et al., 2011). Resveratrol has also been found to exhibit a number of effects in *in vitro* models of endothelial cell interaction including: decreased expression of the adhesive molecules VCAM-1, ICAM-1, MCP-1, and E-selectin; decreased transcription of adhesion molecules; inhibition of NF-κB activation and activator protein-1; reduction in PMN adhesion to thrombin-activated platelets; and reduction in tumour cell (human fibrocarcinoma HT1080) adhesion to endothelial cells; reduction of angiotensin-II induced production of ROS; vasodilation of small arteries; increased formation of NO; and inhibition of growth factors (EGF and TGF-β). (Gresele et al., 2011)

Although several studies have demonstrated the effects of resveratrol and other wine polyphenols in inhibiting platelet aggregation, there are others that show contradictory results. Some have reported no change in platelet aggregation after administration of red or white wine in a group of healthy subjects. Others have also found no difference in platelet aggregation between those who consumed a light or moderate intake of wine compared to those who never consumed wine (Gresele et al., 2011). The discrepancies in these results could be due to the different techniques and variability used to study platelet function (Gresele et al., 2011). Despite the attention given to the beneficial effects of resveratrol, some *in vitro* studies have found that resveratrol is generally a

relatively weaker anti-oxidant compared to the other wine phenolics catechin, epicatechin and quercetin, which are generally found to have better anti-oxidant activity (Yoo et al., 2010).

In cell culture studies, 1-50 μM of resveratrol was found to inhibit cell growth, arrested G1-phase, and induced apoptosis in human epidermoid carcinoma A431 cells (Nassiri-Asl and Hosseinzadeh, 2009; Opie and Lecour, 2007). Resveratrol was also shown to exhibit strong cytotoxic activity towards polyomavirus in mouse fibroblast line 3T6 and human tumour line HL60 cells. Antiviral activity was possibly due to membrane damage and inhibition of movement of the polyomavirus from the ER to cytoplasm (Berardi et al., 2009). There are problems, however, with interpreting data obtained from cell culture studies. Firstly, cells in culture are exposed to a high degree of oxidative stress compared to normal physiological levels (approximately 150 mm Hg under culture compared to 1-10 mm Hg in the human body). These high levels of oxidative stress may change the properties of cells or promote proliferation. Secondly, anti-oxidants are not usually added to cell culture media, thus when polyphenols are added to cultured cells, this can lead to over-interpretations of any beneficial effects observed of the added anti-oxidants. Polyphenols are also known to oxidise easily in various culture media, particularly DMEM, because of the presence of added ferric nitrate which acts as a pro-oxidant (Halliwell, 2008).

Pharmacological studies on grape seed proanthocyanidin extract (GSPE) have demonstrated their anti-oxidant, cardioprotective, hepatoprotective, antimicrobial/antiviral, anticarcinogenic, and CNS effects. It was reported that concentrations of 2 mg/L, 50 mg/L, and 100 mg/kg GSPE provided protection against free radical-induced damage. They have been found to inhibit xanthine oxidase activity,

and at low concentrations of 10 μM and 25 μM, have been reported to protect against DNA oxidative damage. However, DNA damage at higher concentrations of 150 μM has also been reported. Consumption of GSPE has been found to provide cardio-protection by reducing ischemia/reperfusion damage in rats. Endothelium dependent relaxation of blood vessels has been suggested to be due to activation of the AKT/PI3 kinase signalling pathway by GSPE (Leifert and Abeywardena, 2008; Nassiri-Asl and Hosseinzadeh, 2009).

The effect of a 300 mg proanthocyanidin-rich grape seed extract was investigated in two groups of healthy volunteers who consumed a lipid-rich meal with or without (control) the extract. It was found that levels of plasma lipid hydroperoxide and LDL oxidation were significantly reduced (Natella et al., 2002). Similarly, it has also been reported that intake of 240 mL of red wine daily in healthy individuals who consumed a high-fat diet, inhibited endothelial dysfunction. Red grape polyphenol extract also improved endothelial function in patients with coronary artery disease (CAD) (Gresele et al., 2011). Jayaprakasha et al., (2001) investigated the anti-oxidant activity of grape seed extracts using the β-carotene-linoleate model and linoleic acid peroxidation method. It was found that 100 ppm grape seed extracts exhibited good anti-oxidant activity of 65-90 %, and also showed good reducing power at a concentration of 500 μg/mL. The results suggested that these activities were due to the presence of phenolic compounds, which contributed to the high anti-oxidant effects observed.

Supplementation with purple grape juice, grape powder, grape seed proanthocyanidins, and red grape juice has also been found to improve coronary blood flow, decrease plasma cholesterol, decrease platelet aggregation, and decrease atherosclerotic lesions (Leifert and Abeywardena, 2008; Opie and Lecour, 2007). Clinical studies have shown

that administration of 4-8 mL/kg/day of purple grape juice or red grape polyphenol extract to adults with CAD, improved flow-mediated vasodilation (FMD), reduced LDL oxidation susceptibility, increased plasma anti-oxidant capacity, and improved endothelial function. The anticarcinogenic effects of extracts and compounds from grapes are also well documented. 30 μg/mL of GSPE was found to inhibit UVB–induced H_2O_2, lipid peroxidation, and DNA damage in UVB-induced photocarcinogenesis of normal human epidermal keratinocyte (NHEK) cells (Nassiri-Asl and Hosseinzadeh, 2009).

A number of *in vitro* anti-oxidant activity assays have been employed for investigating red wine. Results differ between studies due to variation in polyphenolic content of wines arising from differences in geographical origin, grape variety and type of wine. Tabart et al., (2009) employed five different methods (TEAC, DPPH, ORAC, haemolysis, and electron spin resonance (ESR)) to measure the anti-oxidant capacity of selected beverages. A standardised method of reporting anti-oxidant capacity by using a weighted average of the four methods, found that red wine had the highest anti-oxidant activity. The order of anti-oxidant activities for the beverages were: apple (84 μmol TE/100mL) < vegetable juice (117 μmol TE/100mL) < grape juice (176 μmol TE/100mL) < orange juice (198 μmol TE/100mL) < ice green tea (256 μmol TE/100mL) < red wine (402 μmol TE/100mL).

A study by Seeram et al., (2008) found that pomegranate juice had the greatest anti-oxidant capacity, with an overall anti-oxidant index of at least 20 % higher compared to other beverages. Red wine exhibited the next highest anti-oxidant capacity. Using similar assays as above, it was shown that the anti-oxidant potency of each beverage was in the order: pomegranate juice > red wine > Concord grape juice > blueberry juice

> black cherry juice > açai juice > cranberry juice > orange juice, iced tea beverages, apple juice.

However, although these studies demonstrate the many beneficial health effects of red wine and other grape-based products, there is still a need for further research into the absorption, distribution, and metabolism of phenolic compounds after consumption. In addition, characterisation of the compounds in various foodstuffs needs to be investigated to find out which components are responsible for exerting these beneficial health effects. The low bioavailability of polyphenols, after undergoing metabolism, means that any described health effects may be due to their metabolites or degradation products, rather than the compound tested (Del Rio et al., 2010). As well as this, further research *in vivo*, such as human clinical trials, is needed to fully evaluate the effects of phenolic compounds to health. Limited information exists about the toxicology of excess flavonoid intake, with excessive intake possibly leading to pro-oxidant and mutagenic affects. Therefore establishing the safe range of consumption of these compounds is needed (Skibola and Smith, 2000). Emerging research is focussed on identifying the cellular targets responsible for the effects of polyphenols, and developing target-specific therapies with enhanced efficacy and low potential adverse effects (Chung et al., 2012).

1.8. *In vitro* studies on the pro-oxidant activity of natural products

Much research has demonstrated the anti-oxidant benefits of consuming a diet rich in polyphenolic compounds obtained from fruits and vegetables among others. However, the number of studies on the pro-oxidant activity of these compounds is less well studied. Pro-oxidant activity is characterised by an imbalance between oxidants and anti-oxidants in favour of the former, and is determined by a number of factors,

including the structure of anti-oxidant compounds, chemical environment, and experimental conditions (such as presence of transition metal ions) (Halliwell, 2008). Phenolic compounds reduce the oxidised states of metal ions such as Cu^{2+} or Fe^{3+} to Cu^{+} or Fe^{2+}, respectively (Procházková et al., 2011). The reduced forms of these metal ions are subsequently capable of catalysing the formation of the highly toxic hydroxyl radical from hydrogen peroxide through a Fenton-like or Haber Weiss system (Bartosz, 2009; Gaetke and Chow, 2003). However, metal ions present in many foods can also contribute to ROS formation and oxidative stress.

Epidemiological studies have found a decreased risk of developing oxidative stress-related diseases and consumption of vitamins C and E. However these vitamins and carotenoids have been found to act as pro-oxidants in many studies (Rietjens et al., 2002). In fact the pro-oxidant activity of vitamin C, in combination with a transition metal ion, is used as a test for inducing oxidative stress. In addition, the anti-carcinogenic and apoptosis-inducing activity of vitamin C has been attributed to its pro-oxidant effects. Results vary though, with some large-scale studies reporting beneficial effects, whereas others report adverse effects of these anti-oxidant vitamins (Rietjens et al., 2002). In a study by Suh et al., (2003), human plasma was either treated with ascorbate or left untreated, and subsequently incubated with Fe^{2+}, Cu^{2+}, and/or H_2O_2. Rather than acting as a pro-oxidant, ascorbate was found to exhibit anti-oxidant effects by inhibiting lipid peroxidation as well as protein oxidation in human plasma.

The pro-oxidant effects of Fe^{3+} and Cu^{2+} metal ions was studied by Letelier et al., (2010) using rat liver cytosol and microsomes as biological systems, in order to determine the pro-oxidant activities and non-specific binding properties of these metal

ions. The authors found that Cu^{2+} displayed more pro-oxidant effects than Fe^{3+}, thus promoting lipid peroxidation.

Many studies have reported on the pro-oxidant effects of tea, particularly its key compounds. Epicatechin (EC) and epigallocatechin-3-gallate (EGCG), two flavonoids present in green teas, were found to exhibit pro-oxidant effects in the presence of Cu^{2+} ions, which led to oxidative DNA degradation. In addition, EGCG led to a greater rate of DNA cleavage compared to EC (Azam et al., 2004). Hayakawa et al., (2004) reported similar findings. The pro-oxidative ability of tea catechins was measured by monitoring the generation of H_2O_2 and $^{\cdot}OH$ in the presence of Cu^{2+}, Fe^{3+}, and Fe^{2+} ions. EGC was found to accelerate the generation of H_2O_2 and $^{\cdot}OH$ in the presence of Cu^{2+}, while EGCG displayed less pro-oxidative activity than EGC.

The pro-oxidant activity of tea has also been measured using carbonyl formation in human serum albumin (Ishii et al., 2010). Catechins possessing a galloyl group induced a greater formation of protein carbonyl in human serum albumin than those catechins lacking this group. In a study on the effects of (+)-catechin on haemoglobin-induced damage, Lu et al., (2011) found that catechin was able to efficiently act as a free radical scavenger to remove cytotoxic ferryl haemoglobin. However, haemoglobin-H_2O_2-induced protein oxidation was significantly increased in the presence of lower concentrations of catechin (0.005-0.1 mM), but was inhibited at higher concentrations (0.5 mM or higher).

Some cell culture studies have shown that EGCG or green tea extract exert pro-oxidant effects in several types of cells including Jurkat T cells, oral cell carcinoma cell lines, ovarian cancer cells, PC12, H260 and RAW264.7 cells (Halliwell, 2008). Ascorbate has also been found to induce apoptosis in HL-60 cells, acute myeloid leukemia cells, and

human fibroblasts. Other studies have found myricetin to exhibit toxic effects in Chinese hamster lung fibroblast V79 cells, quercetin and catechin towards pancreatic β cells, cyanidn-3-rutoside towards HL60 cells, and grape seed extract to Caco-2 cells (Halliwell, 2008). Babich et al., (2008) used the neutral red cytotoxicity assay, intracellular glutathione assay, cell-free assay for authentic glutathione, H_2O_2 assay and lipid peroxidation to study the black tea theaflavin monomers, theaflavin-3-gallate and theaflavin-3'-gallate. The authors found that these compounds acted as pro-oxidants and induced oxidative stress, with more pronounced cytotoxicity towards cancerous cells compared to normal cells. However, the problem with interpreting these data is that these pro-oxidant effects occur mainly through generation of H_2O_2 when polphenols are added to cell culture media. Thus, the toxic effects observed may not be a result of the anti-oxidant test compound, but rather the generation of H_2O_2 (Halliwell, 2008).

As well as redox-active metal ions, the pro-oxidant activity of phenolic compounds and mixtures is dependent upon their concentration. Some compounds have been found to exhibit anti-oxidant effects at low doses, whereas the same compounds in other studies have been found to exert pro-oxidant effects at high doses (Bouayed and Bohn, 2010). A study by Huang et al., (2011) demonstrated the effect of concentration on the anti-oxidant and pro-oxidant activity of curcumin. Cu^{2+}-induced damage to neuronal cells was employed as a test to measure pro-oxidant activity. At a high dosage curcumin caused an increase in intracellular ROS in the presence of Cu^{2+}, but at a lower dose, curcumin reduced oxidative damage intracellularly. In another report, microsome membrane rat hepatocytes challenged with CCl_4 were used to study the anti-oxidant effects of six vegetables belonging to the *Cichorium* genus. The results showed that red vegetables contained stronger anti-oxidant properties than green vegetables. High molecular weight compounds (> 3500 Da) exhibited higher anti-oxidant activity than

those of a low molecular weight. However, the low molecular weight fractions, i.e. < 3500 Da, displayed pro-oxidant capacity in the microsome membrane rat hepatocytes (Papetti et al., 2002).

Food processing, such as thermal treatment, dehydration, and storage, can also affect the anti-oxidant and pro-oxidant activity of phenolic compounds (Andueza et al., 2009). A study by López-Galilea et al., (2006) looked at the anti-oxidant and pro-oxidant activity of different coffee blends. Commercial roasted coffees and torrefacto (roasting process carried out in the presence of sugar) roasted blends were compared to determine the effect various roasting processes had on the anti-oxidant capacity of coffee. The DPPH assay was used to determine anti-oxidant capacity and the crocin bleaching method was used to determine pro-oxidant activity. It was found that the addition of sugar at the end of the roasting process had a greater effect on anti-oxidant capacity compared to the commercial blends. This was thought to be because sugar is involved in the formation of Maillard reaction products (MRPs), formed as a consequence of degradation of the natural anti-oxidants such as caffeic and chlorogenic acids present in coffee.

Similarly, in another study using the crocin bleaching assay, it was found that short heat treatments reduced the anti-oxidant properties of milk, whereas applying higher temperatures resulted in an increase, and possible recovery of anti-oxidant capacity (Calligaris et al., 2004). This is in agreement with a study by Roy et al., (2007) who studied the effects of different thermal treatments on the anti-oxidant and pro-oxidant activity of selected allium vegetables. Subjecting these extracts to temperatures of 75 or 100 °C resulted in enhanced total anti-oxidant activity, and decreased levels of pro-oxidant components. Manzocco et al., (2002) found different results however. Commonly consumed foods such as bread and pasteurised skimmed milk, were found to

exert significant pro-oxidant activities upon heat treatment using kinetic analysis of crocin bleaching. Milk exhibited significantly higher pro-oxidant activity than that of the bread extract. The non-enzymatic browning products formed during the heating process were thought to contribute to these pro-oxidant activities.

Andueza et al., (2009) studied the anti-oxidant and pro-oxidant activity of caffeic acid subjected to thermal treatment at 90° over an increasing time period. Initial tests showed a significant increase in pro-oxidant activity and related pro-oxidant compounds. However, further heating showed an increase in anti-oxidant activity, and thus decrease in pro-oxidant degradation products, possibly due to formation of polymers with higher anti-oxidant activity.

Gazzani et al., (1998) found that some commonly consumed vegetables, including carrot, cauliflower, celery, eggplant, garlic, mushroom, onion, white cabbage, white potato, tomato, yellow bell pepper, and zucchini had increased anti-oxidant activity over time at increasing levels of thermal treatment using the β-carotene-linoleic acid test. However, it was found that tomato and yellow bell pepper always exhibited pro-oxidant activity. Another study (Girard-Lalancette et al., 2009) used a cell-based assay using 2′,7′-dichlorofluorescin-diacetate and ORAC assay to measure the anti-oxidant/pro-oxidant activity of fruit and vegetable juices. The cell-based assay revealed a pro-oxidant effect of broccoli and carrot juices, which was not seen in the ORAC assay, and suggested that the carotenoids present in both these juices could possibly be responsible for these pro-oxidant effects. However, it was also found that boiling these juices inhibited the pro-oxidant effect, and led to a recovery of anti-oxidant capacity.

Dorman and Hiltunen, (2011) studied various herbs and spices using the FRAP, $DMPD^{\cdot+}$, β-carotene-linoleic acid, hydroxyl radical-mediated phospholipid degradation, and

alkylperoxyl radical-mediated protein degradation methods. All extracts were found to prevent hydroxyl radical-mediated DNA degradation. Juniper, laurel and basil, which had a high phenolic content and anti-oxidant activity, also had a pro-oxidative effect on BSA. These three herbs were also more efficient than the other extracts in the FRAP, $DMPD^{\cdot+}$, β-carotene-linoleic acid and hydroxyl radical scavenging assays.

A study of ten plant extracts using various assays including DPPH, xanthine-oxidase, inhibition of lipid peroxidation, and deoxyribose degradation assays showed that all extracts exhibited anti-oxidant activity in at least one assay, except for few flowered garlic and cherry plum. However, oregano and horse mint were among those that showed significant pro-oxidant effects in the deoxyribose degradation assay (Motamed and Naghibi, 2010). Another study (Ling et al., 2010) used the DPPH and the ferric reducing anti-oxidant power assay to measure anti-oxidant and pro-oxidant activity of various plant extracts, respectively. A ratio of anti-oxidant/pro-oxidant activity was developed to evaluate net anti-oxidant capacity (called the ProAntidex). The lower the ProAntidex of a sample, the more efficient the anti-oxidant potential. Among the ethanolic extracts, Nephelium lappaceum peel, Fragaria x ananassa leaf, Lawsonia inermis leaf, Syzygium aqueum leaf and grape seed showed lower pro-oxidant activity compared to Emblica (an anti-oxidant agent with very low pro-oxidant activity). Aqueous extract of Nephelium mutobile also showed a lower pro-oxidant capacity than Emblica. Most of the extracts also exhibited a lower pro-oxidant potential compared to vitamin C.

Other experimental conditions such as pH can also affect anti-oxidant and pro-oxidant activity. A study by Keceli and Gordon, (2002) on the anti-oxidant activity of olive oil, found that in the presence of Fe^{3+} ions, there was marked reduction in anti-oxidant

activity of the extracts, whereas, in the absence of Fe^{3+}, olive oil demonstrated strong anti-oxidant effects. However, the phenolic compounds exhibited a pro-oxidant effect at pH 5.4 in the presence of Fe^{3+}, probably due to the oxidation of the *o*-diphenolic structure of caffeic acid and related molecules present in olive oil. Moran et al., (1997) measured the anti-oxidant and pro-oxidant properties of phenolic compounds from soybean nodules towards inhibition of DNA, deoxyribose and linoleic acid oxidation. All phenolics tested were found to chelate Fe^{3+} ions and subsequently inhibit deoxyribose degradation in the absence of EDTA. Like the study by Keceli and Gordon, (2002), pH was found to affect anti-oxidant activity, with phenolics possessing catechol, pyrogallol, or 3-hydroxy-4-carbonyl groups, showing a more potent reducing or chelating activity at pH 5.5. However, these phenolics were observed to promote DNA and deoxyribose degradation (in the presence of EDTA), but prevented linolenic acid peroxidation.

Catalytic metals are present in salt and buffer solutions used in many experimental studies. It has been found that these concentrations can range from 1-10 μM for iron, and ~ 0.1 μM for copper (Buettner and Jurkiewicz, 1996). Although the levels of copper are lower than that of iron, copper is ≈ 80 times more efficient at oxidising ascorbate compared to iron. Thus, copper is the main metal ion involved in catalysing ascorbate oxidation in a typical phosphate buffer. It was reported that the metals present in the Krebs-Henseleit buffer resulted in accumulation of high levels of iron and copper in isolated hearts. However, when these metals were removed using a chelating resin, no detrimental effects occurred (Buettner and Jurkiewicz, 1996). In addition to buffers, equipment and glassware also contain substantial amounts of iron. These findings demonstrate that experimental results have to be interpreted with caution when studying the role of redox-active metal ions in free-radical oxidation reactions.

1.9. Aim and objectives

Aim:

This study aimed to contribute to existing knowledge by determining the anti-oxidant and pro-oxidant profiles of red wine and its individual phenolic compounds in the presence of various oxidant systems, involving hydrogen peroxide and metal ions (Fe^{3+} and Cu^{2+}).

Objectives:

- Develop a new functional-based TLC screening method to monitor the loss of phenolic compounds in wine upon challenge with five model oxidant systems, including hydrogen peroxide and redox-active metal ions. Compare and contrast individual red wine anti-oxidant efficacies with standard phenolic compounds. Determine effect of matrix and dose on activity. Rank anti-oxidant efficacy of these phenolic compounds, and oxidant system strength.

- Develop a new quantitative reversed-phase HPLC method to assess the anti-oxidant efficacies of both the red wine phenolic compounds and standard phenolic compounds in the presence of the five oxidant sytstems. Use spectrophotometric analysis to determine the Fe^{2+} and Cu^{2+} metal binding activities of these phenolics. Determine effect of matrix and dose on activity. Rank anti-oxidant efficacy of these phenolic compounds, and oxidant system strength.

- Assess the pro-oxidant activity of red wine and phenolic standards using the hydroxyl radical-mediated deoxyribose degradation and linoleic acid peroxidation assays. Use RP-HPLC to assess the anti-oxidant activity of red wine and phenolic standards. Use spectrophotometric analysis to determine the Fe^{2+} and Cu^{2+} metal binding activities of these phenolics. Determine effect of matrix and dose on activity. Rank anti-oxidant and pro-oxidant activities of these phenolic compounds, and oxidant system strength.

- Evaluate the oxidant profiles of other grape-based products using modifications of the well known DPPH and ABTS assays, to measure the effect of Fe^{3+} and Cu^{2+} redox-active metal ions on anti-oxidant activity.

Chapter 2

MATERIALS AND METHODS

2.1. Chemicals and reagents

All chemicals, solvents, and phenolic standards were of HPLC or of analytical grade, purchased from Sigma Aldrich (Poole, UK). See Table 2.

Table 2: List of chemicals, reagents, and phenolic standards.

Category	Chemical name	Purity grade
Solvents	Methanol	HPLC grade ≥ 99.9 %
	Water	HPLC grade
	Acetonitrile	HPLC grade ≥ 99.9 %
	Toluene	HPLC grade ≥ 99.9 %
	Formic acid	~ 98 %
	Ethyl acetate	HPLC grade ≥ 99.9 %
	Acetic acid	≥ 99.0 %
	1-Butanol	HPLC grade ≥ 99.7 %
	Orthophosphoric acid	HPLC grade 85-90 %
	Diethyl ether	HPLC grade ≥ 99.9 %
Inorganic salts	Ferric chloride dihydrate	Reagent grade, 97.0 %
	Ferrous chloride tetrahydrate	≥ 99.0 %
	Cupric chloride dihydrate	ACS reagent, ≥ 99.0 %
	Cuprous chloride	Reagent grade, 97.0 %
	Ethylenediaminetetraacetic acid (EDTA)	≥ 98.0 %

Table 2 (continued): List of chemicals, reagents, and phenolic standards.

TLC derivatisation reagents	2-Aminoethyl diphenylborinate & polyethylene glycol 4000	
	Phosphomolybdic acid solution	
Phenolic standards	Gallic acid, Protocatechuic acid, Syringic acid, Vanillic acid, Caffeic acid, *p*-Coumaric acid, Chlorogenic acid, Sinapic acid, Ferulic acid, Luteolin, Apigenin, Naringenin, Trans-resveratrol, Quercetin, Quercitrin, Myricetin, Kaempferol, (+)-Catechin, (-)-Epicatechin, Malvidin-3-galactoside chloride, Cyanidin chloride, Delphinidin chloride, L-ascorbic acid, Morin, Rutin, Trolox	≥ 90% - ≥ 99%
Assay reagents	2,2-Diphenyl-1-picrylhydrazyl (DPPH)	
	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	≥ 99.0 %
	Folin & Ciocalteau's phenol reagent	
	2-Deoxy-D-ribose	≥ 99.0 %
	Linoleic acid	≥ 99.0 %
	2-Thiobarbituric acid	≥ 98.0 %
	Trichloroacetic acid	≥ 99.0 %
	Ammonium thiocyanate	99.99 %
	Hydrogen peroxide (30%) *w/w* in water	
	Potassium persulphate	≥ 99.0 %
	Phosphate buffered saline (PBS) tablets	

2.2. Instrumentation

A list of instrumentation used in this study is given in Table 3.

Table 3: List of instrumentation and equipment.

TLC plates	10 x 10 cm TLC glass-backed silica gel 60 F_{254} plates-0.25 mm layer thickness (Sigma Aldrich, Poole, UK); 20 x 20 cm Analtech reversed-phase TLC silica gel C_{18} plates-250 μm layer thickness (Sigma Aldrich, Poole, UK); 10 x 10 cm Nano silica gel Adamant on TLC plates-0.2 mm layer thickness (Sigma Aldrich, Poole, UK)
TLC developing tank	All-glass rectangular TLC developing tank
UV lamp	UVP UVGL-58 Handheld UV lamp 254/365 nm
HPLC instrument	Perkin Elmer HPLC system 200 Series (Cambridgeshire, UK) equipped with: quaternary LC pump; vacuum degasser; automatic injector; auto sampler; diode array-UV-visible detector; totalChrom data software
HPLC columns	250 mm x 4.6 mm i.d., 5 μm Ascentis RP-amide C18 (Sigma Aldrich, Poole, UK); 150 mm x 4.6 mm i.d., 5 μm, Ascentis C-18 (Sigma Aldrich, Poole, UK)
LC-MS	Waters 2487 dual wavelength UV absorbance detector;Waters 2690 separations module
Syringe filters	0.45 μm Whatman Puradisc PTFE filters
HPLC vials	2 mL amber glass screw top vials
UV-vis spectrophotometer	Varian UV-visible spectrophotometer (Cary 300 BIO)
Cuvettes	Plastibrand 1.5-3.0 mL standard disposable cuvettes
ICP-OES	Jobin Yvon Ultima 2C ICP spectrometer

2.3. Wine, grape juice, and grape samples

Three wines: red wine (Jacob's Creek Shiraz Cabernet, vintage 2006 and 2008, product of Australia), white wine (Jacob's Creek Semillon Chardonnay, vintage 2007, product of Australia) and rosé wine (J.P. Chenet Cinsault-Grenache, vintage 2007, product of France); two commercial grape juices: red grape juice (Sunpride Pure Pressed Red Grape) and white grape juice (Tesco Pure Pressed White Grape); and two varieties of grapes: red seedless, Flame and green seedless, Thompson were tested. Oenological analyses of the wines are given in Table 4 which was obtained from the company's website.

Table 4: Oenological analyses of the wines.

Analysis	Red wine	White wine	Rosé wine
Ethanol (% *v/v*)	14.0	10.0	12.5
pH	3.50	3.18	3.30
Total acidity (g/L)	6.0	5.8	5.4
Volatile acidity (g/L)	0.38	0.29	0.40
Free SO_2 (mg/L)	36	30	40
Total SO_2 (mg/L)	90	103	120

2.4. Thin layer chromatography (Chapter 3)

2.4.1. Preparation of samples and phenolic standards

Standard phenolic solutions were prepared at a concentration of 1.0 mg in 10mL methanol.

Preparation of the red wine sample was carried out according to a previous method with slight modifications (Rastija et al., 2004). All standards and sample solutions were stored at 4°C. The wine was prepared using the two methods shown in Figure 8. Method two was found to be better in terms of clearer resolution of spots, and was therefore used. After evaporating to dryness, the weight of the dried red wine residue was 30 mg/mL.

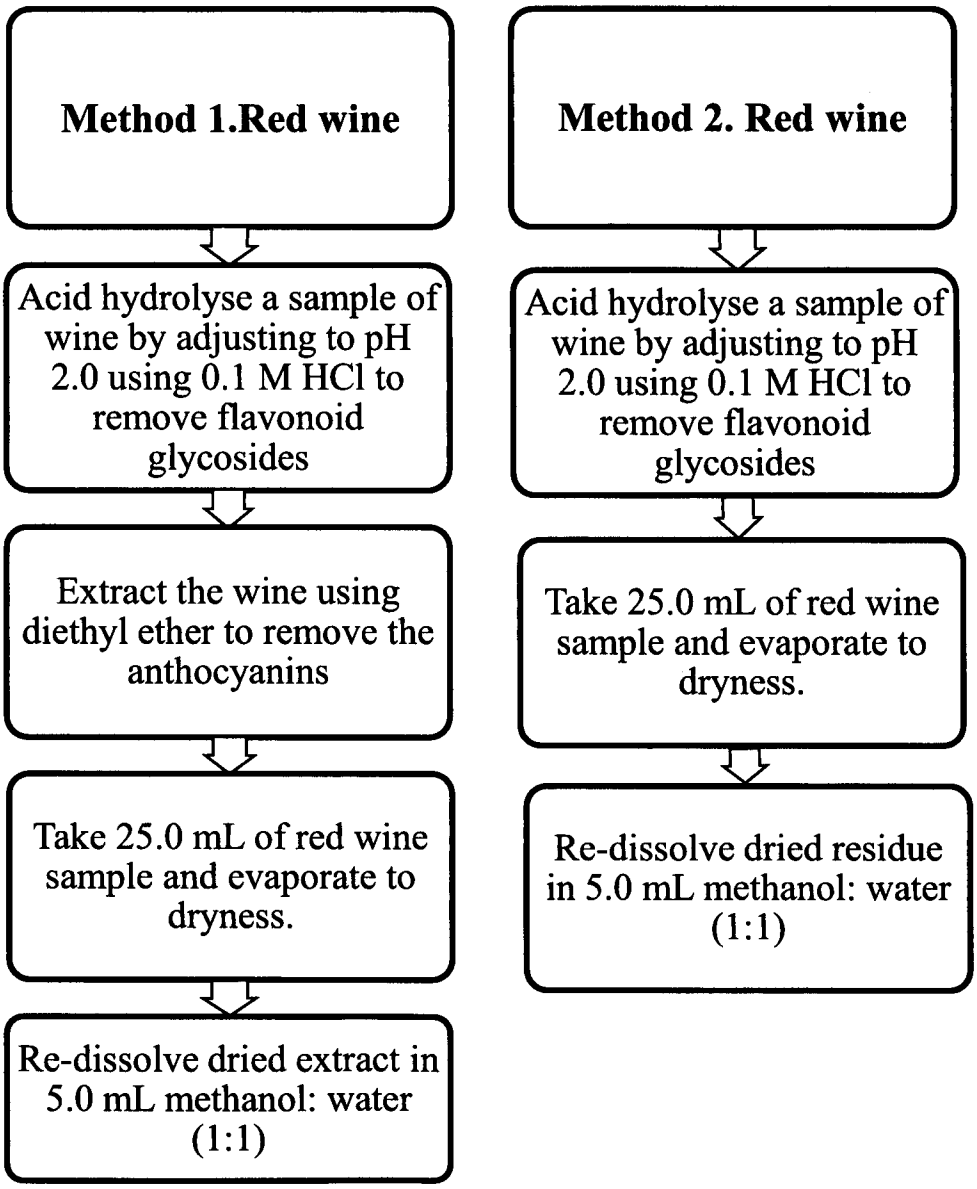


Figure 8: Preparation of red wine sample showing the two methods used.

For the white wine, rosé wine, red grape juice, white grape juice, and grape samples, sample preparation and extraction were performed according to Figure 9.

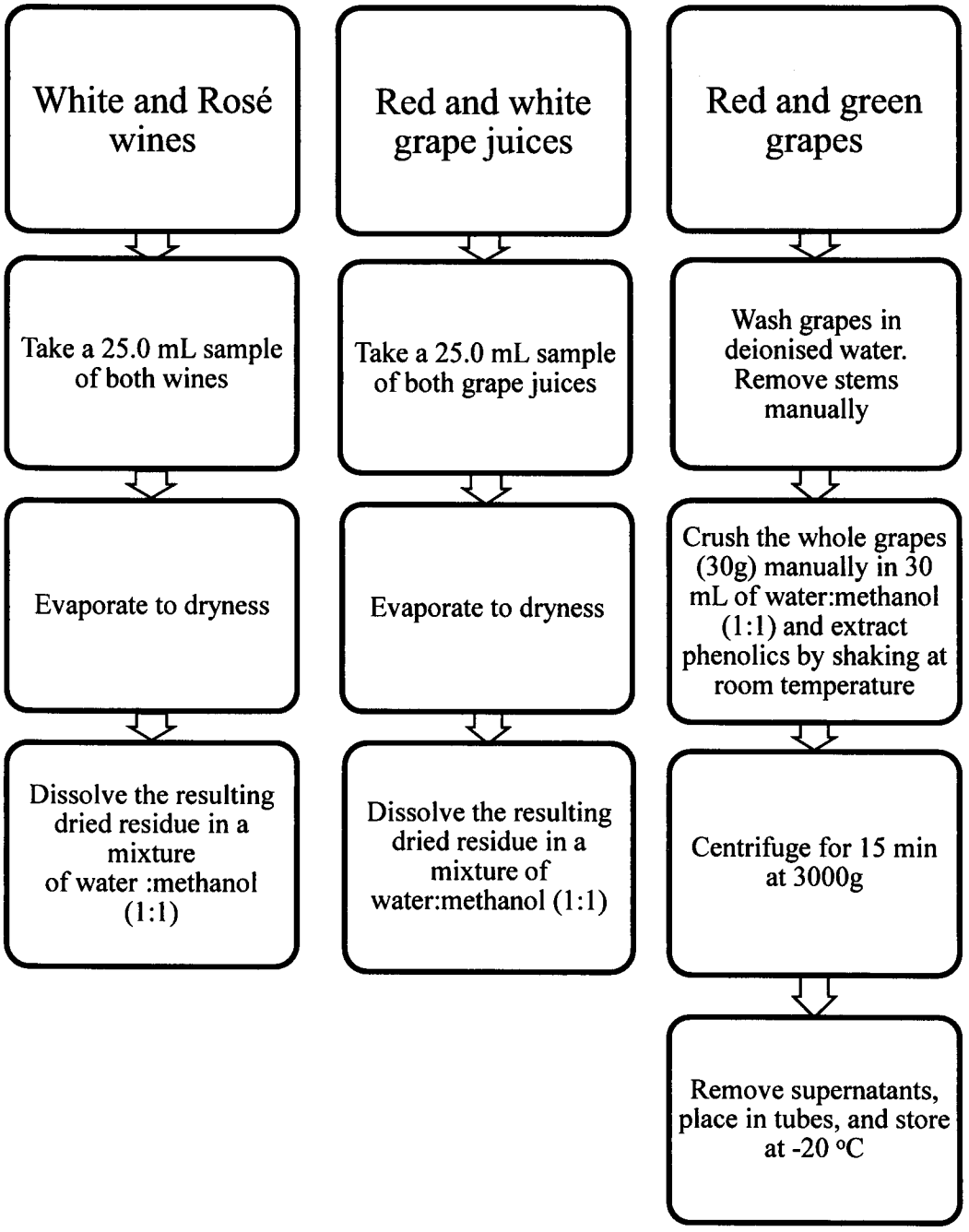


Figure 9: Preparation and extraction of white and rosé wines, red and white grape juices, and red and green grapes.

2.4.2. TLC mobile phases

Samples and phenolic standards were applied onto plates as spots using volumes ranging from 2-10 μL. Different mobile phases at varying ratios were tested according to previous methods (Anderson and Markham, 2006; Medić-Šarić et al., 2009; Sherma, 2000; Wagner and Bladt, 2001) as shown in Table 5. 2D TLC of the red wine was also conducted, and the list of mobile phases is shown in Table 6.

Table 5: List of the various TLC mobile phases tested.

Sample	Eluent	Volume ratio
Flavonoid aglycones	TOL-HCOOEt-HCOOH	5:4:1
	TOL-EtOAc-HCOOH	10:4:1
	TOL-EtOAc-HCOOH	58:33:9
	TOL-EtOAc-HCOOH	30:25:5
Flavonoid glycosides	EtOAc-MeOH-H_2O	50:3:10
	EtOAc-MeOH-H_2O-HCOOH	50:7:5:5
	EtOAc-MeOH-HCOOH-H_2O	100:11:11:26
	EtOAc-MeOH-HCOOH-H_2O	50:2:3:6
	EtOAc-HCOOH-H_2O	9:1:1
	1-BuOH-HOAc-H_2O	65:15:25
	EtOAc-MeOH-H_2O	100:13.5:10
Anthocyanidins and anthocyanins	EtOAc-HCOOH-2*M* HCl	85:6:9
	1-BuOH-HOAc-H_2O	4:1:2

Table 6: List of the various mobile phases tested for 2D TLC of red wine.

1st Direction	2nd Direction
TOL-EtOAc-MeOH (85:10:5)	EtOAc-MeOH (9:1)
EtOAc-MeOH-H_2O-HCOOH (50:7:5:1)	TOL-EtOAc-HCOOH (30:25:5)
EtOAc-MeOH-H_2O-TOL (100:4:5:8)	TOL-EtOAc-HCOOH (50:40:10)
EtOAc-MeOH-HCOOH (100:7:2)	EtOAc-MeOH-HCOOH (50:7:5)
EtOAc-MeOH-HCOOH (50:25:2)	EtOAc-MeOH-HCOOH (50:25:2)
HA-EtOAc-HCOOH (30:15:5)	EtOAc-HCOOH (15:2)

2.4.3. Plate development and analysis

Plates were developed at room temperature in an all-glass rectangular TLC developing tank previously left to equilibrate for 60 minutes, and were developed to a distance of 8.5cm. After development, plates were left to dry, and then visualised under (i) short wave UV light (λ=254 nm), and (ii) long wave UV light (λ=365 nm). Components were visualised by spraying a derivatisation reagent onto the plates - natural product reagent (NP/PEG) - which is used for detection of flavonoids. This was prepared using 1% methanolic 2-aminoethyl diphenylborinate followed by 5% ethanolic polyethylene glycol. Plates were then viewed under 365 nm UV light. Identification of compounds was facilitated by comparing R_f values and colours of zones with those of commercial phenolic standards, as well as reference to literature (Rastija et al., 2004; Rastija et al., 2009; Wagner and Bladt, 2001).

2.4.4. DPPH screening

In order to facilitate identification of antioxidant loss upon addition of the oxidant systems, a 0.04% methanolic solution of DPPH was used to spray the chromatograms. Yellow/white spots on a purple background indicated anti-oxidant activity.

2.4.5. Determination of metal ion content in red wine using ICP-OES

The endogenous content of the redox-active metal ions Fe and Cu in the wine, was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). The sample was diluted one in ten in water prior to analysis. The results of metal ion determination, coupled with prior literature results, were used to aid the selection and concentrations of the oxidant system to be used. The metal ion content was also measured in four of the standards: gallic acid, caffeic acid, *p*-coumaric acid, and quercetin.

2.4.6. Preparation of red wine- and phenolic standard-oxidant reaction mixtures

The red wine sample was challenged with five oxidant systems in separate experiments. These were: (1) H_2O_2, (2) Fe^{3+} and (3) Cu^{2+} at concentrations of 10, 20, 30, 40, and 50 mM, and two hydroxyl radical generator model systems (Fenton systems) (4) Fe^{2+}–H_2O_2, and (5) Cu_2^{+}–H_2O_2 at concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 mM. The Fe^{3+}, Cu^{2+}, and Fe^{2+} metal ions were prepared in water but Cu^{+} was prepared in 0.1 M HCl due to insolubility in water. All reaction mixtures were incubated at room temperature and results taken at 0, 60, 120 and 240 minutes. The phenolic standards mixture was prepared at a concentration of 1 mg/mL and challenged with the five oxidant systems as

described for the wine. However, the concentrations of all oxidant systems used were: 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 mM. R_f values were calculated according to the following equation:

$$Rf = \frac{Distance\ travelled\ by\ component}{Distance\ travelled\ by\ solvent\ front}$$

2.5. Measurement of anti-oxidant activity using reversed-phase high performance liquid chromatography (Chapter 4)

2.5.1. Preparation of wine sample and phenolic standards

Stock solutions of phenolic standards were dissolved in methanol to a concentration of 1 mg/mL and stored at -20°C. Working standards in the range 25-800 mg/L were prepared by dilution of the stock solutions in a mixture of methanol and 0.1 % othophosphoric acid in water (1:1). The red wine was prepared using method 2 as shown in section 2.4.1. Calibration curves were constructed using peak area *vs.* concentration of standard to quantify the levels of the phenolic compounds in the wine. Both were filtered through a 0.45 μm Whatman Puradisc PTFE filter device prior to injection into the HPLC system.

2.5.2. Preparation of red wine-and phenolic standard-oxidant reaction mixtures

A concentration of 1.0 mM of each oxidant (100 μL) was incubated with the wine sample (100 μL). For the standards mix, 400 mg/L of each standard (100 μL) was incubated with 1.0 mM of each oxidant (100 μL). The reaction mixtures were incubated

in the dark at room temperature for 60 minutes. The five oxidant systems were added to the wine and standards in separate experiments. Peak area measurements were analysed immediately after this time using HPLC.

2.5.3. Chromatographic conditions

Gradient elution was used to separate compounds using different ratios of 0.1 % orthophosphoric acid in water (solvent A) and methanol (solvent B). The gradient conditions were as shown in Table 7.

Table 7: Chromatographic conditions used in HPLC analysis (chapter 4).

	Composition of mobile phase (%)	
Time (min)	**A**	**B**
0	80	20
10	60	40
20	50	50
30	45	55
50	35	65

This was followed by a 10 minute equilibration period using initial conditions prior to injection of the next sample.

The flow rate was 1.0 mL/min and the injection volume was 10 µL. Identification and quantification was carried out at 280 nm. Phenolic compounds in the wine sample were identified by comparing the retention times with the pure phenolic standards.

2.5.4. Calculation of anti-oxidant activity

Anti-oxidant activity (%) of each phenolic compound was calculated using the difference in peak area (PA) of the control and the treated red wine sample/standard according to the following equation:

$$\%\ Anti-oxidant\ activity = \frac{PA_{\text{Control}} - PA_{\text{Sample}}}{PA_{\text{Control}}}\ x\ 100$$

The efficiencies of the anti-oxidants were classified according to low, medium, or high anti-oxidant activity as shown on the following scale (Caillet et al., 2007):

< 40 %	Low
40-70 %	Medium
> 70 %	High

2.5.5. Metal chelation activities of gallic acid, caffeic acid, *p*-coumaric acid, and quercetin

In order to evaluate the binding capacities of the four phenolic compounds to the Fe^{3+}, Fe^{2+}, and Cu^{2+} metal ions, UV-visible spectrophotometry was used. Stock solutions of each phenolic compound were prepared in methanol. A concentration of 100 µM was prepared for each phenolic compound in PBS (10 mM, pH 7.4). Aliquots of Fe^{3+}, Fe^{2+}, or Cu^{2+} solutions were added at the same concentration (100 µM) to each compound at a ratio of 1:1. The reaction mixtures were left to incubate in the dark at room temperature for 10 minutes, and spectra were recorded immediately after this time. A 4.0-fold EDTA concentration (400 µM) was subsequently added to these phenolic-metal ion complexes,

and left to incubate for a further 10 minutes. Spectra were recorded between 200-600 nm.

HPLC was also used to monitor metal ion interaction at a 1:1 ratio of metal ion: phenolic (1.0 mM) and 400 μM EDTA.

2.6. Hydroxyl radical–mediated deoxyribose assay, linoleic acid peroxidation assay, and RP-HPLC (Chapter 5)

2.6.1. Preparation of wine sample and phenolic standards

The red wine sample (same brand, but 2008 vintage) was used undiluted without extraction and/or hydrolysis. Working solutions of the standards were prepared in methanol: orthophosphoric acid in water (1:1 *v/v*) in the range of 10-800 mg/L.

2.6.2. HPLC analysis of red wine-and phenolic standard-oxidant reaction mixtures

For the RP-HPLC measurements, the concentrations of the eleven phenolic compounds in wine were quantified in order to prepare the same concentrations of standards. To achieve this, calibration curves were constructed using peak area *vs.* concentration of standard (mg/L). Different concentrations of each oxidant (H_2O_2 and metal ions) were prepared. Each of the five oxidant systems (0.2 mL), i.e. (i) H_2O_2; (ii) Fe^{3+}; (iii) Cu^{2+}; and two Fenton systems (iv) H_2O_2-Fe^{2+} and (v) H_2O_2-Cu^{+}, were incubated with the wine sample and standards (0.2 mL) in separate experiments. These solutions were left to incubate in the dark, and peak area measurements were taken at different time intervals

from 0-120 minutes. Phenolic loss was calculated using the standard curves, and the concentration in mg/L was converted to mM. The data were presented as phenolic loss (mM) of each compound relative to the control. Values lower than the control indicated anti-oxidant activity.

2.6.3. Chromatographic conditions

Gradient elution was used to separate compounds using 0.1 % orthophosphoric acid in water (solvent A) and methanol (solvent B). The gradient conditions were as shown in Table 8.

Table 8: Chromatographic conditions used in HPLC analysis (chapter 5).

	Composition of mobile phase (%)	
Time (min)	**A**	**B**
0	80	20
10	60	40
20	50	50
30	30	70

2.6.4. Hydroxyl radical-mediated deoxyribose degradation assay

Hydroxyl radical-mediated deoxyribose degradation of red wine and phenolic standards was measured according to the method of Halliwell et al., (1987) with minor modifications. Concentration effects were tested using: (i) fixed concentrations of standards determined in Table 17 and 20 mg/L wine, and (ii) increasing concentrations of red wine and standards (20-640 mg/L). To prepare these concentrations of red wine, a

sample of wine was taken and evaporated to dryness. The resulting dried residue was then weighed and re-dissolved in water to make a stock solution of 1 mg/mL. This stock solution was then used to prepare the working solutions for the assay. Stock solutions of the standards were prepared at a concentration of 1 mg/mL, and subsequently used to make the working standard solutions.

Two Fenton systems were tested. The first Fenton system (Ascorbic acid-Fe^{3+}-EDTA-H_2O_2) was prepared as follows: 0.1 mL deoxyribose (20 mM), 0.1 mL Fe^{3+} (0.5 mM), 0.1 mL EDTA (1mM), 0.5 mL wine or standards, and 0.1 mL ascorbic acid (1 mM) in 50 mM KH_2PO_4–KOH buffer (pH 7.4). The reaction was initiated after the addition of 0.1 mL 1 mM H_2O_2. The second Fenton system (H_2O_2-Fe^{3+}) was prepared using 0.1 mL Fe^{3+} (0.5 mM), 0.1 mL H_2O_2 (1 mM), and 0.5 mL wine or standards. The reaction mixtures were subsequently incubated in a water bath at 37 °C for 60 minutes.

After this time, the reaction was stopped by the addition of 0.1 mL 2.0 % *(w/v)* trichloroacetic acid in water and 0.1 mL 1.0 % *(w/v)* 2-thiobarbituric acid in 0.05 M NaOH. The reaction media were then incubated in a boiling water bath for 15 minutes to allow development of the chromogen. After a short incubation on ice to stop the reaction, the chromogen was determined spectrophotometrically at 532 nm.

The control contained the reaction mixture without sample added. Possible interferences of red wine in the assay were checked prior to conducting the experiment, by measuring absorbance values of the wine alone as well as in the presence of deoxyribose. Data were presented as absorbance at 532 nm. A high absorbance relative to the control indicated pro-oxidant activity, reflecting enhanced damage to the substrate.

2.6.5. Linoleic acid peroxidation assay

The anti-oxidant activity of the wine and phenolic standards in a linoleic acid system was determined according to the method of Yen and Hsieh, (1998). 0.5 mL undiluted wine or standards (at the concentrations in Table 17) in methanol were mixed with 2.5 mL linoleic acid emulsion (0.02 M), 5 mL ethanol (99.8 %), and 5 mL phosphate buffer (0.2 M, pH 7.0).

Reaction mixtures were also incubated with the same Fenton reagents as the hydroxyl radical-mediated deoxyribose degradation assay. The first Fenton system (Ascorbic acid-Fe^{3+}-EDTA-H_2O_2) was prepared as follows: 50 µL EDTA (1 mM), 50 µL Fe^{3+} (0.5 mM), 50 µL ascorbic acid (1 mM) and 50 µL H_2O_2 (1 mM). The second Fenton system (H_2O_2-Fe^{3+}) was prepared using 50 µL Fe^{3+} (0.5 mM) and 50 µL H_2O_2 (1 mM). These solutions were then incubated at 37 °C, with aliquots taken at various intervals. After these time intervals, 0.1 mL of the reaction mixture was added to 5 mL of 75 % (*v/v*) ethanol, followed by 0.1 mL of 0.02 M ferrous chloride (prepared in 3.5 % HCl *v/v*) and ammonium thiocyanate (0.1 mL, 30 % *w/v*). The solutions were left to incubate for 3 minutes before measuring the absorbance at 500 nm.

The control contained the reaction mixture without sample added. Possible interferences of red wine in the assay were checked prior to conducting the experiment, by measuring absorbance values of the wine alone as well as in the presence of linoleic acid. Anti-oxidant activity was presented as (%) Inhibition calculated as follows:

$$\%\ Inhibition = \frac{A_0 - A_1}{A_0}\ x\ 100$$

Where A_0 is the absorbance of the control (in the absence of the wine or standards) and A_1 is the absorbance in the presence of the wine or standards. A negative result relative to the control indicated pro-oxidant activity, reflecting enhanced oxidative damage to the substrate.

2.6.6. Metal chelation activities of protocatechuic acid, syringic acid, catechin, epicatechin, resveratrol, myricetin, and kaempferol standards

In order to evaluate the binding capacities of the standard phenolic compounds to the Fe^{2+} and Cu^{2+} metal ions, UV-visible spectrophotometry was used. Stock solutions of each phenolic compound were prepared in methanol. A concentration of 100 μM was prepared for each phenolic compound in PBS (10 mM, pH 7.4). Aliquots of Fe^{2+} or Cu^{2+} solutions were added at the same concentration (100 μM) to each compound at a ratio of 1:1. The reaction mixtures were left to incubate in the dark at room temperature for 10 minutes, and spectra were recorded immediately after this time. A 4.0-fold EDTA concentration (400 μM) was subsequently added to these phenolic-metal ion complexes, and left to incubate for a further 10 minutes. Spectra were recorded between 200-600 nm.

2.7. Measurement of anti-oxidant activity using modified $ABTS^{\cdot+}$ and DPPH assays (Chapter 6)

2.7.1. Total phenol assay

A calibration curve was constructed using gallic acid as standard at a concentration range of 0, 50, 100, 150, 250, and 500 mg/L. A 1:10 dilution of red wine was prepared, while white wine, rosé wine, red grape juice and white grape juice was used undiluted. 20 µL of each sample was added to separate cuvettes, followed by 1.58 mL of water, and 100 µL of Folin-Ciocalteau reagent and mixed well. After 1 minute, 300 µL of a 20 % (*w/v*) sodium carbonate solution was added and mixed. After 120 minutes of incubation, the absorbance of all the solutions was measured at 760 nm. Total phenols were reported as gallic acid equivalents (GAE).

2.7.2. DPPH radical-scavenging assay

This assay was carried out according to the method of Brand-Williams et al., (1995). A concentration of 0.025 g/L DPPH· radical was prepared in methanol. Red wine was diluted 1:20 in a 1:1 mixture of ethanol/water. White wine, rosé wine, and white grape juice were used undiluted. Red grape juice was diluted 1:3. Trolox standards were prepared in methanol in the concentration range of 0-180 mg/L. A 2.9 mL volume of DPPH radical solution was mixed with 100 µL standard or sample, and the absorbance was measured over 30 minutes at 517 nm. The blank control contained a water/methanol mixture.

The effect of adding increasing concentrations of Fe^{3+} and Cu^{2+} to the wine and grape juice samples was assessed. A concentration range of 0.01-0.01 mM metal ions was prepared. These metal ion solutions were added at a volume of 50 µL to 50 µL of samples. Then 2.9 mL of DPPH radical was subsequently added to the metal ion: sample mixtures, and absorbances were measured over 30 minutes. Anti-oxidant activity was presented as mg TE/L.

2.7.3. $ABTS^{\cdot+}$ radical-scavenging assay

This assay was carried out according to the procedure by Re et al., (1999). The $ABTS^{\cdot+}$ radical was produced by reacting 7 mM aqueous ABTS with 2.45 mM potassium persulphate and leaving this mixture in the dark at room temperature for 16 hours. The $ABTS^{\cdot+}$ radical solution formed after this time was diluted with PBS to obtain an absorbance of 0.70 ± 0.02 at 730 nm. Trolox standards were prepared in ethanol in the concentration range of 0-80 mg/L. Red wine was diluted 1:100 in a 1:1 mixture of ethanol/water. White wine, rose wine, red grape juice, and white grape juice were diluted 1:20 in water. 2.9 mL of the $ABTS^{\cdot+}$ radical solution was mixed with 100 µL of standard or sample, and the absorbance was measured after 6 minutes incubation at 734 nm. The blank control contained a water/ethanol mixture.

The effect of adding increasing concentrations of Fe^{3+} and Cu^{2+} to the wine and grape juice samples was assessed. A concentration range of 0.01-0.01 mM metal ions was prepared. These metal ion solutions were added at a volume of 50 µL to 50 µL of samples. Then 2.9 mL of $ABTS^{\cdot+}$ radical was subsequently added to the metal ion: sample mixtures, and absorbances were measured over 30 minutes. Anti-oxidant activity was presented as TEAC (mg/L).

2.8. Statistical analysis

All experimental data reported were obtained by triplicate determinations and mean values were calculated using Microsoft Excel (2007).

For the HPLC analysis in chapter four and the hydroxyl radical-mediated deoxyribose degradation assay in chapter five, data were subjected to a two-way ANOVA (analysis of variance). The linoleic peroxidation assay in chapter five, and the $ABTS^{\cdot+}$ and DPPH assays in chapter six, were subjected to repeated measures ANOVA. Significant differences were determined by Bonferroni's post test. The statistical significance was determined at $p < 0.05$. Analysis was carried out using GraphPad (version 5.0).

Chapter 3

DEVELOPMENT OF A NEW FUNCTIONAL THIN-LAYER CHROMATOGRAPHIC METHOD TO ASSESS THE ANTI-OXIDANT EFFICACIES OF RED WINE AND ITS COMPONENTS

3.1. INTRODUCTION

Among the many analytical methods used to study the anti-oxidant activity of wine such as UV-vis absorption spectrometry, electron spin resonance (ESR) (Staško et al., 2006), flow injection analysis (Milardovic et al., 2007), and Fourier transform infrared spectroscopy (FTIR) (Versari et al., 2010), UV-vis- based methods are the commonest techniques used due to their rapidity and ease of use in relation to the other methods. UV-vis based methods usually involve measuring the reaction between different chromogenic free radical species and a test compound to determine anti-oxidant activity.

Thin layer chromatography is another technique that is commonly used in natural product analysis. There have been many uses of this technique in screening the biological activity of a sample, such as anti-oxidant activity, anti-microbial, and enzyme inhibition tests (Hosu et al., 2010). Inhibition of enzymes involved in ROS formation, such as xanthine oxidase, can be screened using TLC. Anti-oxidant testing can involve monitoring the inhibition of β-carotene bleaching. However, the use of DPPH to detect radical scavenging of a sample is a common method (Hosu et al., 2010). 'Dot-blot' tests

have been used, whereby the sample under study is spotted on the TLC plate without migration, and then dipped in a DPPH solution. However, the samples are not separated using this method. ABTS is another radical which can be used to screen for radical scavenging activity, but DPPH is more stable on TLC plates (Marston, 2011).

Hosu et al., (2010) used TLC to determine the anti-oxidant activity of natural and commercial juices. The commercial juices used were (1) orange + grapefruit, (2) orange + apple + carrot, and (3) a 'multi-fruit' juice. The natural juices were (1) orange + apple + carrot and (2) orange. DPPH was added to various juices and a vitamin C standard. These reaction mixtures were applied to TLC plates to determine the anti-oxidant activity of the spots. Using this TLC method, it was found that the anti-oxidant activity of natural juices was higher than those of commercial juices. Multi-component juices, i.e. those containing mixtures of fruits and vegetables, had a higher anti-oxidant efficacy than mono-component juices, due to the different classes of phenolic anti-oxidants present in mixtures of fruits and vegetables.

Two-dimensional TLC has also been used in phytochemistry to separate complex mixtures which might not be adequately separated using one-dimensional techniques (Cieśla and Waksmundzka-Hajnos, 2009).

Although there are numerous studies on the use of TLC in the analysis of various plant extracts, there are limited studies demonstrating the use of this technique on wine. Rastija et al., (2004) and Cimpoiu et al., (2007) separated the components of wine using both normal phase silica gel and reversed phase silica gel sorbents, respectively. Rastija et al., (2004) were able to identify six phenolic compounds in red wine using a mobile phase of benzene-ethyl actetate-formic acid (30:15:5 *v/v*). Cimpoiu et al., (2007), however, used acetonitrile-water-formic acid (40:58:2 *v/v*) to separate the compounds of

different wines. DPPH was used determine the anti-oxidant activity, but identification of the compounds was not given. Studies on the use of TLC in separating other grape-based products, such as grape juices, white wines, and rosé wines are lacking.

As noted earlier, the presence of high levels of metal ions in wine can have potential pro-oxidant effects in the body. However, the number of studies investigating the effect of adding metal ions to wines, and evaluating anti-oxidant activity is limited. Espinoza et al., (2009) found that the addition of copper and iron to red wines, resulted in a reduced free radical scavenging capacity for all the wines tested using the DPPH assay and ESR methods. Similarly, Argyri et al., (2006) found that the anti-oxidant capacity of red wine was reduced using the FRAP assay in the presence of iron, under conditions of *in vitro* digestion. Mixtures of iron, red wine, ascorbic acid, meat and casein were tested to determine their effect on the anti-oxidant behaviour of wine polyphenols. Whilst ascorbic acid increased the anti-oxidant capacity, protein was found to lower the anti-oxidant efficacy of wine. Total phenolic content was also found to be reduced.

However, there are no studies using functional TLC to assess the effect of challenging red wine with different oxidants. The advantage of using this method to measure anti-oxidant activity over using standard anti-oxidant assays is that TLC is a good screening method for demonstrating which components are consumed upon treatment with the various oxidant systems. Rather than measuring the anti-oxidant activity of red wine on an oxidisable substrate, the principle of this method is based on the rate of loss of the phenolic components. Thus, the main aim of this chapter was to develop a new TLC method to monitor the loss of red wine phenolic anti-oxidants upon challenge with various oxidant systems.

3.2. AIMS

The aims of this chapter are:

- To employ different mobile and stationary phases to determine the most suitable TLC method for separating the components of the selected foodstuffs, namely red wine, white wine, rosé wine, red grape juice, white grape juice, red grapes, and green grapes as detailed in chapter two.
- To identify and characterise the separated phenolic components using commercial phenolic standards.
- To measure the native level of metal ions in wine using ICP-OES.
- To challenge the red wine and standard compounds with five different oxidant model systems namely: (1) H_2O_2, (2) Fe^{3+}, (3) Cu^{2+}, and two hydroxyl radical generator model systems (Fenton systems): (4) Fe^{2+}-H_2O_2, and (5) Cu^{2+}-H_2O_2. Determine the possible effect of matrix on anti-oxidant activity.
- To monitor the loss of phenolic components and rank these according to their individual anti-oxidant efficacies, as well as rank the five oxidant systems according to their oxidising power.

3.3. METHODS

The methods are given in chapter two. Briefly:

- A number of different eluent systems were tested to separate the components of wines, grape juices, and grape samples.
- ICP-OES was conducted to determine the elemental composition of red wine.
- For anti-oxidant testing of the red wine, the sample was challenged with five oxidant systems in separate experiments at concentrations of 10-50 mM for

H_2O_2, Fe^{3+}, and Cu^{2+},and 0.5–8.0 mM for both Fenton systems. The red wine was applied onto plates as spots using volumes ranging from 2-10 µL, and developed using TOL-EtOAc-HCOOH (30:25:5 *v/v*) as mobile phase. R_f values were recorded at different time intervals from 0-240 minutes. For the phenolic standards, 1mg/mL of each standard was treated with the five oxidant systems at a concentration range of 0.5-32 mM.

- A methanolic solution of DPPH was applied to further confirm anti-oxidant loss upon treatment with each of the oxidants.

3.4. RESULTS

3.4.1. TLC of white wine, rosé wine, grape juices and grapes

For the grape juices, white wine, rosé wine, and grape samples, no separation was achieved using the eluent systems in chapter two. Therefore, other mobile phases were tested. The list of eluents that showed separation of rosé and white wines, and both grape juices is shown in Table 9. Of the three stationary phases used, i.e. normal phase, reversed-phase C_{18}, and nano-silica gel plates, normal phase plates exhibited the best separation of compounds.

Table 9: List of eluent systems tested for rosé wine, white wine, red grape juice, and white grape juice which showed separation of one component.

Sample	Eluent	Volume ratio
Rosé wine	1-BuOH-HOAc-H_2O	50:10:40
	EtOAc-HA	30:100
	HA- EtOAc-H_2O	50:40:10
	1-BuOH- HA- HOAc	50:50:10
White wine	1-BuOH-HOAc-H_2O	50:10:40
Red grape juice	MeOH-HOAc	80:20
	1-BuOH-HOAc-H_2O	50:10:40
	1-BuOH-HOAc	100:10
White grape juice	MeOH-HOAc	80:20
	1-BuOH-HOAc	100:10

The different mobile phases used in Table 9 showed that only one spot was observed for all samples. This was shown as a light blue fluorescent zone for each sample, which could suggest it was a phenol carboxylic acid. It could also suggest the same compound, however further analysis would need to be carried out to determine the identification of this compound. Different TLC derivatisation reagents were used to visualise any other spots not visible under UV, including natural product reagent, phosphomolybdic acid, and iodine crystals, but these did not show the presence of any additional spots for the samples.

3.4.2. TLC of red wine

An initial TLC of the untreated red wine showed unclear separation of spots using the mobile phases listed in chapter two. Extraction of the red wine sample with diethyl ether after acid hydrolysis (method one), led to the appearance of fewer spots. Therefore, this step was removed from sample preparation, and method two was used. Reversed phase C_{18} plates, using polar solvents only, did not produce clear spots; therefore, normal phase plates were selected as the optimum stationary phase.

The more polar mobile phases showed all phenolic standards migrated to near the solvent front, with less polar solvents showing compounds near the baseline. Of the various eluent systems, EtOAc-MeOH-H_2O- HCOOH (50:7:5:5) and EtOAc-EtOH-HCOOH-H_2O (100:11:11:26) effected separation of the wine sample, with one anthocyanin identified as malvidin (as determined by comparison with the phenolic standard). However, the best resolution of compounds was achieved with the TOL-EtOAc-HCOOH (30:25:5) eluent, although malvidin was not identified using this solvent system. In addition, optimisation for sample loading was achieved with application of a 2 μL volume of the sample showing clearer individual spots compared to a 10 μL volume (Figure 10). This volume was chosen for further analysis of the anti-oxidant activity of individual components. In addition to performing 1D-TLC of the red wine, 2D-TLC was also conducted, however additional zones were not observed.

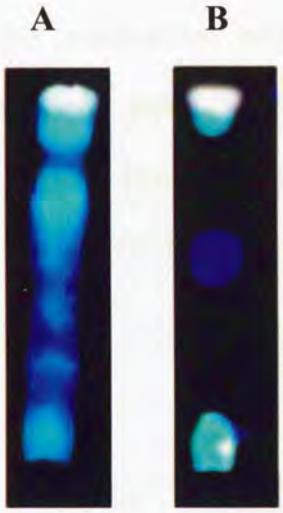


Figure 10: Chromatograms of red wine (RW) shown under long wave UV light showing: (**A**) 10 µL application volume and (**B**) 2 µL application volume.

As red wine produced the best separation of compounds using TOL-EtOAc-HCOOH (30:25:5), this sample was chosen for subsequent analysis on assessing its anti-oxidant profile. Anthocyanins were not separated using this eluent system, therefore the focus of this experiment was on monitoring the loss of the phenolic acids and flavonoids that were separated and identified using this mobile phase.

3.4.3. Determination of metal ion content in red wine using ICP-OES

The metal ion content of the red wine was measured in order to determine the elemental composition of the wine, as well as to aid the concentrations of oxidant system to use. Twenty elements were detected, with major metals present at a concentration of >10 µg/mL, and minor and trace metals at a concentration of < 1.8 µg/mL. As seen in Figure 11A, the levels of metals K, Na, Mg, and Ca were found to be in agreement with the concentrations reported previously for wine (10–10^3 µg/mL) (Pohl, 2007). In contrast,

the concentrations of the key redox-active metals [Fe and Cu] were below the previously reported range of 0.1–10 µg/mL (Figure 11B). In the selected red wine sample, Fe was below the working limit of quantification for the instrument (0.06 µg/mL), whereas Cu was not quantifiable in the wine although both elements were detected.

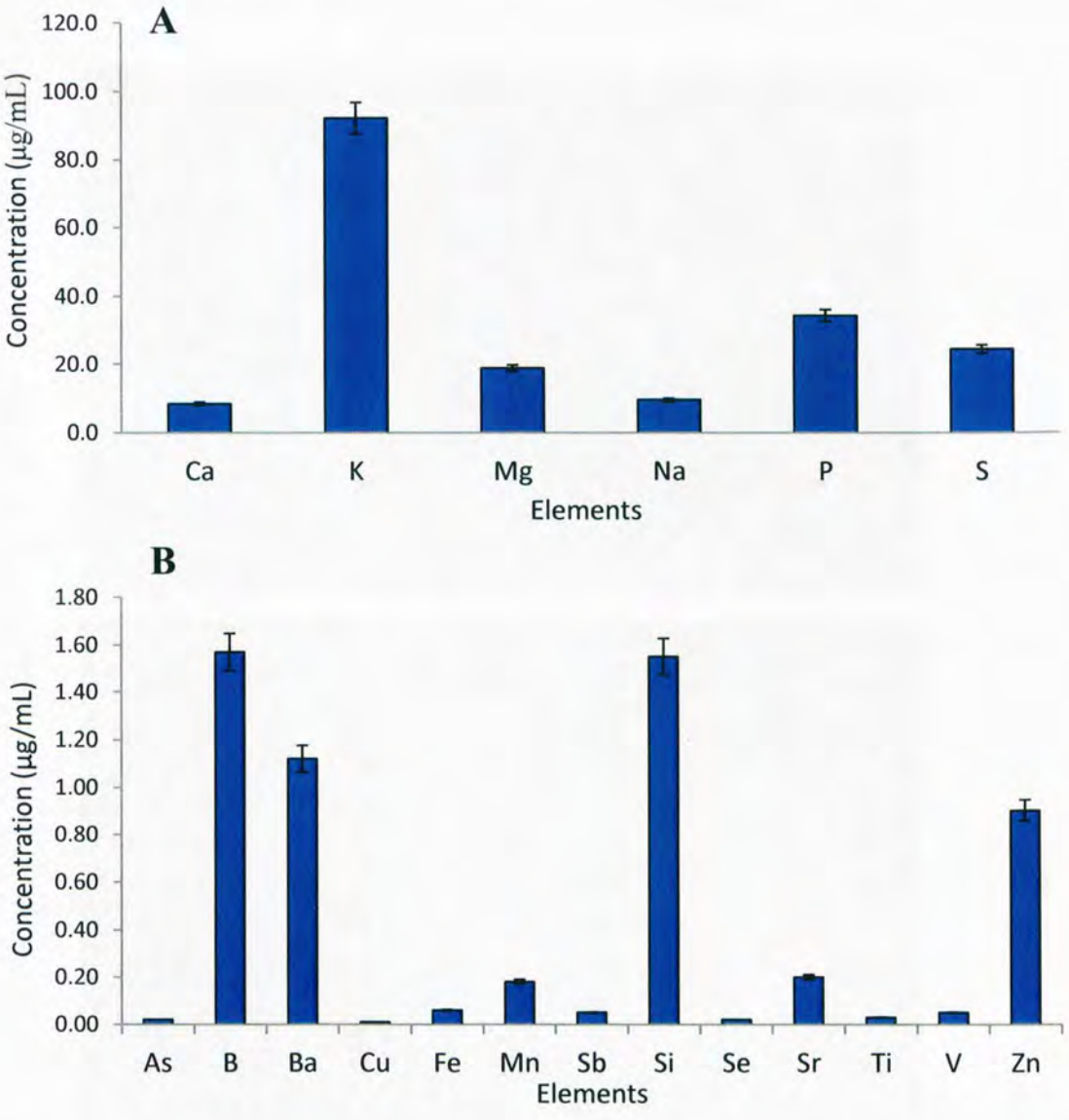


Figure 11: Quantification of metal ions in the red wine using ICP-OES showing (**A**) major elements, and (**B**) minor and trace elements.

3.4.4. Identification of the five phenolic compounds in red wine

Identification of compounds in wine was carried out using a prior literature search (La Torre et al., 2006; Rastija et al., 2004; Wagner and Bladt, 2001). Several phenolic standards were tested as listed in chapter two. Figure 12 shows the chromatograms of the red wine and the different phenolic compounds used to identify the components. Chromatograms are shown under both short wave and long wave UV light.

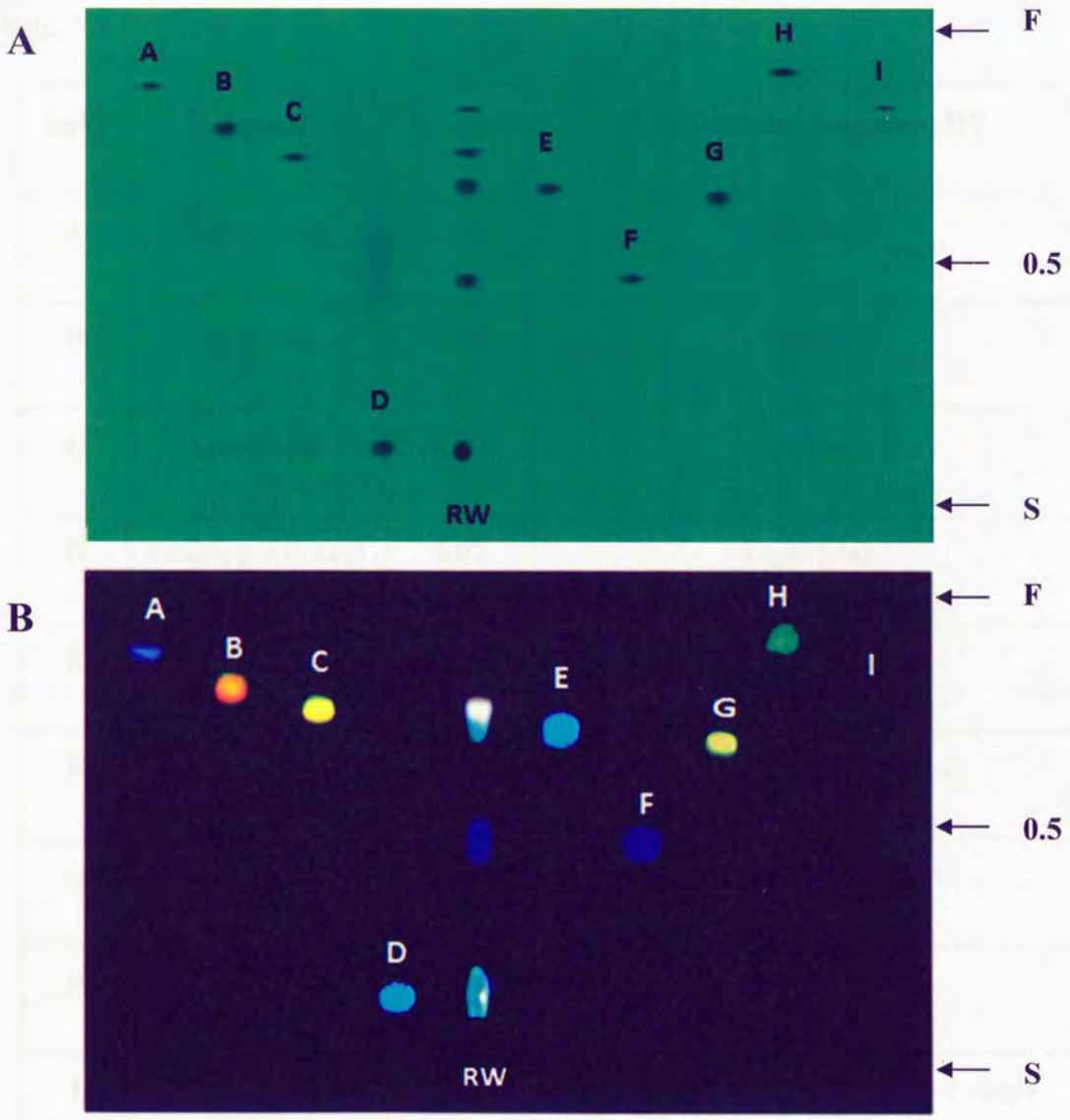


Figure 12: TLC chromatograms under (**A**) SW UV, and (**B**) LW UV of the RW sample and the different standards tested. S=Spot origin, and F=Solvent front.

Of the several commercial phenolic standards tested, five phenolic compounds in the wine corresponded to chlorogenic acid, gallic acid, caffeic acid, quercetin, and *p*-coumaric acid as shown in Table 10.

Table 10: R_f values of the different phenolic standard compounds tested. Identification of the compounds in RW was based on the R_f values and colours of the fluorescent zones under long wave UV. The five compounds identified in wine are highlighted in bold.

Spot	Compound	R_f value	Colour under long wave UV
A	Ferulic acid	0.65	Light blue
B	Apigenin	0.62	Orange
C	**Quercetin**	**0.60**	**Yellow**
D	**Chlorogenic acid**	**0.07**	**Light blue**
E	**Caffeic acid**	**0.57**	**Light blue**
F	**Gallic acid**	**0.47**	**Dark blue**
G	Myricetin	0.55	Pale yellow
H	Kaempferol	0.66	Green
I	***p*-Coumaric acid**	**0.64**	**(visible under short wave UV only)**

In order to visualise anti-oxidant compounds, the plates were sprayed with a methanolic solution of DPPH. The purple chromophore changes to colourless upon reaction with an anti-oxidant as shown in Figure 13.

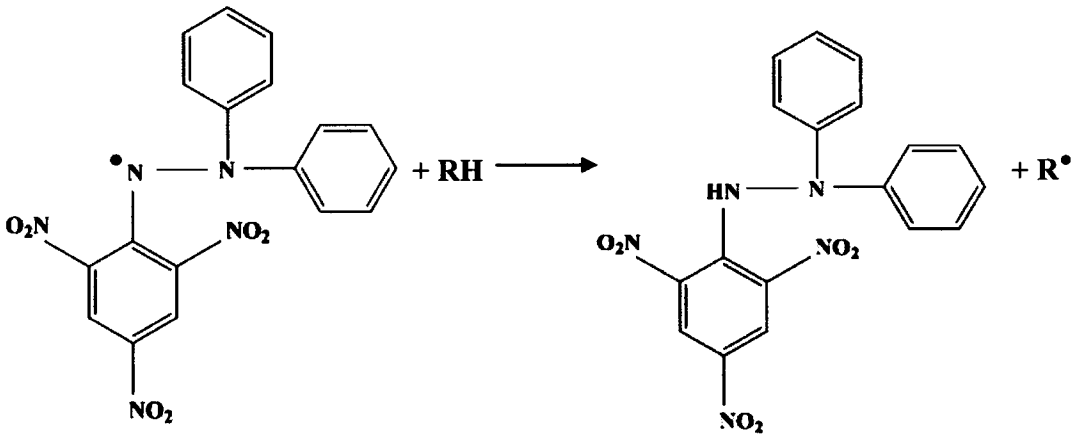


Figure 13: The principle of the DPPH assay showing the change in colour from purple to colourless as the radical is quenched by the anti-oxidant (Marston, 2011).

Standards were spotted on either side of the central wine sample in order to remove any parallax errors arising through uneven solvent fronts, and visualised under short wave (Figure 14A) and long wave UV light (Figure 14B). The DPPH-treated chromatogram showing the anti-oxidant components is shown in Figure 14C.

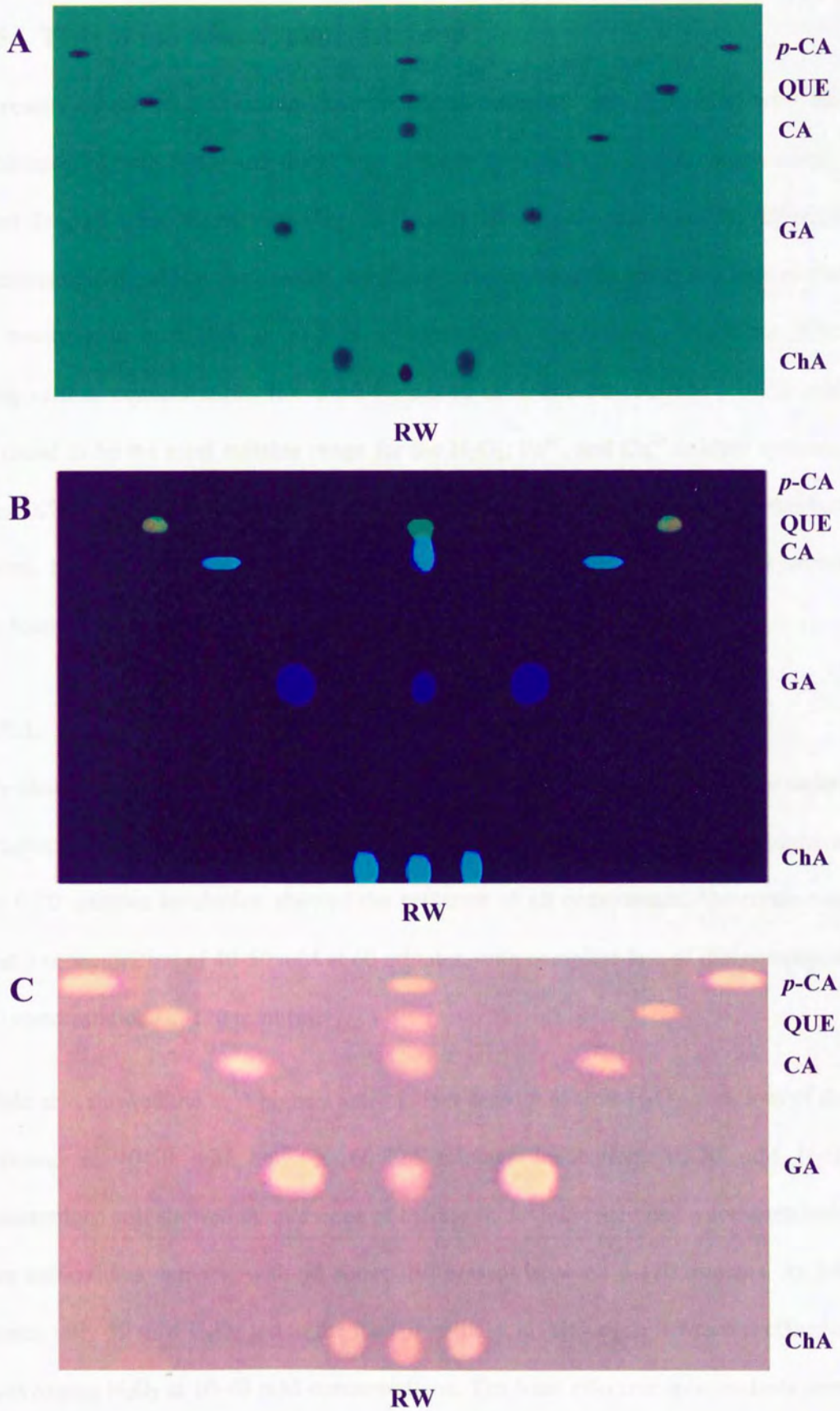


Figure 14: TLC chromatograms of RW and five standards under (**A**) SW UV, (**B**) LW UV, and (**C**) DPPH-treated.

3.4.5. TLC of red wine-oxidant mixtures

The results of the five oxidation experiments all revealed varying results, with the combination of both H_2O_2 and metal ions (Fenton systems) showing a comparatively greater loss of components than the individually added oxidants. Initially, different concentrations of oxidant were tested, but these were found to be either too high or too low, resulting in total loss or no loss of compounds, respectively. Therefore, after testing various concentrations (i.e. 5-30 %, 2.0-10 mM, and 50-150 mM), 10-50 mM was found to be the most suitable range for the H_2O_2, Fe^{3+}, and Cu^{2+} oxidant systems, whilst 0.5-8.0 mM was the most suitable concentration range for the Fenton oxidant systems. For the standards, concentrations of 0.5-32 mM for the five oxidant systems were found to be a suitable range.

3.4.5.1. H_2O_2 addition

H_2O_2-challenged red wine resulted in loss of three of the five compounds in the order: quercetin, caffeic acid, and gallic acid, respectively (Figure 15). The chromatograms from 0-30 minutes incubation showed the presence of all components. Quercetin was lost at a concentration of 40-50 mM at 60 minutes, with complete loss of this compound at all concentrations at 120 minutes.

Caffeic acid showed the next highest anti-oxidant activity against H_2O_2, with loss of the compound at 40-50 mM between 60-120 minutes incubation. 10-30 mM H_2O_2 concentrations still showed the presence of caffeic acid. Gallic acid had a comparatively lower anti-oxidant activity, with all zones still present between 0-120 minutes. At 240 minutes, only 50 mM H_2O_2 led to the loss of gallic acid. However, it was not effective at scavenging H_2O_2 at 10-40 mM concentrations. The least effective anti-oxidants were

chlorogenic acid and *p*-coumaric acid, which were all still present after 240 minutes incubation at all concentrations of H_2O_2.

For the standards, from 0-30 minutes gallic acid showed a loss at 32 mM, caffeic acid at 16-32 mM, and quercetin at 2-32 mM. *p*-Coumaric acid and chlorogenic acid were still present. At 60 minutes there was no change; however, chlorogenic acid disappeared at 16-32 mM H_2O_2. At 120-240 minutes, gallic acid and caffeic acid further diminished at 8-32 mM. Quercetin disappeared at all concentrations of H_2O_2, whilst *p*-coumaric acid was still present at all concentrations.

3.4.5.2. Ferric chloride (Fe^{3+}) addition

Like H_2O_2, Fe^{3+}-challenged wine did not show any change after 30 minutes incubation (Figure 16). Again, quercetin, caffeic acid, and gallic acid, respectively, exhibited anti-oxidant/chelating activity towards the Fe^{3+} ion. Quercetin was lost at concentrations of 40-50 mM at 60 minutes, but gradually diminished over 240 min. Caffeic acid disappeared between 60 and 120 minutes at 50 mM Fe^{3+} concentration. Gallic acid was also lost at this concentration, but only after 240 minutes. Again, chlorogenic acid and *p*-coumaric acid were still present after 240 minutes, indicating possible low anti-oxidant/chelating activity towards Fe^{3+}.

For the standards, from 0-30 minutes gallic acid and caffeic acid showed a loss at 8-32 mM, and quercetin at 4-32 mM Fe^{3+}. *p*-Coumaric acid and chlorogenic acid were still present. At 60 minutes, there was no change; however, chlorogenic acid disappeared at 8-32 mM Fe^{3+}. At 120-240 minutes, gallic acid and caffeic acid further diminished at 1-32 mM. Quercetin disappeared at all concentrations of Fe^{3+}, whilst *p*-coumaric acid was still present at all concentrations.

3.4.5.3. Cupric chloride (Cu^{2+}) addition

Similar to H_2O_2 and Fe^{3+} oxidant systems, interaction of red wine components with Cu^{2+} showed no change in activity between 0-30 minutes incubation (Figure 17). Quercetin, caffeic acid, and gallic acid were the most efficient anti-oxidants in this test system. Loss of quercetin was observed at 40-50 mM Cu^{2+} at 60 minutes, but gradually diminished after 240 minutes. Caffeic acid disappeared between 60 and 120 minutes at 50 mM Cu^{2+}. However, unlike the previous two systems, gallic acid was lost between 40-50 mM at 240 minutes. Again, chlorogenic acid and *p*-coumaric acid were still present after Cu^{2+} treatment indicating possible low anti-oxidant/chelating activity towards Cu^{2+}.

For the standards, from 0-30 minutes loss of caffeic acid and quercetin at 16-32 mM Cu^{2+} addition was observed. *p*-Coumaric acid and chlorogenic acid were still present. At 60 minutes gallic acid showed a loss at 32 mM, whilst chlorogenic acid disappeared at 16-32 mM Cu^{2+}. At 120 minutes, caffeic acid and quercetin further diminished at 4-32 mM. After 240 minutes, loss of quercetin at 1-32 mM and gallic acid at 16-32 mM was seen. *p*-coumaric acid was still present at all concentrations, whilst chlorogenic acid diminished slightly further at 8-32 mM Cu^{2+} addition.

3.4.5.4. Fenton systems

The hydroxyl radical generators (Fenton system models) resulted in a comparably greater loss of all anti-oxidants than each oxidant added independently. Unlike the other models, the Fenton systems showed loss of components from 0 minutes incubation. Similar to the other systems, however, quercetin, caffeic acid, and gallic acid were the most effective anti-oxidants.

Fe^{2+}-H_2O_2 addition

For the Fe^{2+}-H_2O_2 model system, quercetin and caffeic acid were lost at 2-8 mM at 0 minutes, which gradually diminished at all concentrations after 60 minutes incubation (Figure 18). Gallic acid was lost at all concentrations at 60 minutes. Chlorogenic acid and *p*-coumaric acid were still present after 240 minutes at 0.5-2 mM Fe^{2+}-H_2O_2, but not 4-8 mM.

For the standards, from 0-30 minutes loss of quercetin and caffeic acid at 16-32 mM Fe^{2+}-H_2O_2 was observed. These spots gradually diminished at all concentrations over 60 minutes incubation, whereas *p*-coumaric acid and chlorogenic acid were still present. At 60 minutes gallic acid was lost at all concentrations, whilst chlorogenic acid disappeared at 16-32 mM Cu^{2+}. At 120-240 minutes, loss of *p*-coumaric acid and chlorogenic acid was observed at 2-32 mM.

Cu^{2+}-H_2O_2 addition

As with the Fe^{2+}-H_2O_2 Fenton system, Cu^{2+}-H_2O showed a similar ranking order of anti-oxidants (Figure 19). Quercetin and caffeic acid were lost at 2-8 mM at 0 minutes, which gradually diminished at all concentrations after 60 minutes incubation. Gallic acid was present between 0-30 minutes, but all spots disappeared after 60 minutes. However, unlike Fe^{2+}-H_2O_2, *p*-Coumaric acid disappeared at all concentrations at 240 minutes, whereas chlorogenic acid was lost at 1-8 mM.

For the standards, similar results were seen as the Fe^{2+}-H_2O_2 Fenton system. From 0-30 minutes, loss of quercetin and caffeic acid at 16-32 mM Cu^{2+}-H_2O_2 was observed. These spots gradually diminished over 60 minutes incubation, whereas *p*-coumaric acid and chlorogenic acid were still present. At 60 minutes gallic acid was lost at all

concentrations, whilst chlorogenic acid disappeared at 4-32 mM Cu^{2+}. At 120-240 minutes, unlike the Fe^{2+}-H_2O_2 Fentons system, complete loss of *p*-coumaric acid and chlorogenic acid spots was observed at all concentrations.

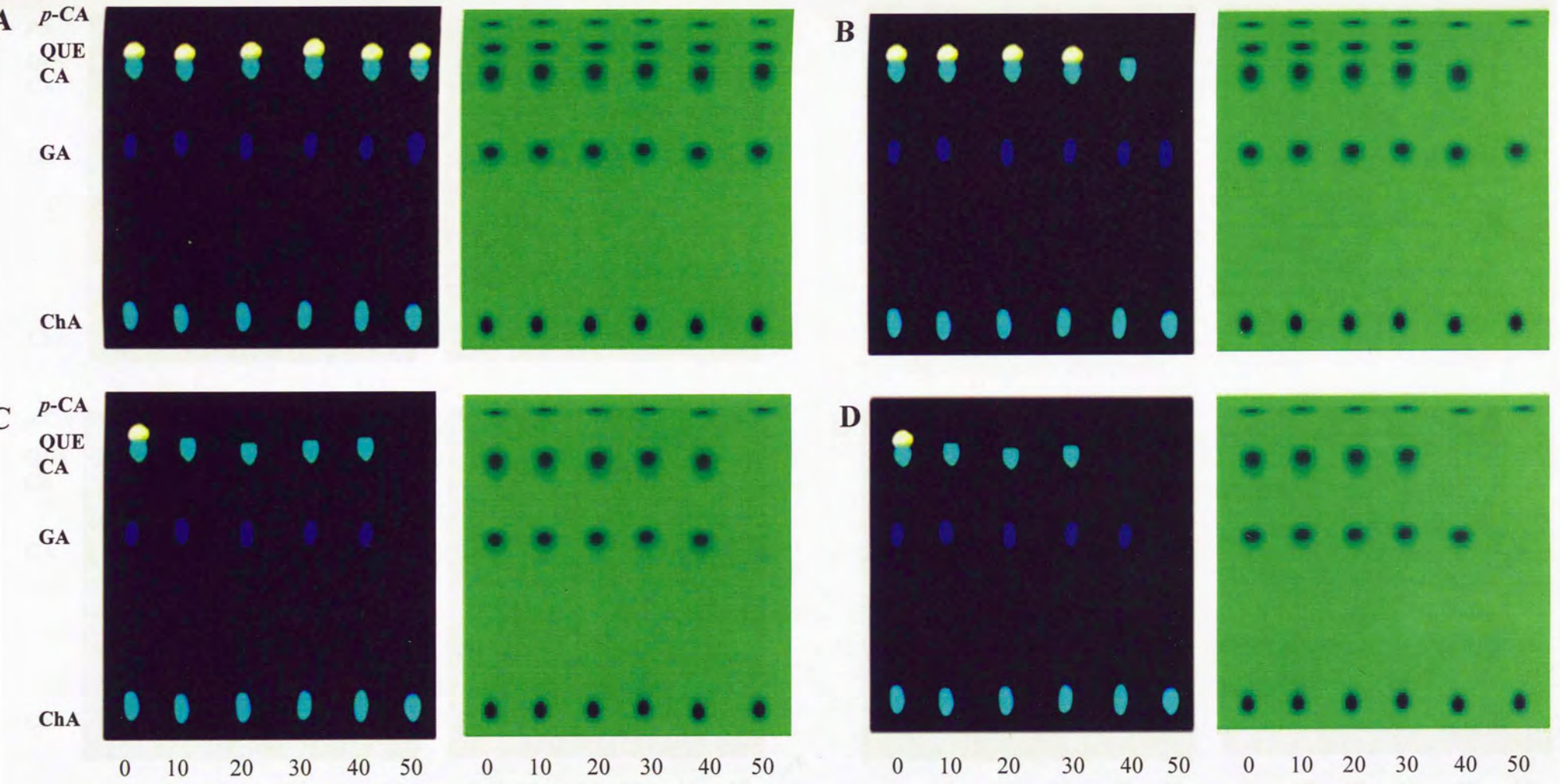


Figure 15: Chromatograms under SW and LW UV showing wine challenged with 10-50 mM H_2O_2 at (**A**) 0; (**B**) 60; (**C**) 120; and (**D**) 240 minutes.

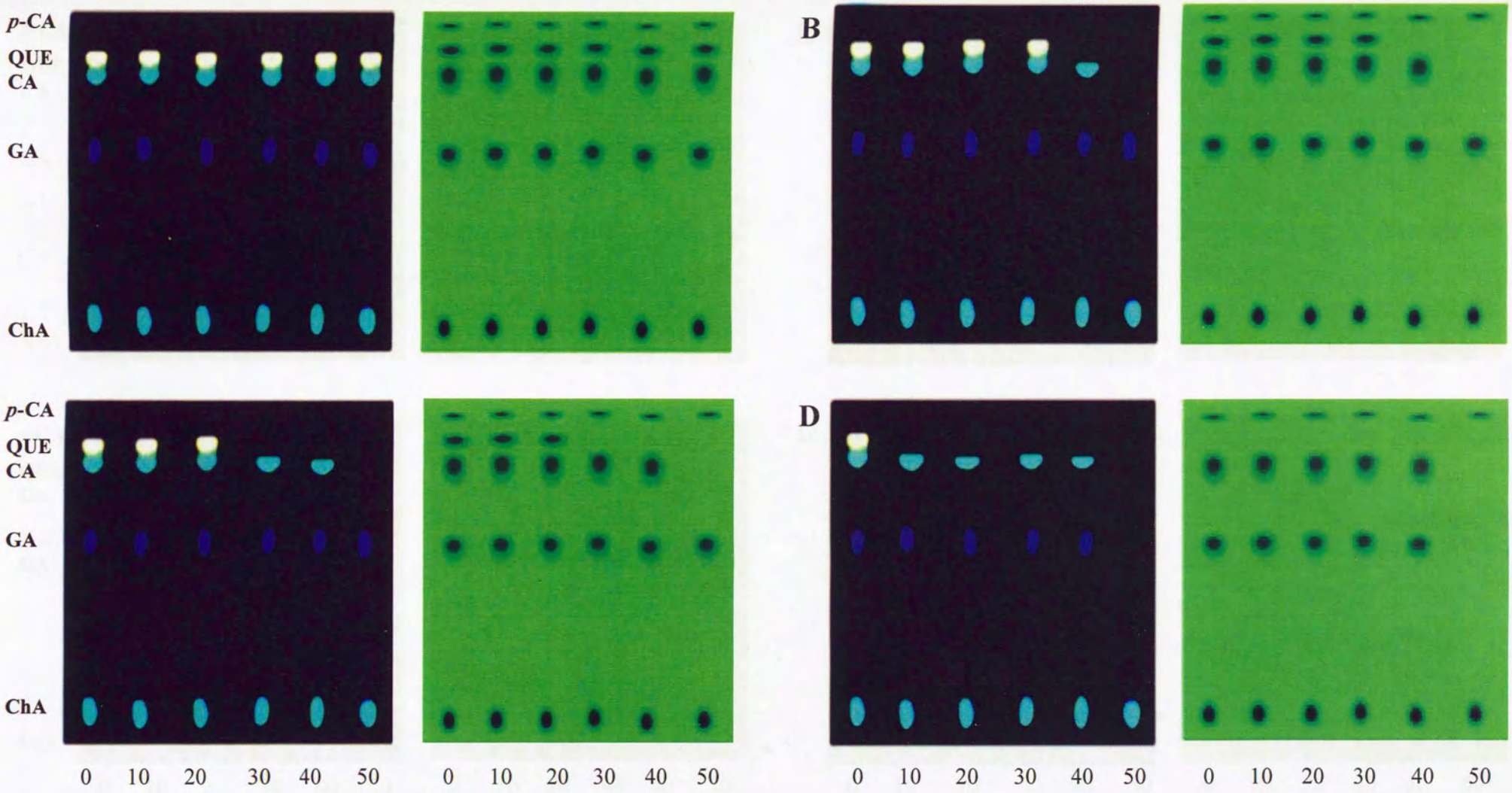


Figure 16: Chromatograms under SW and LW UV showing wine challenged with 10-50 mM Fe^{3+} at (**A**) 0; (**B**) 60; (**C**) 120, and (**D**) 240 minutes.

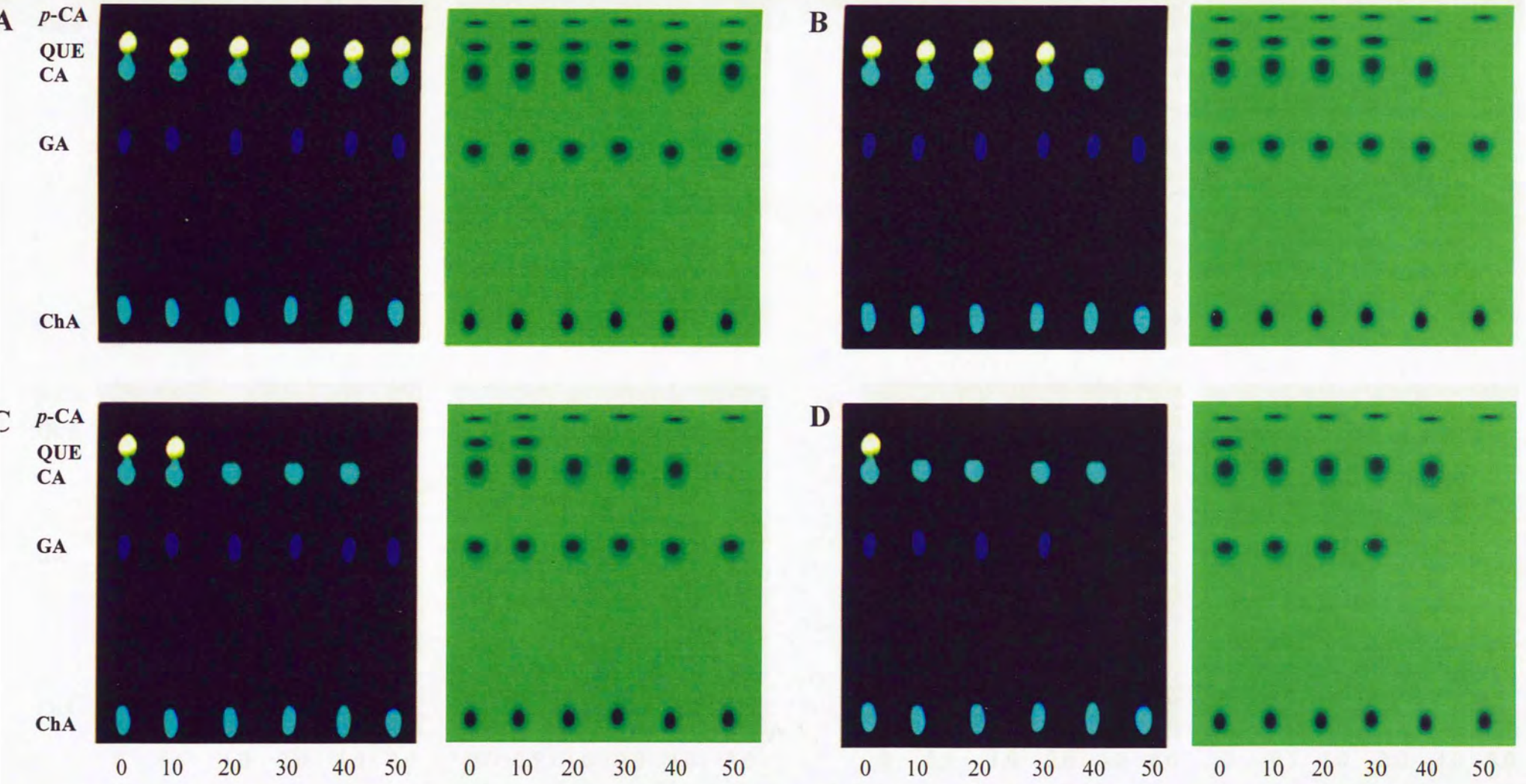


Figure 17: Chromatograms under SW and LW UV showing wine challenged with 10-50 mM Cu^{2+} at (**A**) 0; (**B**) 60; (**C**) 120; and (**D**) 240 minutes.

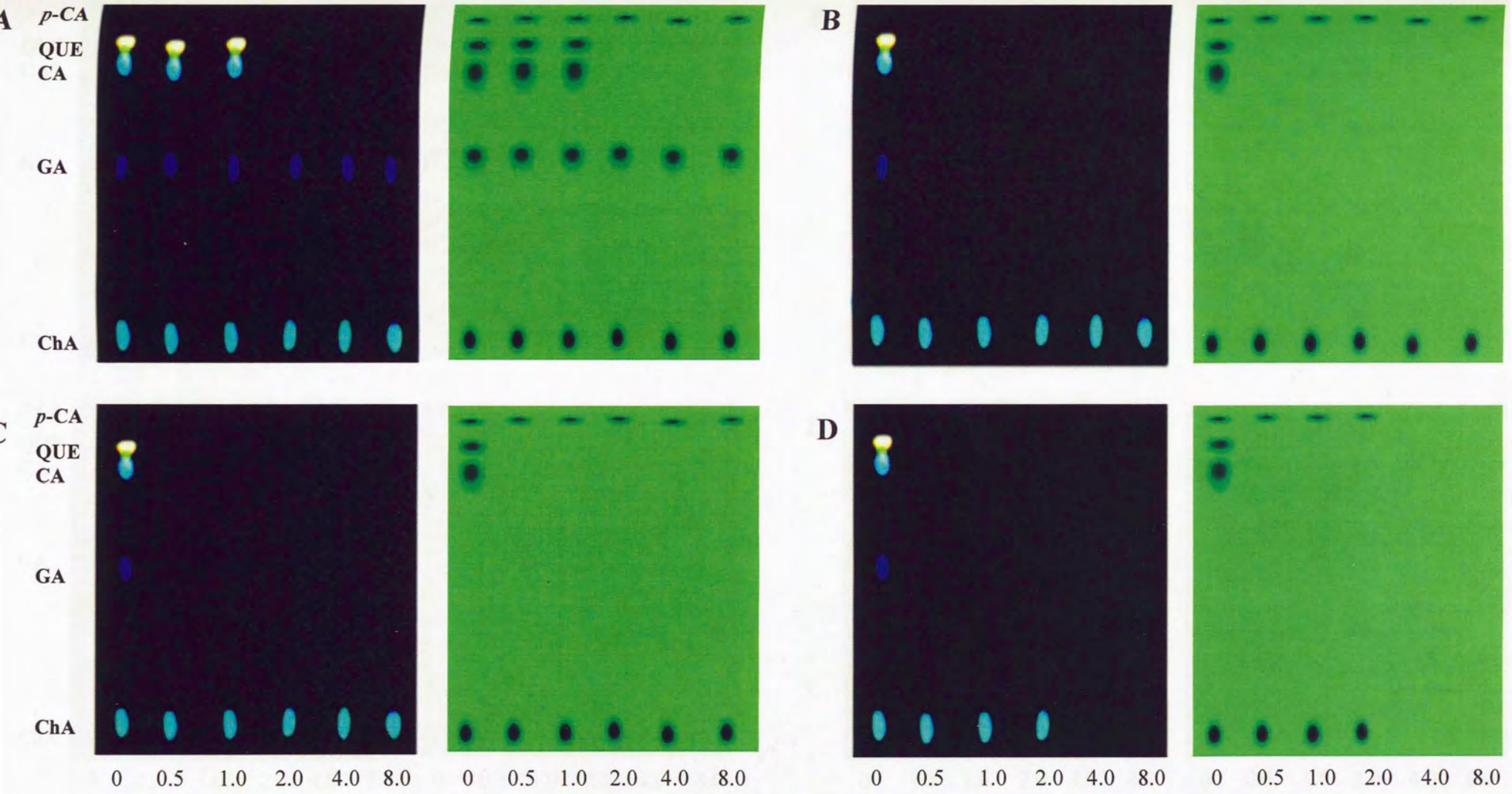


Figure 18: Chromatograms under SW and LW UV showing wine challenged with 0.5-8.0 mM Fe^{2+}-H_2O_2 at (**A**) 0; (**B**) 60; (**C**) 120; and (**D**) 240 minutes.

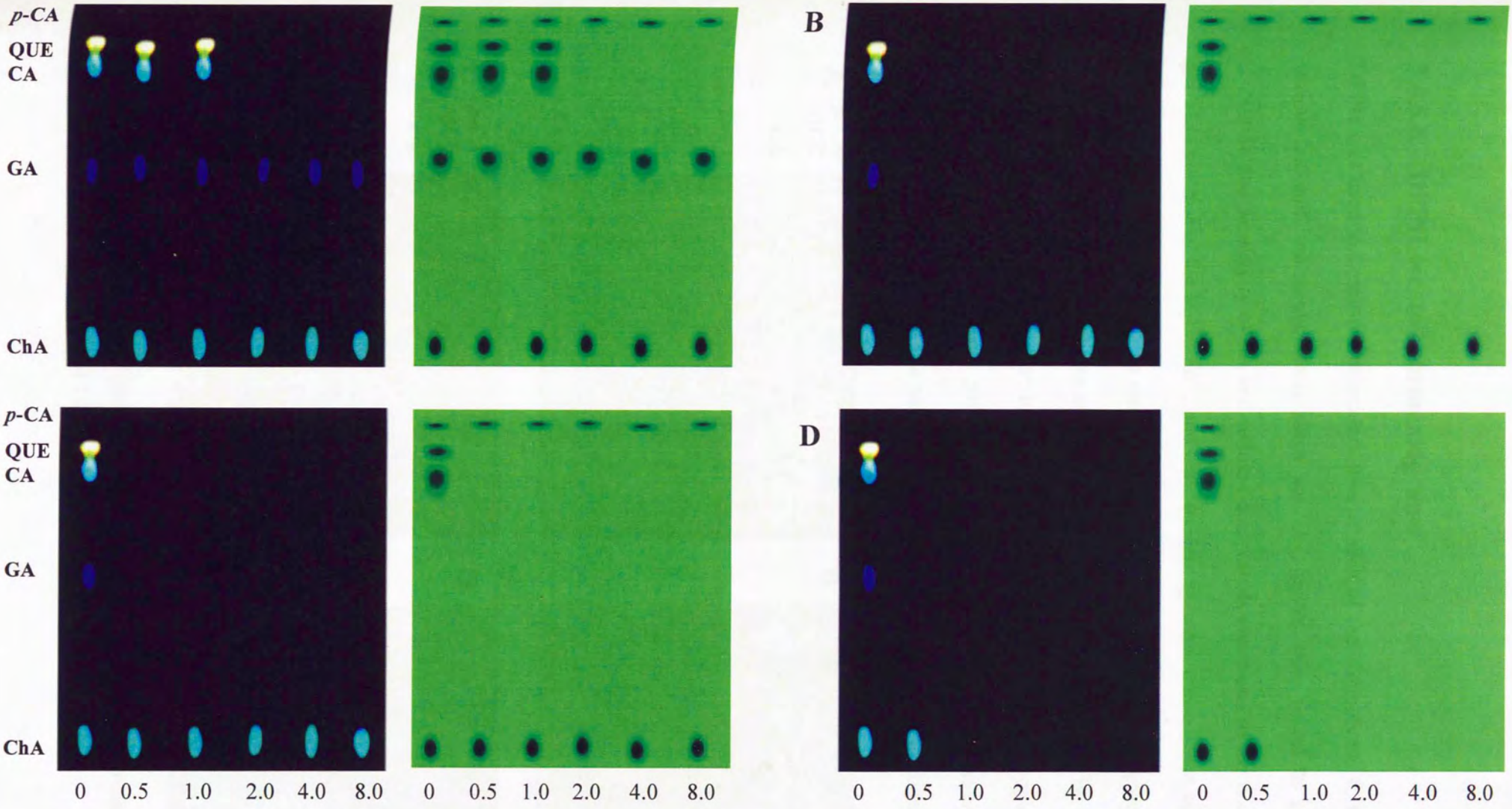


Figure 19: Chromatograms under SW and LW UV showing wine challenged with 0.5-8.0 mM Cu^{2+}-H_2O_2 at (**A**) 0; (**B**) 60; (**C**) 120; and (**D**) 240 minutes.

3.4.5.5. DPPH-treated chromatograms

DPPH was used to confirm anti-oxidant loss of each of the components following the various oxidant treatments. Nano silica gel TLC plates were found to give better resolution and sensitivity of the anti-oxidant compounds compared to the normal silica gel TLC plates. For the H_2O_2, Fe^{3+}, and Cu^{2+} oxidant systems, anti-oxidant activity was still evident at 0-30 minutes, as shown by white spots on a purple background. At 60 minutes, this anti-oxidant activity started to diminish gradually as shown by the fading of spots. At 120 minutes, further loss in activity was observed, with complete loss at 240 minutes incubation (Figure 20). For the two Fenton systems, anti-oxidant activity was evident at 0 minutes, but this activity was lost from 30 minutes incubation.

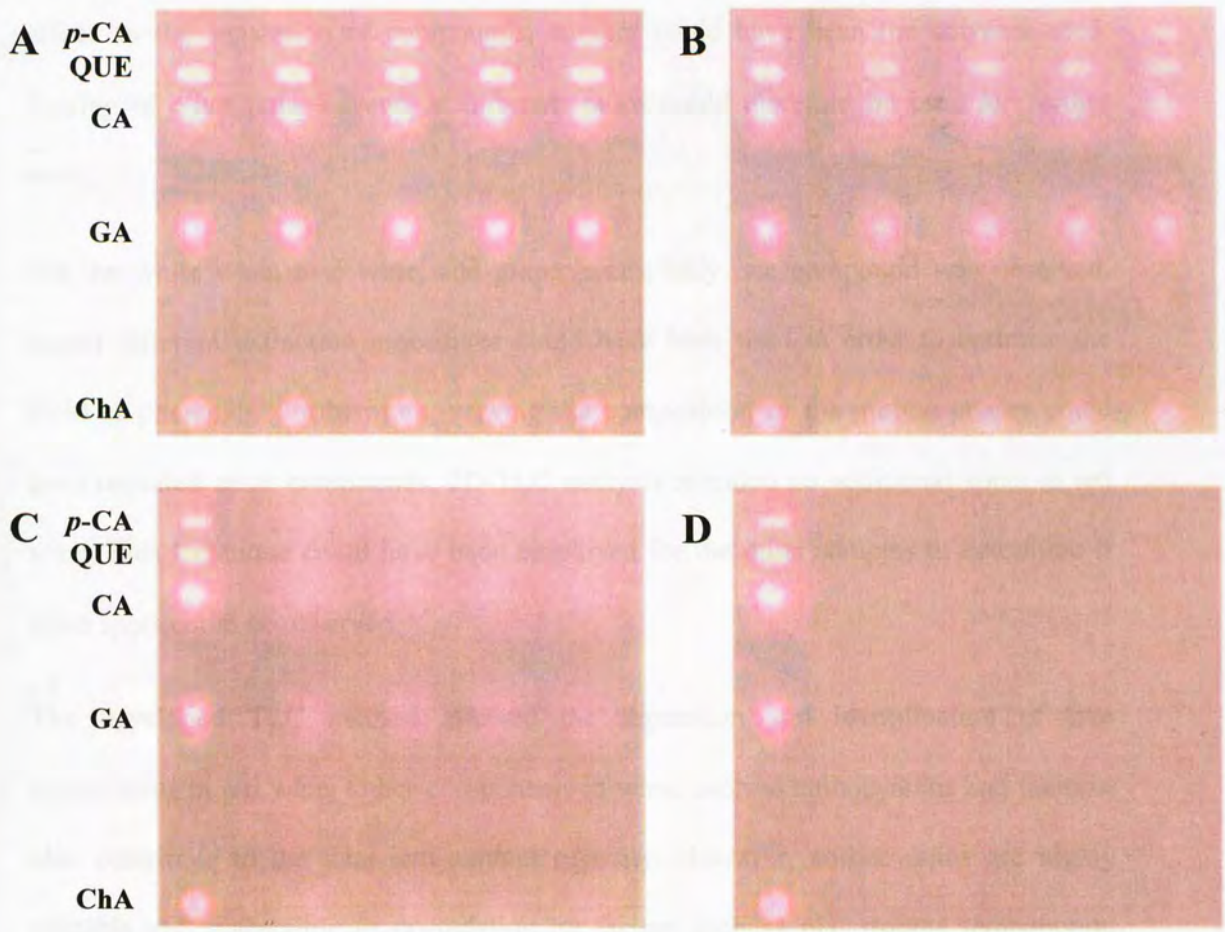


Figure 20: DPPH-sprayed chromatograms of H_2O_2, Fe^{3+}, and Cu^{2+}-treated RW at **(A)** 0 minutes; **(B)** 60 minutes; **(C)** 120 minutes; and **(D)** 240 minutes.

3.5. DISCUSSION

Initial TLC experiments were conducted to separate and identify the components from selected complex mixtures. The results showed that the tested mobile phases were not able to separate the grape samples. This could be due to the method of sample preparation that was used to extract the phenolic compounds from the grapes. Factors such as extraction time, temperature, and solvent can influence total yield of phenolics. In order to optimise the extraction of key phenolic components, different extracation methods have been reported, including supercritical fluid extraction (SFE) (de Campos et al., 2008); pressurised liquid extraction (PLE) (Piñero et al., 2006) and solid phase extraction (SPE) (Palma et al., (2002). Although the extraction method could have an affect on the separation of compounds, another could have been the solvents used. Testing of other polar sovents at different ratios could therefore be used for further work.

For the white wine, rosé wine, and grape juices, only one compound was observed. Again different extraction procedures could have been used in order to optimise the yield of phenolics. Furthermore, varying the composition of the mobile phases could have revealed other compounds. 2D-TLC analysis revealed no additional spots in red wine. This technique could have been employed for the other samples to determine if other spots could be observed.

The developed TLC method allowed the separation and identification of five components in red wine. Other components in wine, such as anthocyanins and tannins, also contribute to the total anti-oxidant capacity. However, anthocyanins are highly unstable and susceptible to degradation by factors such as pH, storage temperature, chemical structure, concentration, light, oxygen, flavonoids, proteins, and metal ions

(Castañeda-Ovando et al., 2009). There are many studies on the use of TLC to separate the anthocyanin pigments of various plant extracts (Lapornik et al., 2004; Nayak et al., 2010; Zhang et al., 2011). Further work could involve the use of TLC to separate anthocyanins and other components of wine, and monitoring anti-oxidant loss upon treatment with oxidants.

The results of this experiment showed that, among the investigated red wine phenolics, quercetin and caffeic acid were the most effective anti-oxidants followed by gallic acid. Overall, *p*-coumaric acid and chlorogenic acid were found to have the lowest anti-oxidant efficacies. In terms of anti-oxidant power of each oxidant test system, both Fenton systems were found to be the most oxidising compared to each oxidant added independently.

The standards exhibited a similar ranking order of compounds relative to the wine. Quercetin and caffeic acid were the most effective anti-oxidants followed by gallic acid, whereas *p*-coumaric acid and chlorogenic acid were also found to have the lowest anti-oxidant efficacies. However, although the standards were at a lower concentration to wine, i.e. 1 mg/mL, greater loss of compounds was observed in the presence of all oxidant systems. Whereas the red wine compounds were all still present over 30 minutes incubation in the presence of H_2O_2, Fe^{3+}, and Cu^{2+}, the standard compounds started to diminish over this time period even at a considerably lower concentration of these three oxidant systems.

Both Fe^{3+} and Cu^{2+} addition revealed similar results to the H_2O_2 system. The same binding sites involved in efficient radical scavenging could also account for the anti-oxidant efficacy of quercetin, and could explain the efficient chelation of these metal

ions. The results suggested that chlorogenic acid and *p*-coumaric acid were less efficient in sequestering these metal ions.

The Cu^{2+}-H_2O_2 system was slightly more oxidising compared to Fe^{2+}-H_2O_2. Unlike the oxidants added independently, all compounds gradually diminished in the presence of both Fenton systems. Both wine and standards showed similar results in terms of the order of loss of compounds. However, the chlorogenic acid standard was more efficient than the wine in that loss of this compound was seen after 60 minutes, compared to 240 minutes in wine. In addition, complete loss of this compound was observed in the Cu^{2+}-H_2O_2 system. The *p*-coumaric acid standard also appeared to be more efficient than the red wine phenolic. The greater loss of quercetin, caffeic acid, and gallic acid in both wine and standards over time suggested that these compounds were more efficient in inhibiting hydroxyl radical-mediated oxidation compared to *p*-coumaric and chlorogenic acids. Again, the mechanism of action could be through chelation or reduction of the metal ions.

The greater anti-oxidant efficacy of quercetin relative to gallic acid, *p*-coumaric acid, and chlorogenic acid can be attributed to the phenolic structure of the compound. It possesses the three components thought to be required for efficient radical scavenging: (1) the 2,3 double bond in combination with a 4-oxo function, (2) the 3'4'-di-hydroxy group on the B ring, and (3) the presence of hydroxyl groups in positions 3 and 5 (Procházková et al., 2011). It also possesses an additional OH group in ring B at the 5' position, which has also been found to enhance anti-oxidant activity (Rice-Evans et al., 1996).

For the two hydroxycinnamic acids, caffeic acid was more effective than *p*-coumaric acid. Although both have similar structures, *p*-coumaric acid has one hydroxyl group in

its structure, whereas dihydroxylation in the 3,4 position of caffeic acid could explain the higher anti-oxidant efficacy. Comparing caffeic acid to the hydroxybenzoic acid, gallic acid, the structures of both could account for the higher activity of the former. Caffeic acid possesses a CH=CH-COOH group, which is thought to exhibit better anti-oxidant efficiency than the COOH group in gallic acid (Soobrattee et al., 2005).

The standards mixture exerted greater anti-oxidant efficacies than the red wine phenolics even though these compounds were present at a lower concentration compared to the wine. The anti-oxidant activity of phenolic compounds is greatly affected by interactions with other constituents in the red wine matrix. Synergistic and/or antagonistic interactions occur between compounds which could affect total anti-oxidant capacity (Cheynier, 2005). For the pure standards mixture, however, there are less interfering substances such as proteins and other phenolic compounds. Metal ions can form complexes with the phenolic compounds present in wine which can form anti-oxidant enzyme mimetics or metal chelators able to scavenge radical species. However, metal ion binding has also been found to reduce anti-oxidant activity (Argyri et al., 2006).

3.6. CONCLUSION

Overall, the results demonstrated the use of functional TLC in monitoring the loss of red wine compounds in the presence of various oxidants. The anti-oxidant efficacies of the red wine phenolic compounds investigated decreased in the order: quercetin > caffeic acid > gallic acid > *p*-coumaric acid ≈ chlorogenic acid. For the phenolic standards, similar results were observed, but chlorogenic acid was a more efficient anti-oxidant compared to *p*-coumaric acid as follows: quercetin > caffeic acid > gallic acid >

chlorogenic acid > *p*-coumaric acid. In terms of oxidising power, the oxidant systems decreased in the order: $H_2O_2 + Fe^{2+} = H_2O_2 + Cu^{+} > H_2O_2 \approx Fe^{3+} \approx Cu^{2+}$, with both Fenton systems exhibiting the greatest oxidising power, and the three individual oxidant systems demonstrating comparable oxidising power.

In this experiment, functional TLC offered a quick and simple method to dissect the anti-oxidant profile of red wine in the presence of oxidant systems involving metals ions and H_2O_2. The use of the more sensitive and accurate method, HPTLC, to improve the separation and resolution power of the wine, could enable further identification and characterisation of the anti-oxidant efficacies of red wine. The advantages of this method over conventional TLC are many including: high throughput with minimal costs; minimal sample preparation; multiple detection using UV-visible, derivatisation; and parallel chromatography under identical environmental conditions (Morlock and Schwack, 2010). In addition hyphenation of HPTLC with other analytical techniques shows considerable potential for many areas of research.

Chapter 4

AN ASSESSMENT OF THE ANTI-OXIDANT ACTIVITY OF RED WINE USING A NEW REVERSED-PHASE HPLC METHOD

4.1. INTRODUCTION

Progress in chromatographic analytical techniques and instrumentation, as well as development of new methods, continues to advance our understanding of wine composition and its properties. HPLC, gas chromatography (GC), and capillary electrophoresis (CE) are some of the methods used in studying wine composition (Stalikas, 2010; Tsao and Deng, 2004). The use of GC in wine analysis, however, is not an ideal technique to use due to the limited volatility of many flavonoids, particularly the glycosides. Thus a derivatisation step is required before GC analysis (Antolovich et al., 2002).

However, to gain more detailed chemical information of complex low-level constituents in wine, hyphenated techniques are commonly employed, and include GC-MS (López et al., 2002), and LC-MS/MS (Jaitz et al., 2010). Other methods used to study wine composition include NMR spectroscopy (Košir and Kidrič, 2002) and two-dimensional LC (Dugo et al., 2009). Due to the speed, accuracy, and robustness of HPLC, quantitative analysis of wine constituents using this technique has grown considerably (de Villiers et al., 2012). Ultra-performance liquid chromatography (UPLC) offers an

improved method for analysis, with higher resolution and sensitivity, and lower analysis time than conventional HPLC.

There are numerous literature reports on the use of HPLC in quantitative analysis of the phenolic compounds in wine as outlined in Table 11. The levels of phenolic compounds reflect the variability in wines according to a number of factors, including: climate, soil type, grape variety, and processing methods (Pohl, 2007).

Table 11: *A selection of studies showing the levels of different phenolic compounds quantified in red wines using HPLC.*

eference	**Baroni et al., (2012)**	**Fang et al., (2007)**	**Fanzone et al., (2010)**	**Gambelli and Santaroni, (2004)**	**Milano et al., (2009)**	**Porgali and Büyüktune, (2012)**	**Rastija et al., (2009)**	**Roussis et al., (2008)**	**Šeruga et al., (2011)**
allic acid	21.2-34.0	-	13.8-21.7	13.6-90.5	21.29-29.81	24.07-50.89	4.9-26.9	1138-1700	51-179
rotocatechuic id	-	-	2.3-4.1	-	-	1.11-2.84	-	-	-
ydroxybenzoic id	-	-	-	-	-	0.69-1.21	-	-	-
yringic acid	-	-	2.3-4.2	-	-	3.35-5.08	-	-	-
atechin	-	-	24.4-47.0	-	98.31-181.19	154.5-201.20	2.0-4.7	230.8-527.1	31-138
picatechin	-	-	14.5-23.6	-	53.9-102.25	11.97-45.06	-	-	7.8-37.7
hlorogenic acid	-	-	-	-	15.19-32.33	2.00-5.81	-	-	-
affeic acid	7.0-7.9	-	1.3-3.4	2.5-17.9	3.86-20.53	0.96-3.67	13.6-21.6	208-308	3.2-18.6

Table 11 (continued): *A selection of studies showing the levels of different phenolic compounds quantified in red wines using HPLC.*

Caftaric acid	-	-	0.4-5.6	2.8-29.3	-	-	-	-	-
Coutaric acid	-	-	1.2-6.0	1.3-10.9	-	-	-	-	-
GRP (Grape Reaction Product)	-	-	-	2.2-9.2	-	-	-	-	-
p-Coumaric acid	4.5-7.3	-	0.3-3.0	0.9-16.0	-	1.22-8.24	1.7-7.4	-	1.8-4.4
Cinnamic acid	-	-	-	-	72.98-196.84	-	-	-	-
Ferulic acid	2.9-4.1	-	-	-	0.14-0.23	-	0.2-1.8	-	-
Rutin	-	-	-	-	-	3.20-9.03	-	-	-
t-Resveratrol	4.4-11.6	-	0.6-1.3	<0.2-2.2	0.85-1.74	0.31-0.97	0.4-4.9	-	-
Myricetin	-	1.57-4.45	0.7-2.9	1.5-9.7	-	0.78-4.61	0.5-3.3	-	-
Quercetin	-	0.17-4.87	3.2-5.4	1.5-12.8	-	0.60-4.65	3.6-10.4	144-219	1.2-7.0
Kaempferol	-	0.06-0.20	-	-	-	-	0.3-0.8	-	-
Isorhamnetin	-	0.03-0.54	-	-	-	-	-	-	-
Galangin	-	0.01-0.04	-	-	-	-	-	-	-

Table 11 (continued): *A selection of studies showing the levels of different phenolic compounds quantified in red wines using HPLC.*

Luteolin	-	0.18-0.96	-	-	-	-	-	-	-
Apigenin	-	-	-	<0.2-4.7	-	-	0.2	-	-
Malvidin	-	-	189.9-408.8	<0.4-139	-	-	-	224-523	-
Peonidin	-	-	3.5-23.5	<0.4-26.3	-	-	-	-	-
Petunidin	-	-	17.7-83.5	<0.4-2.3	-	-	-	-	-
Cyanidin	-	-	1.6-15.5	<0.4-12.3	-	-	-	-	-
Delphinidin	-	-	12.0-67.8	<0.4-2.7	-	-	-	-	-
Tyrosol	-	-	5.0-7.5	-	-	-	-	-	-
Total phenolic content (mg/L)	**2290-2555**	**3.29-10.22**	**1932.0-3506.8**	-	**2015-2650**	**1836.5-3466.9**	**1156-2619**	**2082-3184**	**1012-3264**

There have also been several studies using on-line HPLC assays for anti-oxidant screening-the most popular approach using DPPH and ABTS as stable radical reagents (Niederländer et al., 2008). A study by Yasuda et al., (2012) investigated the effect of adding Fe^{3+}, Fe^{2+}, and Cu^{2+} ions to catechins using HPLC coupled to an electrochemical detector. The peaks of epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) decreased as the ratio of Fe^{2+} increased, with complete disappearance of EGCG at $[Fe^{2+}]$/[catechin]=2. The effects of EDTA on the metal ion-catechin complexes were also evaluated. It was found that the addition of low concentrations of EDTA (100 μM) to the metal-catechin solutions (20 μM) gradually decreased the HPLC intensities over time. This was thought to indicate that the longer the standing time before the addition of EDTA, the more likely oxidation of catechins would occur. When a higher concentration of EDTA (1 mM) was applied to the metal ion-catechin complexes, the HPLC intensities of all catechins were completely recovered, indicating that EDTA readily removed the metal ions from the metal-catechin complexes.

HPLC was also used by Ruenroengklin et al., (2009) to monitor the degradation of anthocyanins from litchi fruit pericarp in the presence of H_2O_2 and hydroxyl radical. Degradation of anthocyanin solutions at 0.5 μM was observed at increasing concentrations of H_2O_2 (0, 0.1 % and 1 %). However, in the presence of Fenton's reagent ($FeSO_4/H_2O_2$), a greater effect on anthocyanin degradation was observed (as shown by a greater decrease in peak height).

However, there are no reports on the use of this technique to study the anti-oxidant activity of wine. Chapter three demonstrated the use of functional TLC to rank the anti-oxidant efficacies of five phenolic compounds in red wine. The aim of this chapter is to develop a quantitative approach using reversed-phase HPLC to further evaluate the anti-oxidant activity of four of these components, i.e. gallic acid, caffeic acid, *p*-coumaric

acid, and quercetin. The selection of these compounds rather than others such as anthocyanins, was informed by the TLC experiments in the previous chapter. As well as being present in wines (with gallic acid being the predominant phenolic acid in red wine), these compounds are also found in a variety of other foods including fruits and vegetables. Gallic acid widely occurs in gallnuts, grapes, tea, and oak bark, whilst caffeic acid and *p*-coumaric acid are found in coffee beans, tea, and olive oil. The anti-oxidant activities of gallic, caffeic, and *p*-coumaric acids have been widely reported (Ferk et al., 2011; Cheng et al., 2007). Quercetin is one of the most common flavonoids, and is the most predominant flavonoid in the diet, widely distributed in plant and plant-derived products. It is therefore a frequently studied anti-oxidant, with a wide range of reported pharmacological and biological benefits (Russo et al., 2012).

4.2. AIMS

The aims of this chapter are:

- Development, optimisation, and validation of an analytical HPLC method to measure the selected phenolic compounds in red wine.
- To use RP-HPLC to assess the anti-oxidant efficacies of four of the red wine anti-oxidants: gallic acid, caffeic acid, *p*-coumaric acid, and quercetin using the previous five oxidant systems: (1) H_2O_2, (2) Fe^{3+}, (3) Cu^{2+}, and two Fenton systems: (4) Fe^{2+}-H_2O_2, and (5) Cu^{+}-H_2O_2. Compare and contrast anti-oxidant efficacies with those of the standard phenolic compounds, and discuss possible matrix effects.
- Rank anti-oxidant efficacy of these phenolic compounds, and oxidant system strength.

- Measure the Fe^{2+} and Cu^{2+} metal binding activities of the phenolic compounds using UV-vis spectrophotometry. Monitor the effect of competitive chelation between redox-active metal ions and excess EDTA. Detail these spectral changes.

4.3. METHODS

The methods are given in chapter two. Briefly:

- For the HPLC analysis, a concentration of 1.0 mM of each of the five oxidants (100 μL) was incubated with the wine sample (100 μL). For the standards, 400 mg/L of each standard was mixed together and 100 μL of this mixture was incubated with 1.0 mM of each oxidant (100 μL). The reaction mixtures were incubated in the dark at room temperature for 60 minutes. The five oxidant systems were added to the wine and standards in separate experiments. Peak area measurements were analysed immediately after this time using HPLC.
- For the metal chelation activities, a concentration of 100 μM was prepared for each phenolic compound in PBS (10 mM, pH 7.4). Aliquots of Fe^{3+} or Cu^{2+} were added at the same concentration (100 μM) to each compound at a ratio of 1:1. The reaction mixtures were left to incubate in the dark at room temperature for 10 minutes, and spectra were recorded immediately after this time. A 4.0-fold EDTA concentration (400 μM) was subsequently added to these phenolic-metal ion complexes, and left to incubate for a further 10 minutes.

4.4. RESULTS

4.4.1. Method validation

Different mobile phases were tested using acetonitrile, acetic acid, and formic acid at different ratios. However, these did not show efficient separation of peaks. Methanol and othophosphoric acid were found to give optimum separation and resolution of the four phenolic compounds and red wine. This RP-HPLC method was therefore used for further anti-oxidant testing. The chromatograms of the phenolic standards and red wine are shown in Figure 21A and B, respectively.

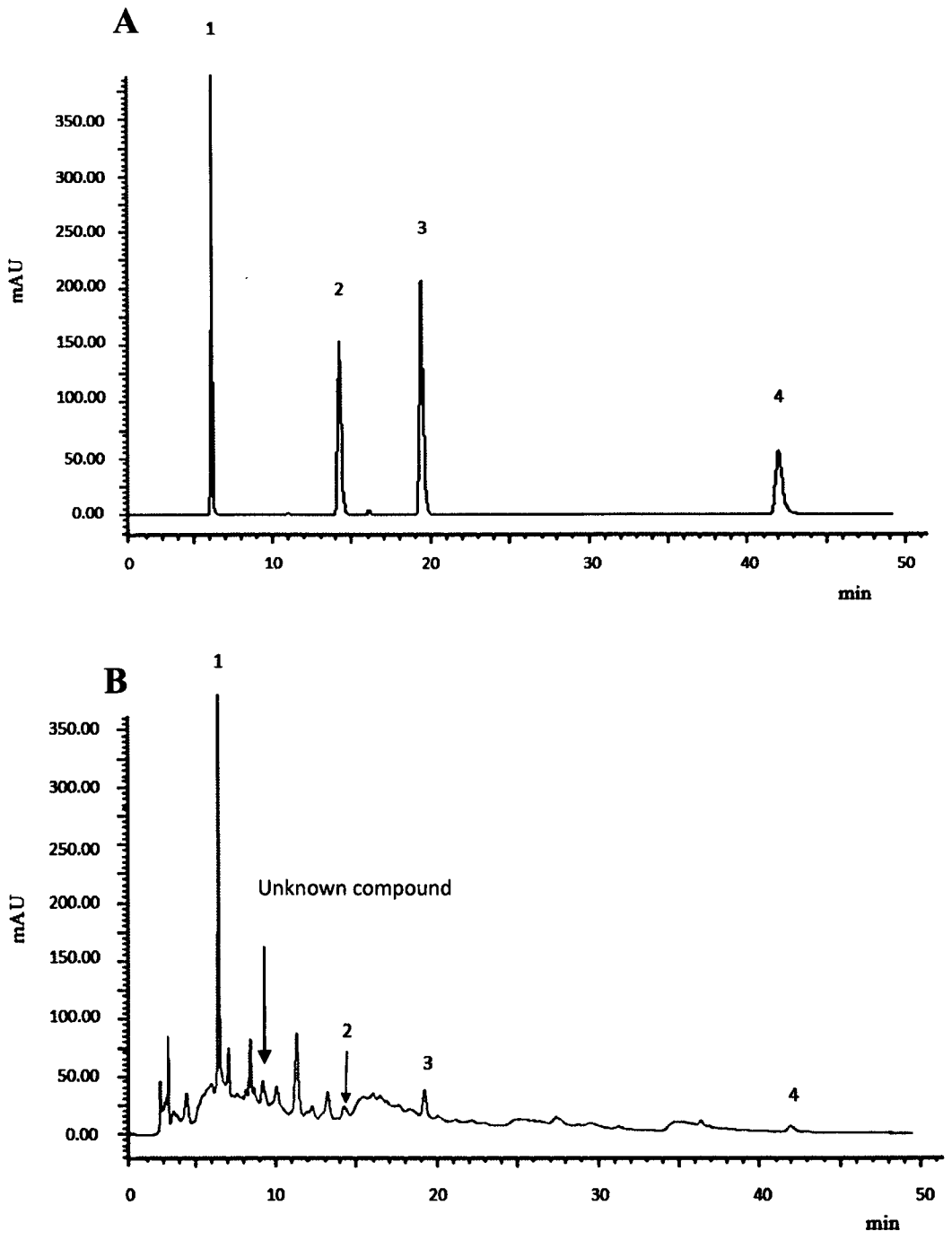


Figure 21: HPLC chromatograms of **(A)** phenolic standards, and **(B)** RW, showing peaks of the four phenolics measured: 1: gallic acid, 2: caffeic acid, 3: *p*-coumaric acid, 4: quercetin, and the unknown compound.

Table 12 shows the regression equations and other characteristic parameters for determination of the concentrations of the phenolic standards. The correlation coefficients ranged from 0.998-0.999. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the calibration curves. LOD ranged between 0.93-13.50 mg/L, and LOQ was 1.61-13.75 mg/L.

Table 12: Parameters of linearity, retention times, LOD, and LOQ for the standard phenolic compounds. Limit of detection (LOD) = (3*SD)/slope and limit of quantification (LOQ) = (10*SD)/slope.

Peak no.	Compound	t_r (min)	Range of linearity (mg/L)	Regression equation	R^2	LOD (mg/L)	LOQ (mg/L)
1	Gallic acid	5.91	25-800	Y=5598.6*x*-48420	0.999	8.81	9.18
	Unknown compound	9.68	25-800	Y=1956.7x+12979	0.998	7.09	8.15
2	Caffeic acid	13.85	25-800	Y=5145.7*x*+16751	0.999	3.43	3.83
3	*p*-Coumaric acid	18.99	25-800	Y=8365.9*x*-112066	0.999	13.50	13.75
4	Quercetin	41.91	25-800	Y=3057.8*x*+1960.5	0.999	0.93	1.61

Intraday repeatability, which was determined by analysing six consecutive replicates, was found to be 0.46-2.93 %. Interday reproducibility, which was calculated using six measurements on six separate days, was 0.56-6.90 % (Table 13).

Table 13: Repeatabilities intra- and inter-day at three concentration levels. Intraday and interday precision expressed as % RSD = (SD/mean) × 100%.

Compound	**Concentration (mg/L)**	**Intraday % RSD**	**Interday % RSD**
Gallic acid	100	2.36	2.25
	200	0.92	2.94
	400	0.93	1.20
Unknown compound	100	2.93	3.71
	200	0.46	1.95
	400	0.87	0.81
Caffeic acid	100	2.59	2.01
	200	0.91	2.08
	400	0.88	0.56
p-Coumaric acid	100	2.49	1.79
	200	0.97	2.24
	400	0.92	0.60
Quercetin	100	2.23	6.90
	200	1.33	3.63
	400	1.15	1.38

Quantification of the phenolic compounds showed gallic acid was the most predominant. The unknown compound had the next highest level in wine, whereas caffeic acid showed the lowest overall concentration. Levels of *p*-coumaric acid, caffeic acid, and quercetin were consistent with previously reported levels in red wine (Table 14) (Paixão et al., 2008).

Table 14: Content of phenolic compounds in the red wine sample (mean ± SEM).

Phenolic compound	Concentration (mg/L)
Gallic acid	749.26 ± 21.94
Unknown compound	719.74 ± 54.06
Caffeic acid	31.19 ± 2.10
p-Coumaric acid	69.26 ± 1.26
Quercetin	53.43 ± 1.28

4.4.2. Tentative identification of unknown compound using LC-MS

As shown in the chromatograms, initital HPLC analysis of the unknown compound was thought to be chlorogenic acid. The concentration of this compound was calculated to be 719.74 ± 54.06. However, the level of the compound found was above the concentration range reported in some studies (Milano et al., 2009; Porgali and Büyüktune, 2012). The fraction corresponding to the elution time of the compound was

therefore collected, and subjected to further analysis using LC-MS to determine its identification.

LC-MS found that the compound in that particular fraction was not chlorogenic acid, as shown in Figure 22A for the red wine fraction, compared to the chlorogenic acid standard in Figure 22B. However, chlorogenic acid was identified in the wine using TLC as shown in chapter three, and several studies have reported the presence of chlorogenic acid in red wines (Milano et al., 2009; Porgali and Büyüktune, 2012).

The mass spectrum of the red wine fraction shows the main peaks at m/z 311, 351, and 557. The peak at m/z 311 could be a fragment of the molecular ion m/z 557, suggesting an anthocyanin (Ginjom et al., 2011). The peak at m/z 221 suggests that this could be a flavanol (possibly catechin). The results indicate that the compound could be a flavanol-anthocyanin condensed pigment (González-Paramás et al., 2006; Vivar-Quintana et al., 2002). In addition, the concentration of this compound suggests it may be a polymeric condensed tannin, which has been reported to constitute the majority of wine phenolics (Waterhouse, 2002).

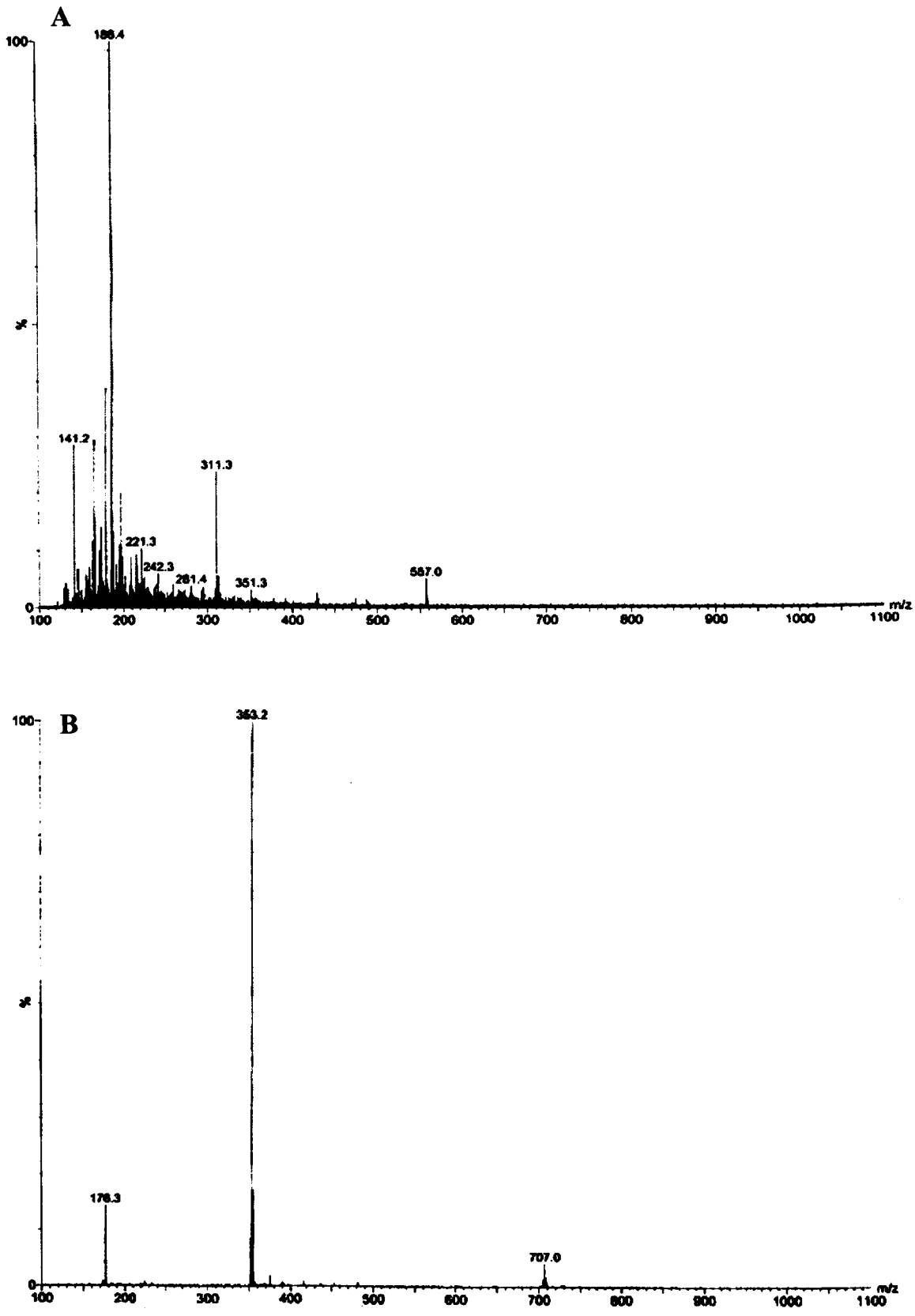


Figure 22: Mass spectra of (A) red wine fraction eluted between 9-10 minutes to determine identification of unknown compound with retention time of 9.68 minutes, and (B) chlorogenic acid standard.

4.4.3. HPLC of red wine-and standard-oxidant mixtures

4.4.3.1. H_2O_2 addition

In this oxidant system caffeic acid exhibited the highest anti-oxidant activity, while gallic acid was the least effective at scavenging H_2O_2 (Figure 23A). The anti-oxidant activitites of the individual phenolic compounds decreased in the order: caffeic acid (77.4 %) > quercetin (47.0 %) > *p*-coumaric acid (43.1 %) > gallic acid (39.4 %). These activities were statistically significant ($p < 0.01$) between all red wine compounds.

For the standards, quercetin was the most effective anti-oxidant, with the other standards showing similar activities: quercetin (59.7 % > gallic acid (50.4 %) ≈ caffeic acid (49.6 %) ≈ *p*-coumaric acid (48.8 %). There were no significant differences between the standards.

Overall, for both the red wine and standards there was no significant difference between both groups, except for the caffeic acid red wine phenolic.

4.4.3.2. Ferric chloride (Fe^{3+}) addition

Addition of Fe^{3+} ions to wine revealed similar results as the H_2O_2 system (Figure 23B). Although all red wine phenolics exhibited similar activities, caffeic acid and quercetin exhibited slightly higher anti-oxidant activities than *p*-coumaric acid and gallic acid. The ranking order was: quercetin (51.3 %) ≈ caffeic acid (51.3 %) > *p*-coumaric acid (48.6 %) ≈ gallic acid (46.8 %). There were no significant differences between the compounds in red wine.

The standards showed *p*-coumaric acid to have slightly better anti-oxidant activity than caffeic acid and gallic acid. Again, quercetin was the most effective anti-oxidant as follows: quercetin (58.6 %) > *p*-coumaric acid (51.8 %) ≈ caffeic acid (49.4 %) ≈ gallic acid (47.9 %). Like H_2O_2, there were no significant differences between the standards.

Overall, for both the wine and standards, there was no statistically significant difference between both groups.

4.4.3.3. Cupric chloride (Cu^{2+}) addition

Wine challenged with Cu^{2+} ions reflected the anti-oxidant ranking found for Fe^{3+} addition, with variations in the efficacies of the compounds (Figure 23C). Again, quercetin and caffeic acid were the most efficient, and *p*-coumaric acid exhibited the weakest activity. The anti-oxidant activities of the wine phenolics decreased in the order: quercetin (51.0 %) ≈ caffeic acid (50.8 %) > gallic acid (46.2 %) > *p*-coumaric acid (27.2 %). These results suggest that for the wine phenolics, Cu^{2+} oxidation/chelation appeared to be less effective than Fe^{3+}-catalysed oxidation/chelation. The anti-oxidant activities of quercetin, gallic acid, and caffeic acid were not statistically significant. *p*-Coumaric acid, however, was statistically significant ($p < 0.01$).

The standards showed a different order of anti-oxidant activity, with caffeic, gallic and *p*-coumaric acids displaying similar activities. These were in the order: quercetin (57.4 %) > *p*-coumaric acid (52.2 %) ≈ caffeic acid (50.2 %) ≈ gallic acid (48.9 %). Again, like H_2O_2 and Fe^{3+}, there were no significant differences between the standards.

Overall, there was no significant difference between the standards and red wine, except for the *p*-coumaric acid wine phenolic.

4.4.3.4. Fenton systems

Treatment with both Fenton reagents revealed a more marked reduction in peak area of all compounds compared to the oxidants added alone. When both systems were applied, quercetin and caffeic acid were found to possess the greatest anti-oxidant efficacies in the wine in relation to the standards, as shown by complete loss of peaks on the chromatograms.

Fe^{2+}-H_2O_2 addition

In the presence of the Fe^{2+}-H_2O_2 Fenton system, the anti-oxidant activities of the wine phenolics were in the order: quercetin (100.0 %) ≈ caffeic acid (100.0 %) > gallic acid (62.7 %) ≈ *p*-coumaric acid (60.6 %) (Figure 23D). The difference between the activities of quercetin, caffeic acid, gallic acid, and *p*-coumaric acid were statistically significant ($p < 0.001$).

For the standards, the anti-oxidant efficacies of quercetin and caffeic acid were less efficient, but gallic acid and *p*-coumaric acid standards exhibited comparatively higher activities than those in the wine. The ranking order was: quercetin (88.7 %) > gallic acid (71.6 %) > caffeic acid (68.8 %) ≈ *p*-coumaric acid (67.6 %). The difference in activities between quercetin and the other three standards was statistically significant ($p < 0.001$).

Overall, the activities of the gallic acid and *p*-coumaric acid wine phenolics, and caffeic acid and *p*-coumaric acid standards were not significantly different.

Cu^{+}-H_2O_2 addition

The Cu^{+}-H_2O_2 Fenton system showed similar results to Fe^{2+}-H_2O_2. The anti-oxidant activities of the wine compounds decreased in the order: quercetin (100.0 %) ≈ caffeic acid (100.0 %) > gallic acid (68.3 %) > *p*-coumaric acid (66.5 %) (Figure 23E). The difference between the activities of querectin, caffeic acid, gallic acid, and *p*-coumaric acid were statistically significant ($p < 0.001$).

For the standards, the anti-oxidant efficacies of quercetin and caffeic acid were less efficient, but gallic acid and *p*-coumaric acid standards exhibited comparatively higher activities than those in the wine. The anti-oxidant activities of the standards in the presence of Cu^{+}-H_2O_2 were in the order: quercetin (68.3 %) ≈ *p*-coumaric acid (67.1 %) ≈ caffeic acid (66.8 %) ≈ gallic acid (65.8 %). Unlike the H_2O_2 + Fe^{2+} Fenton system, there were no significant differences between the standards.

Overall, for both the wine and standards, there was a significant difference between both groups ($p < 0.001$).

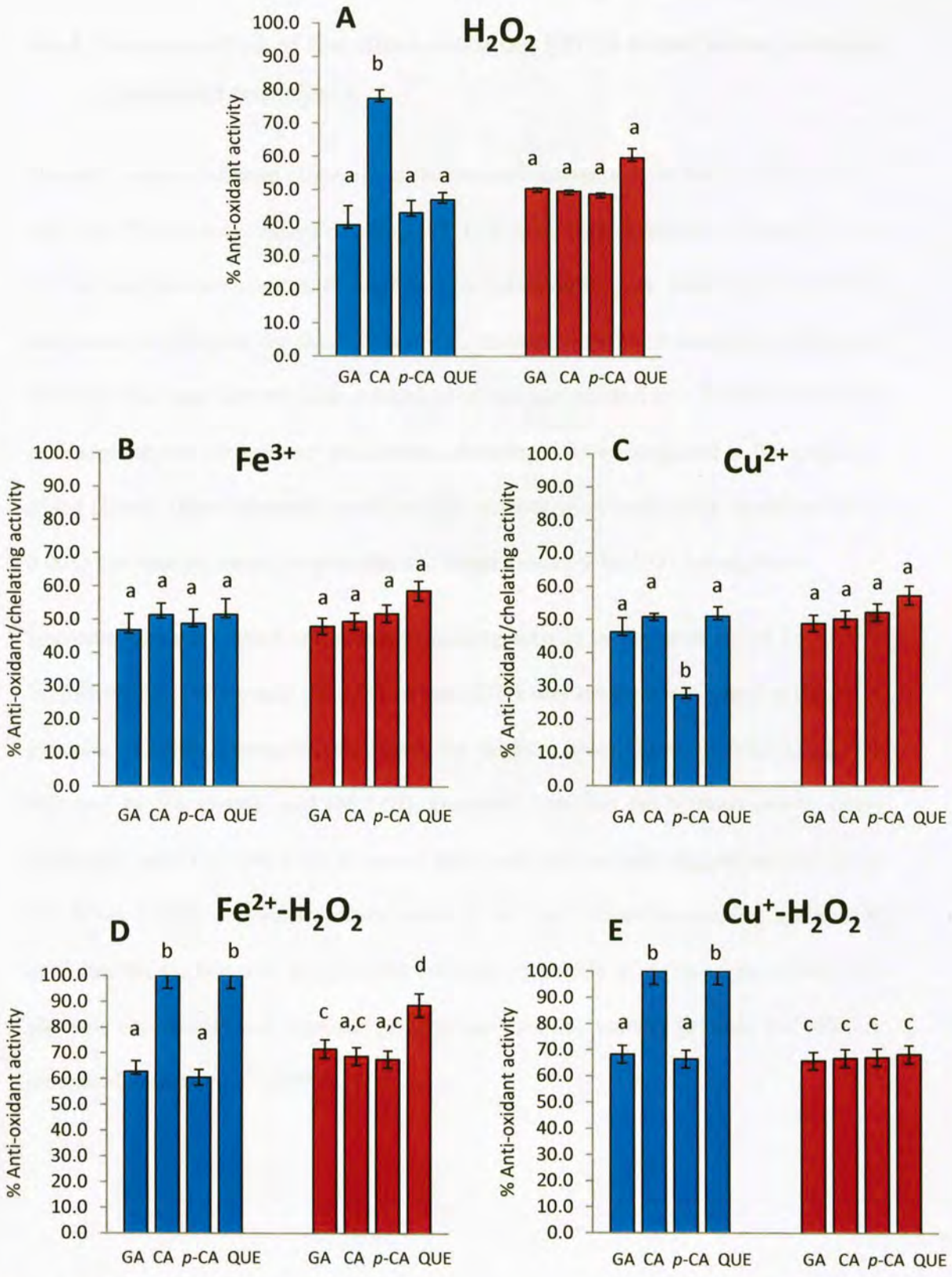


Figure 23: % Anti-oxidant activity of RW (Blue bars) and standard phenolic compounds (Red bars) challenged with **(A)** H_2O_2; **(B)** Fe^{3+}; **(C)** Cu^{2+}; **(D)** Fe^{2+}-H_2O_2; and **(E)** Cu^{+}-H_2O_2 after 60 minutes incubation. Bars with the same lowercase letter are not significantly different between treated samples ($p > 0.05$).

4.4.4. Determination of the effect of adding EDTA to metal-ion phenolic compound complexes

The anti-oxidant/chelating capacities of the phenolic compounds in the presence of Fe^{2+}, Cu^{2+} and EDTA were measured using HPLC (Figure 24). Addition of either Fe^{2+} or Cu^{2+} to the phenolic compound resulted in a reduction in peak area after 10 minutes incubation as observed for the Fe^{2+} and Cu^{2+} oxidant systems. Subsequent addition of EDTA to the metal ion-phenolic compound complexes resulted in a further decrease in peak area (shown by a greater anti-oxidant/chelating activity compared to the metal ion added alone). These increases in anti-oxidant activity were statistically significant ($p < 0.001$) between the metal ion-phenolic and metal ion-phenolic-EDTA complexes.

Quercetin had the highest anti-oxidant/chelating activity in the presence of Fe^{2+} (59.7 %) followed by caffeic acid (50.8 %). When EDTA was subsequently added to the Fe^{2+}-phenolic complex, quercetin exhibited the highest anti-oxidant activity (71.5 %) followed by *p*-coumaric acid (65.5 %). Quercetin also had the highest activity when challenged with Cu^{2+} (60.6 %), however gallic acid had the next highest activity (57.6 %). When EDTA was subsequently added to the Cu^{2+}-phenolic complex, gallic acid exhibited the highest activity (73.4 %) followed by caffeic acid (70.1 %). Overall, the phenolic compounds had a greater anti-oxidant/chelating activity towards Cu^{2+}-EDTA compared to that of Fe^{2+}-EDTA.

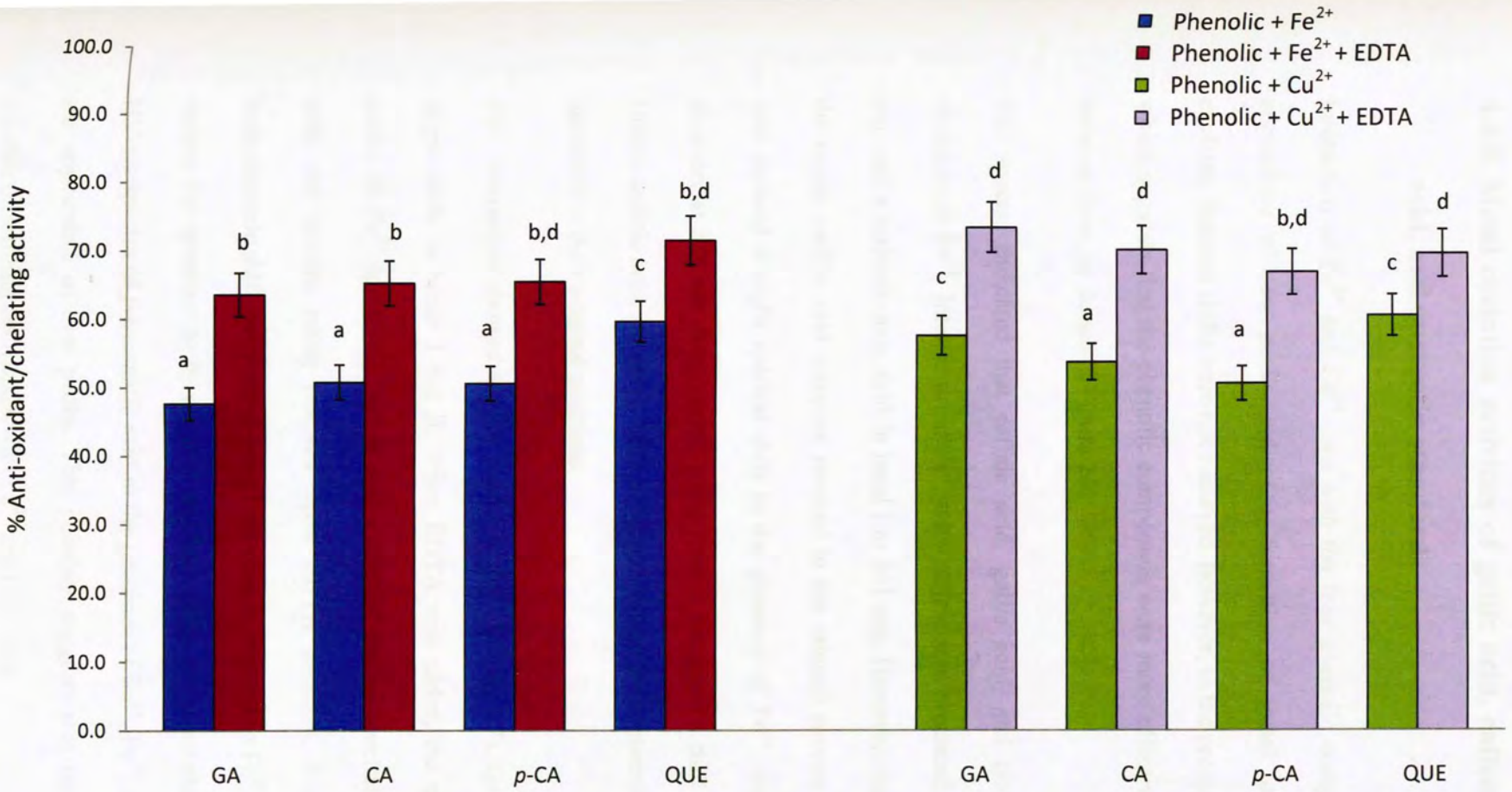


Figure 24: % Anti-oxidant/chelation activity of the standard phenolic compounds challenged with Fe^{2+}, Cu^{2+} and EDTA using HPLC.

4.4.5. Metal chelation activities of gallic acid, caffeic acid, *p*-coumaric acid, and quercetin standards

Interaction of Fe^{2+} and Cu^{2+} ions with the four phenolic compounds resulted in the appearance of new peaks, indicating formation of metal ion-phenolic compound chelates. Spectral shifts were not observed, however, in the presence of Fe^{3+} (Figure 25) which suggests that the phenolic compounds were more effective at binding Fe in the bivalent form, as shown in Figure 26.

The spectra indicated that caffeic acid, gallic acid, and quercetin were effective chelators of Fe^{2+}. Interaction of Fe^{2+} with caffeic acid produced two new peaks at 261 nm, and a bathochromic shift in band I to 341 nm. However, the addition of EDTA to the metal: caffeic acid complex resulted in the original spectra being restored. Gallic acid showed a slight spectral shift in the presence of Fe^{2+}. For quercetin, the small shoulder at 328 nm disappeared, while band I exhibited a shift between 350-450 nm. Unlike caffeic acid, however, EDTA did not restore the spectra for both gallic acid and quercetin to their original positions.

Cu^{2+} interaction showed similar results to Fe^{2+} (Figure 27). Gallic acid demonstrated slight shifts in bands I and II. When EDTA was added, the spectrum was restored, unlike its Fe^{2+} spectrum. Caffeic acid exhibited similar spectral shifts to that of Fe^{2+}, with the spectra being restored upon EDTA addition. For quercetin, a larger bathochromic shift was seen in band I maxima in contrast to Fe^{2+}. Again EDTA did not restore the spectrum to its original profile. Unlike the other phenolic compounds, the UV-vis spectra of *p*-coumaric acid in the presence of Fe^{3+}, Fe^{2+}, and Cu^{2+} did not show the appearance of new peaks. This possibly suggests that this compound was less capable of forming complexes with Fe^{2+} and Cu^{2+} ions.

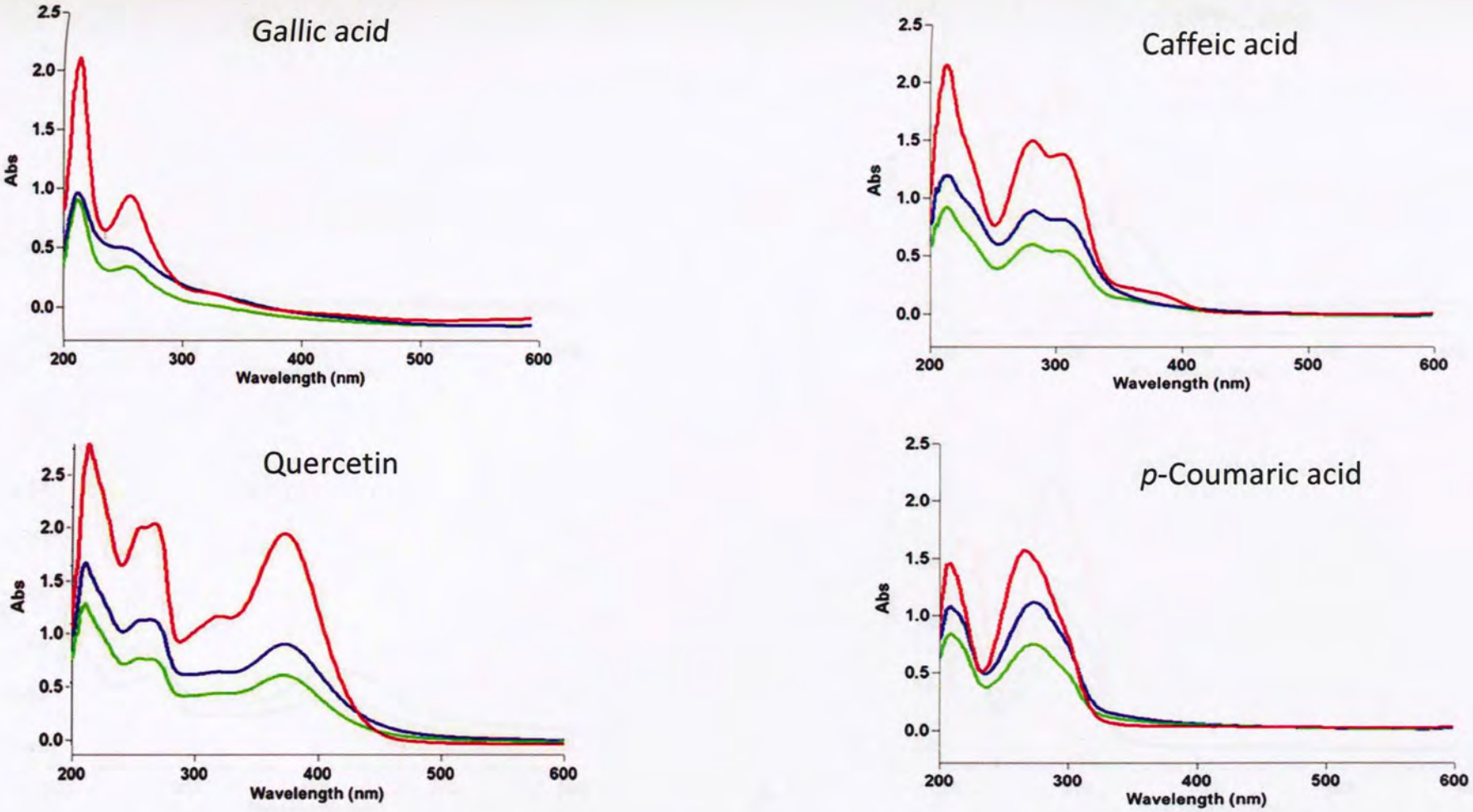


Figure 25: Absorption spectra of the standard phenolic compounds in the presence of Fe^{3+} ions. Red line = Phenolic compound only, Blue line = Phenolic compound + Fe^{3+}, Green line = Phenolic compound + Fe^{3+} + EDTA.

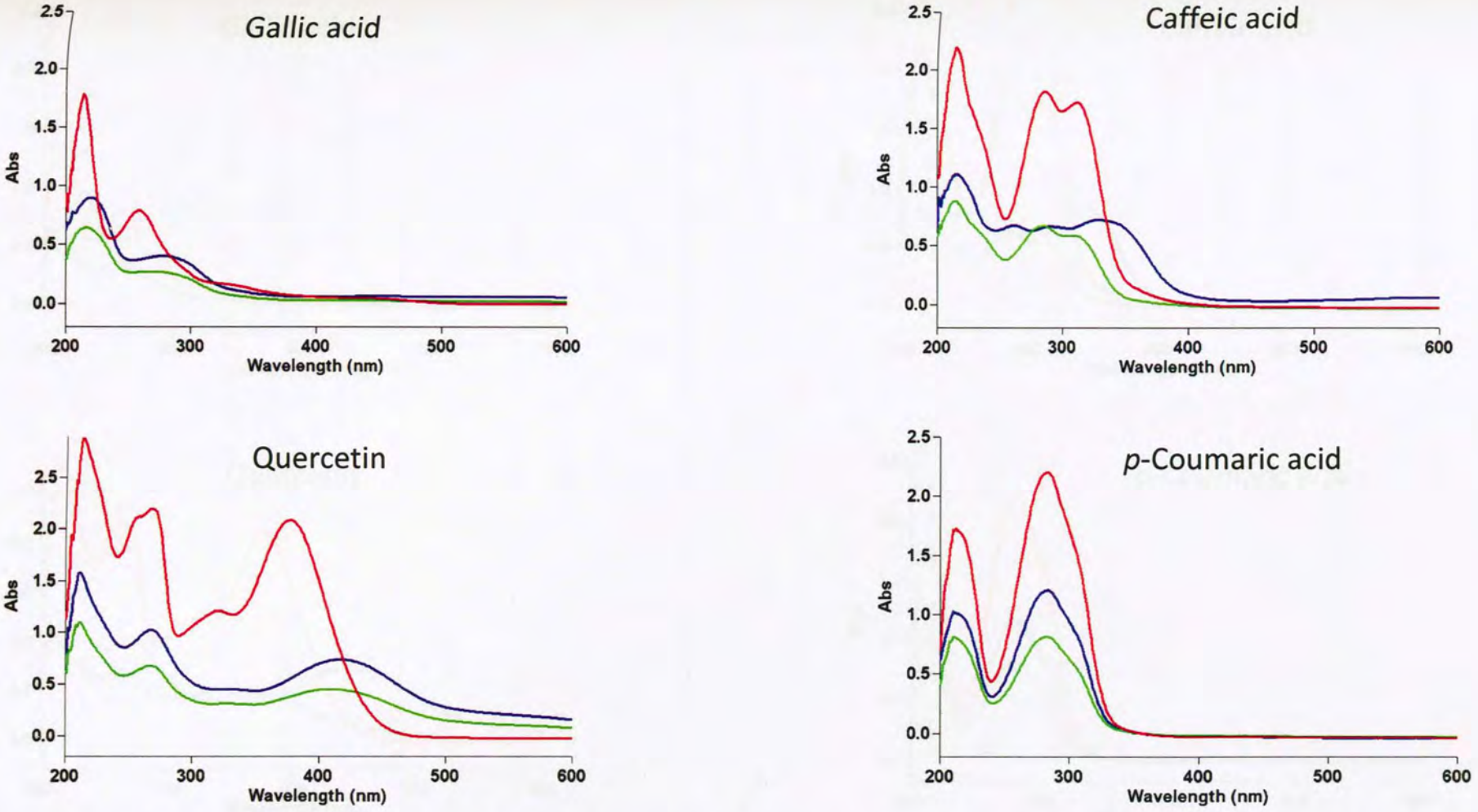


Figure 26: Absorption spectra of the standard phenolic compounds in the presence of Fe^{2+} ions. Red line = Phenolic compound only, Blue line = Phenolic compound + Fe^{2+}, Green line = Phenolic compound + Fe^{2+} + EDTA.

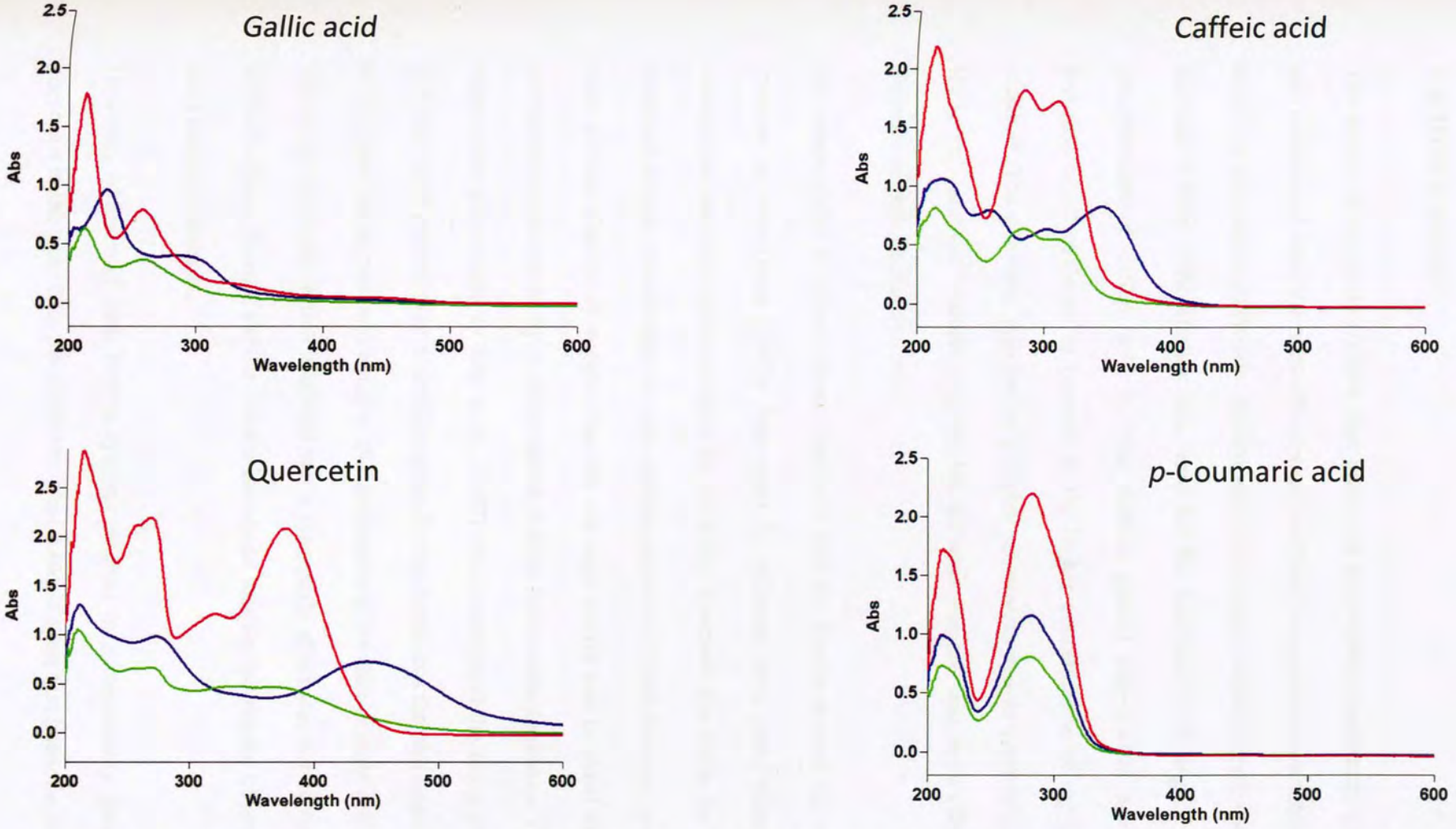


Figure 27: Absorption spectra of the standard phenolic compounds in the presence of Cu^{2+} ions. Red line = Phenolic compound only, Blue line = Phenolic compound + Cu^{2+}, Green line = Phenolic compound + Cu^{2+} + EDTA.

4.5. DISCUSSION

The results of this study showed that, under the investigated concentrations, quercetin and caffeic acid were the most efficient anti-oxidants in agreement with data in chapter three. As mentioned previously, the red wine compounds varied in their concentration, whereas a fixed concentration was used for the standards (400 mg/L). The lower concentration of caffeic acid in wine showed greater anti-oxidant activity in the presence of all oxidants, in contrast to the higher concentration of the caffeic acid standard. The quercetin standard at a higher concentration was more efficient in the H_2O_2, Fe^{3+}, and Cu^{2+} oxidant systems, but the wine phenolic was more effective in the presence of both Fenton systems.

As demonstrated in chapter three, treatment wih the Fenton regents led to a greater increase in anti-oxidant activity than when the oxidants were added separately. This observation was more pronounced in the red wine, however. The H_2O_2, Fe^{3+}, and Cu^{2+} treatment groups showed that the anti-oxidant activities of both the wine and standards were similar. This could suggest that the red wine matrix had minimal effect on the overall anti-oxidant activity in the presence of these three oxidant systems. The effect of matrix was also studied by Heo et al., (2007) who investigated the effect of individual and combined phenolics in a model system. It was found that the anti-oxidant capacity of the phenolic mixture was equal to the summation of the anti-oxidant activities of the individual phenolics, which indicated that a synergistic effect was not occurring but an additive effect. Thus, a similar mechanism could explain the results observed for red wine and the standards.

However, addition of both Fenton systems resulted in a comparably greater loss of phenolics in the wine than the standards. This could suggest that phenolic complexes of

the metal ions in wine can function as catalysts or anti-oxidant enzyme mimetics, which can scavenge these hydroxyl radicals or function as chelators of Fe^{3+} and Cu^{2+} metal ions.

Gallic acid was the most abundant phenolic compound in wine, but was less efficient compared to the lower concentration of the standard compound in the H_2O_2, Fe^{3+}, Cu^{2+}, and H_2O_2 + Fe^{2+} oxidant systems. However, *p*-coumaric acid was more efficient at a higher concentration in relation to the lower concentration found in the wine. Sroka and Cisowski, (2003) showed that gallic acid exhibited the strongest anti-oxidant activity towards lipid peroxidation, hydrogen peroxide, and DPPH. Caffeic acid with two hydroxyl groups bonded to the aromatic ring in the *ortho* position, was shown to have strong anti-oxidant activity, but was less effective than gallic acid. These results are in disagreement with the present study which found that gallic acid had a lower anti-oxidant efficacy than caffeic acid, although this could be due to the different methods used. It has been suggested that the -CH=CH-COOH moiety of caffeic acid could account for the higher anti-oxidant efficacy compared to the carboxylate group present in gallic acid. The former structural group thought to confer higher H-donating and radical stabilisation compared to the latter group (Rice-Evans et al., 1996).

Quercetin was also shown in chapter three to possess a high anti-oxidant activity in all oxidant systems. This could be due to the structural properties of the flavonoid molecule, which possess all three criteria for radical scavenging and metal chelating abilities, i.e. (i) 3′,4′-catechol structure in the B ring, (ii) the 2,3 double bond in conjugation with a 4-oxo function in the C ring, and (iii) the 3- and 5-OH groups with 4-oxo function in the A and C rings. These structural arrangements are thought to strongly inhibit Fenton-induced oxidation (Heim et al., 2002; Rice-Evans et al., 1996).

The H_2O_2 system was the only oxidant in which caffeic acid showed a much greater capacity towards H_2O_2 scavenging than quercetin.

The medium to low anti-oxidant efficacy of *p*-coumaric acid in all systems can be accounted for by the phenolic structure. *p*-Coumaric acid possesses a monophenolic structure, whereas caffeic acid has a second hydroxyl group in the *ortho* position (Rice-Evans et al., 1996). Similar findings were reported by Villaño et al., (2005) who carried out a comparative study of the anti-oxidant activity of standard phenolic compounds found in wine using DPPH, ABTS, and ORAC anti-oxidant tests. It was found that the number of hydroxyl groups correlated with anti-oxidant activity, with a higher number of OH groups in the aromatic ring having a larger TEAC value. Thus caffeic acid with two hydroxyl groups exhibited a higher anti-oxidant activity than *p*-coumaric acid with one hydroxyl group, in agreement with the present study.

The overall results of the HPLC study showed that the anti-oxidant efficiencies of the phenolic compounds can be classified according to Table 15. As seen in the table, most phenolics demonstrated medium activity, but in the presence of the Fenton systems, these were high, particularly for the wine phenolics.

Table 15: Classification of the anti-oxidant efficiencies of the phenolic compounds in the HPLC study

Red wine			**Standards**		
Oxidant system	Phenolic	Anti-oxidant efficiency classification*	Oxidant system	Phenolic	Anti-oxidant efficiency classification*
H_2O_2	QUE	Medium	H_2O_2	QUE	Medium
	CA	High		CA	Medium
	GA	Low		GA	Medium
	p-CA	Medium		*p*-CA	Medium
Fe^{3+}	QUE	Medium	Fe^{3+}	QUE	Medium
	CA	Medium		CA	Medium
	GA	Medium		GA	Medium
	p-CA	Medium		*p*-CA	Medium
Cu^{2+}	QUE	Medium	Cu^{2+}	QUE	Medium
	CA	Medium		CA	Medium
	GA	Medium		GA	Medium
	p-CA	Low		*p*-CA	Medium
Fe^{2+}-H_2O_2	QUE	High	Fe^{2+}-H_2O_2	QUE	High
	CA	High		CA	Medium
	GA	Medium		GA	High
	p-CA	Medium		*p*-CA	Medium
Cu^{+}-H_2O_2	QUE	High	Cu^{+}-H_2O_2	QUE	Medium
	CA	High		CA	Medium
	GA	Medium		GA	Medium
	p-CA	Medium		*p*-CA	Medium

*Anti-oxidant efficiency was determined as shown in chapter two.

As shown in Figure 24, the anti-oxidant/chelating activity increased upon exposure to EDTA, with Cu^{2+}-EDTA exhibiting a more oxidising effect. It is thought that chelation of Fe^{2+} or Cu^{2+} metal ions by EDTA does not necessarily prevent these metal ions from entering the Fenton reaction. The high anti-oxidant activities of quercetin and caffeic acid in the wine, as seen in the Fenton systems (Figure 23D and E), can be attributed to a greater capacity to form complexes with Fe^{2+} and Cu^{2+}, by removing Fe^{2+} or Cu^{2+} bound to the co-chelator (EDTA), and thereby suppressing $^{\cdot}OH$ production (Heim et al., 2002; Malešev and Kuntić, 2007; Rice-Evans et al., 1996). Gallic acid and *p*-coumaric acid appeared to be less able to bind these metal ions effectively in the Fenton systems, thereby contributing to $^{\cdot}OH$ formation. These results suggest that in the putative anti-oxidant-metal ion-EDTA chelate complex, both Fe^{2+} and Cu^{2+} ions may function as catalysts in the generation of hydroxyl radicals. These results are in agreement with Yasuda et al., (2012), who also found that after addition of EDTA, free catechins were gradually oxidised by auto-oxidation.

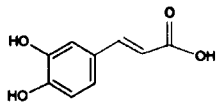
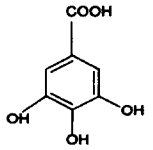
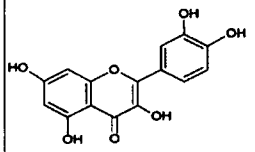
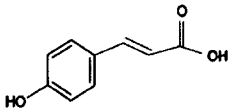
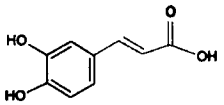
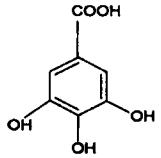
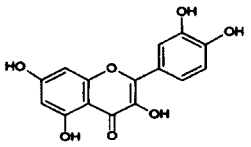
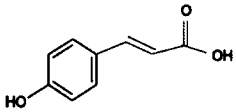
Metal chelation studies of the four phenolics showed that at a ratio of 1:1 metal ion: phenolic compound at pH 7.4, spectral shifts were observed for quercetin, caffeic acid, and gallic acid. *p*-Coumaric acid did not show the appearance of new peaks. The inability of the quercetin/EDTA solutions to recapture the original spectra when both Fe^{2+} and Cu^{2+} were added, suggests that quercetin could have been oxidised perhaps at the 3-hydroxy and 4'-hydroxy groups, which is consistent with previous literature data (Andjelković et al., 2006; Brown et al., 1998).

The number of OH groups as well as the 3',4'-dihydroxy groups on the B-ring of caffeic acid and gallic acid, could have accounted for the ability of these phenolic acids to chelate the metal ions more effectively compared to *p*-coumaric acid (Andjelković et al., 2006; Mira et al., 2002). Andjelković et al., (2006) also found that complex formation

occurred between phenolic compounds bearing galloyl or catechol moieties and Fe^{2+}, whereas phenolic compounds lacking these groups showed no complex formation. The study found that caffeic acid was a stronger chelator than gallic acid.

These results could explain why quercetin and gallic acid were efficient reducing/chelating agents of Fe^{3+} and Cu^{2+} oxidant systems (Figure 23B and C). The inability of *p*-coumaric acid to form complexes with both metal ions could account for the relatively low anti-oxidant activity observed. In addition, the lack of galloyl or catechol moieties on the aromatic ring in contrast to gallic or caffeic acids, could account for the lack of complex formation. Overall, caffeic acid was the most efficient at chelating both metal ions, and restoring spectra upon addition of EDTA. This could account for the high anti-oxidant activity observed for this compound in the HPLC analysis. A summary of the metal binding/EDTA efficiencies of the four standard phenolics is given in Table 16.

Table 16: Summary of the metal binding/EDTA activities of the four standard phenolics: gallic acid, caffeic acid, *p*-coumaric acid, and quercetin

Compound	Chemical structure	Metal binders	Spectra restored upon EDTA addition?
Fe^{2+} ions			
Caffeic acid		√	+
Gallic acid		√	-
Quercetin		√	-
p-Coumaric acid		X	-
Cu^{2+} ions			
Caffeic acid		√	+
Gallic acid		√	+
Quercetin		√	-
p-Coumaric acid		X	-

√: Chelator
+: Spectra restored upon addition of EDTA
X: Non chelator
-: Spectra not restored upon addition of EDTA

4.6. CONCLUSION

In contrast to previous reports, the ranking of anti-oxidant activities, facilitated by this new approach, identified two anti-oxidants (quercetin and caffeic acid) with much greater activities in a complex matrix relative to a simple model system. Overall, the results were in agreement with the TLC study in chapter three. Depending upon the oxidant systems tested and the concentrations of phenolics, red wine demonstrated higher or lower anti-oxidant activities relative to the standard phenolic compounds.

The results showed that the overall ranking for the investigated phenolic anti-oxidants was: quercetin > caffeic acid > gallic acid ≈ *p*-coumaric acid. Similar to the oxidant ranking in chapter three, the two Fenton systems exhibited the greatest oxidising power. However, the other three oxidant systems added independently showed that H_2O_2 possessed the highest oxidising power, as follows: $H_2O_2 + Fe^{2+} = H_2O_2 + Cu^{+} > H_2O_2 > Fe^{3+} > Cu^{2+}$. Potential synergistic and/or antagonistic interactions with other components such as anthocyanins and tannins within a complex mixture such as wine should be taken into account when evaluating anti-oxidant capacity. In addition, as different concentrations of red wine and standards were used in the HPLC experiment, another approach would be to determine the effect of concentration on anti-oxidant activity. Therefore, the focus of the next chapter is to determine the effect of increasing concentrations of oxidants on fixed concentrations of red wine and standard compounds in order to compare and contrast their activities.

Chapter 5

ASSESSMENT OF THE POTENTIAL PRO-OXIDANT ACTIVITY OF RED WINE AND ELEVEN PHENOLIC STANDARDS

5.1. INTRODUCTION

Although there are numerous literature reports on the anti-oxidant activity of foods and natural products, the data on their pro-oxidant capacities is less reported. The structural characteristics of flavonoids, which are responsible for anti-oxidant capacity, can contribute to pro-oxidant effects *in vitro* (Aruoma, 1996; Heim et al., 2002; Procházková et al., 2011). The number of hydroxyl groups on the flavonoid molecule can also affect anti-oxidant/pro-oxidant activity, with multiple hydroxyl groups said to promote hydroxyl radical production (Procházková et al., 2011). As previously discussed, hydroxyl radical generation using the Fenton reagents Fe^{2+} or Cu^{+} and H_2O_2, can be inhibited by formation of complexes of phenolic compounds with these metal ions. Another Fenton system, composed of ascorbate, Fe^{3+}, EDTA and O_2, can yield the following set of reactions (Andrade et al., 2006):

$$Fe^{3+}\text{-EDTA} + \text{ascorbate} \rightarrow Fe^{2+}\text{-EDTA} + \text{ascorbyl}^{\cdot} \quad (1)$$

$$Fe^{2+}\text{-EDTA} + O_2 \rightarrow Fe^{3+}\text{-EDTA} + O_2^{-} \quad (2)$$

$$Fe^{2+}\text{-EDTA} + O_2^{-} + 2H^{+} \rightarrow Fe^{3+}\text{-EDTA} + H_2O_2 \quad (3)$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad (4)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + {}^{\cdot}OH + OH^{-} \quad (5)$$

Thus, addition of ascorbic acid to the reaction can greatly increase ·OH generation, by acting as a catalyst in reducing Fe^{3+} to Fe^{2+}, and thus promoting the Fenton reaction (Caillet et al., 2007).

Cao et al., (1997) studied the anti-oxidant and pro-oxidant behaviour of several flavonoids using the ORAC assay. It was found that flavones, isoflavans, and flavanones acted as anti-oxidants against peroxyl and hydroxyl radicals. However, in the presence of Cu^{2+}, these flavonoids exhibited pro-oxidant behaviour. These observations were attributed to the flavonoid structures, with multiple OH substitutions resulting in stronger anti-oxidant and pro-oxidant activities.

Another study by Beker et al., (2011) used the linoleic acid peroxidation assay to determine the effects of quercetin, morin, and catechin on copper (II) and ascorbic acid-initiated oxidation. It was found that morin exhibited an anti-oxidant effect at all concentrations, whereas quercetin and catechin exhibited pro-oxidant as well as anti-oxidant activities, which were dependent upon their concentrations.

Similarly, the anti-oxidant and pro-oxidant activities of phenolic compounds were also studied by Fukumoto and Mazza, (2000). The DPPH and β-carotene bleaching assays were used to measure anti-oxidant activity, whilst an HPLC method was developed to measure both anti-oxidant and pro-oxidant activity using a Cu^{2+} catalyst and measuring percent malonaldehyde formed. Results showed that most phenolic compounds had pro-oxidant activity at low concentrations, unlike BHT and BHA. Compounds that possessed similar structures showed similar anti-oxidant activities. In addition, anti-oxidant activity was found to increase as the number of hydroxyl groups increased, but glycosylated compounds showed a decrease in activity.

Iwasaki et al., (2011) also studied the anti-oxidant and pro-oxidant activities of various phenolic compounds in the presence of Cu^{2+} ions. ESR measurements were taken, and the DPPH assay was used to measure anti-oxidant activity. Pro-oxidant effects of the phenolic compounds were assessed by measuring 8-OHdG levels formed from oxidative damage to DNA. It was found that phenolics that possess *ortho*-dihydroxyl groups exhibited the greatet pro-oxidant activity due to the ability to chelate Cu^{2+} ions.

Samra et al., (2011) used cyclic voltammetry and chemiluminescence to evaluate the anti-oxidant, pro-oxidant and possible synergistic activities of five phenolic compounds: ascorbic acid, caffeic acid, quercetin, catechin, and hesperetin. Ascorbic acid added to each phenolic compound was found to either increase or decrease anti-oxidant activity. Ascorbic acid + quercetin showed an increase in anti-oxidant activity, but this was reduced when ascorbic acid was mixed with catechin. Caffeic acid and ascorbic acid mixtures were found to exhibit the most pro-oxidant activity compared to the other phenolic compounds and their mixtures. Quercetin + catechin were found to exert a pro-oxidant action compared with each phenolic measured alone. Hesperetin showed a slight increase in anti-oxidant activity when added to caffeic acid, but this effect was not seen when mixed with other compounds or alone.

In a follow-up study by Choueiri et al., (2012), it was found that a mixture of quercetin and ascorbic acid at a 2:1 ratio exhibited the highest anti-oxidant activity. A 1:2 ratio of querectin and caffeic acid was found to show very weak anti-oxidant activity, which possibly suggested pro-oxidant behaviour. Catechin and caffeic acid at a 1:3 ratio also showed weak anti-oxidant activity. Efficient recycling of *o*-quinones was thought to account for the observed anti-oxidant activity of quercetin and ascorbic acid.

Although studies on the anti-oxidant activity and phenolic composition of wine are numerous, studies on the pro-oxidant effects of wine are scarce. However, studies have revealed the presence of high levels of metal ions such as Fe, Cu, Al, Mn, Cr, and Zn, in wine and other beverages which are thought to contribute to oxidative stress (Fiket et al., 2011; Hague et al., 2008; Naughton and Petróczi, 2008). In one study, it was reported that some wines all had a Target Hazard Quotient (THQ) value above 1, indicating potential toxic effects to health. Typical THQ values were found to range from 50 to 200, with Hungarian and Slovakian wines reaching 300. These hazardous levels of metal ions in wines have led to questions of the potential pro-oxidant effects of wine to health (Naughton and Petróczi, 2008).

Hötzer et al., (2005) used chemiluminescence to measure the effects of red wine and its fractions on Cu^{2+}-induced LDL oxidation. It was found that both red wine and its fractions exhibited a protective effect when added before the LDL oxidation process. However, when red wine and its fractions were added after the oxidation process, pro-oxidant behaviour was observed. Laggner et al., (2005) measured the effect of sulphite on LDL oxidation, also using Cu^{2+} as a catalyst to initiate LDL oxidation. The authors found that sulphite was able to facilitate oxidation of LDL at concentrations found *in vivo*. This pro-oxidant action was thought to be due to the formation of Cu^{+}, sulphite radicals, and hydroxyl radicals. However, as this study looked at only one compound in wine, it did not take into account other phenolic compounds in red wine which can exert pro-oxidant activity. In addition, both studies used one method to measure activity, rather than a number of methods to measure the overall activities of individual compounds.

In chapters four and five, a functional TLC approach and quantitative HPLC method to evaluate the anti-oxidant activity of wine components were discussed. The present chapter is concerned with extending these previous observations by looking at the potential pro-oxidant effects of red wine and phenolic standards, as well as establishing the anti-oxidant activities of these compounds. Seven further compounds, in addition to the four in the previous chapter, were selected to compare and contrast their individual oxidant profiles. These eleven particular compounds were selected to represent those that commonly occur in each phenolic class of red wine. These were: three hydroxybenzoic acids (gallic acid, syringic acid, and protocatechuic acid); two hydroxycinnamic acids (caffeic acid and *p*-coumaric acid); two flavan-3-ols (catechin and epicatechin), one stilbene (resveratrol), and three flavonols (quercetin, myricetin, and kaempferol). In addition, the selection of these compounds was informed by the TLC and HPLC experiments.

The pharmacological properties of these particular phenolic compounds are well known. Flavanols (catechin and epicatechin) are found in a number of foods, with high concentrations in green tea and cocoa. However, catechins are present at greater concentrations in red wine than all the other flavonoid compounds. In particular, green tea catechins have been the subject of much research (Crespy and Williamson, 2004). Of the flavonols, myricetin and kaempferol are less well-studied anti-oxidants compared to quercetin. Like quercetin, these flavonols are abundant in fruits, vegetables, herbs, and other plants. The anti-oxidant and other health properties of both myricetin and kaempferol have been described (Mahat et al., 2010; Wang et al., 2010). For the hydroxybenzoic acids, the number of studies on the anti-oxidant activity of syringic acid and protocatechuic acid are less well-known compared to gallic acid. However, of all the red wine phenolic compounds, resveratrol has received the most attention. Berries,

peanuts, and dark chocolate are among the other sources of resveratrol, but red wine is the most notable dietary source. Along with quercetin, the health benefits of resveratrol have been widely reported (Pandey and Rizvi, 2011).

The HPLC method was used to measure the anti-oxidant activities of the wine and standard phenolic compounds in the presence of the five oxidant systems as described previously. To measure both pro-oxidant and anti-oxidant activity, the hydroxyl radical-mediated deoxyribose degradation and linoleic acid peroxidation assays were chosen. For these two assays two different model Fenton systems were applied in order to test for pro-oxidant activity: (i) Ascorbic acid-Fe^{3+}-EDTA-H_2O_2 and (ii) Fe^{3+}-H_2O_2. Whereas the previous chapters focussed on measuring the effect of adding a fixed concentration of oxidant to different concentrations of phenolic compounds, this chapter aimed to measure the effect of adding increasing concentrations of oxidant to fixed concentrations of red wine and phenolic standards in order to compare and contrast activities.

5.2. AIMS

The aims of this chapter are:

- To use RP-HPLC to measure the anti-oxidant activities of wine and eleven standard phenolic compounds. Determine the effects of matrix and dose on activity.
- To measure the pro-oxidant and anti-oxidant activities of red wine and standard phenolic compounds using the hydroxyl radical-mediated deoxyribose degradation assay in the presence of two different Fenton systems: (i) Ascorbic

acid-Fe^{3+}-EDTA-H_2O_2 and (ii) Fe^{3+}-H_2O_2. Determine the effects of matrix and dose on activity.

- To measure the pro-oxidant and anti-oxidant activity of red wine and standard phenolic compounds towards inhibition of linoleic acid peroxidation using two different Fenton systems: (i) Ascorbic acid-Fe^{3+}-EDTA-H_2O_2 and (ii) Fe^{3+}-H_2O_2. Determine the effects of matrix and dose on activity.
- Measure the Fe^{2+} and Cu^{2+} metal binding activities of the phenolic compounds using UV-vis spectrophotometry. Monitor the effect of competitive chelation between redox-active metal ions and excess EDTA. Detail these spectral changes.

5.3. METHODS

The methods are given in chapter two. Briefly:

- For the RP-HPLC analysis, the wine and eleven standard phenolic compounds (at the concentrations in Table 17) were challenged with the five oxidant systems, using similar chromatographic conditions in chapter four.
- For the hydroxyl radical-mediated deoxyribose degradation assay, red wine and standards (both tested at increasing concentrations of 20-640 mg/L, as well as at fixed concentrations determined in Table 17) were added to the reaction mixture containing deoxyribose (20 mM) and the Fenton system reagents (Ascorbic acid-Fe^{3+}-EDTA-H_2O_2 or Fe^{3+}-H_2O_2). After incubation, the absorbance of the reaction mixtures was measured at 532 nm.
- For the linoleic acid peroxidation assay, the undiluted wine and standards (tested at the concentrations in Table 17) in methanol (0.5 mL) were mixed with 0.02 M linoleic acid emulsion (2.5 mL), 99.8 % ethanol (5 mL) and 5 mL phosphate

buffer (0.2 M, pH 7.0). This reaction mixture was incubated with two Fenton system models (Ascorbic acid-Fe^{3+}-EDTA-H_2O_2 or Fe^{3+}-H_2O_2). After incubation, the absorbance of the reaction mixtures was measured at 500 nm.

- For the metal chelation activities, the seven standard phenolic compounds were incubated with Fe^{2+}, Cu^{2+} and EDTA, and recorded spectrophotometrically between 200-600 nm.

5.4. RESULTS

5.4.1. Phenolic content of red wine

Parameters of linearity, retention times, LOD, and LOQ for the standard phenolic compounds, together with the concentrations of the individual phenolic compounds in wine are presented in Table 17. As previously mentioned, the red wine used in this experiment was a 2008 vintage, whereas in chapters four and five, a 2006 vintage was tested. In agreement with the previous HPLC experiment, gallic acid was found to be the most abundant phenolic in wine. However, caffeic acid was nearly 5-fold higher than previous results. *p*-Coumaric acid was at a lower concentration than the previous wine. Quercetin, however, was found at similar levels. Amongst the flavan-3-ols catechin was the more predominant, with myricetin the most abundant of the flavonols. Resveratrol had the lowest concentration in the wine.

Table 17: *Parameters of linearity, retention times, LOD, and LOQ for the standard phenolic compounds. Concentration of phenolic compounds in wine shown in mM ± SEM. Limit of detection (LOD) = (3*SD)/slope and limit of quantification (LOQ) = (10*SD)/slope.*

Phenolic compound	**Concentration in red wine (mM)**	**t_r (min)**	**Range of linearity (mM)**	**Regression equation**	**R_2**	**LOD (mM)**	**LOQ (mM)**
Gallic acid	4.41 ± 0.27	4.76	0.06-4.70	y=3161.9x-846.45	0.990	0.003	0.007
Protocatechuic acid	0.65 ± 0.04	5.85	0.06-1.04	y=4715.3x+20645	0.999	0.030	0.033
Catechin	2.42 ± 0.04	6.11	0.03-2.76	y=583.88x-2465.5	0.989	0.020	0.033
Epicatechin	1.05 ± 0.03	7.08	0.03-2.20	y=1230.2x-5402	1.000	0.018	0.024
Syringic acid	0.33 ± 0.09	8.78	0.05-0.81	y=7539.9x-79044	0.999	0.053	0.055
Caffeic acid	0.84 ± 0.06	9.13	0.06-0.89	y=3081.8x-9726.8	0.997	0.019	0.024
p-Coumaric acid	0.24 ± 0.01	13.64	0.06-0.49	y=4731.3x-24696	0.994	0.033	0.035
trans-Resveratrol	0.10 ± 0.01	16.47	0.04-0.35	y=6896.7x-28942	0.993	0.052	0.053
Myricetin	0.39 ± 0.08	20.75	0.03-0.50	y=889.78x-11078	0.998	0.044	0.054
Quercetin	0.18 ± 0.00	25.99	0.03-0.26	y=4922.2x-16984	0.991	0.012	0.013
Kaempferol	0.11 ± 0.00	28.74	0.03-0.28	y=2242.4x-7252.4	0.991	0.013	0.016

5.4.2. H_2O_2, metal ion and Fenton-mediated oxidation of red wine and phenolic standards

Epicatechin was found to be the most potent anti-oxidant in wine, whilst resveratrol was the most efficient anti-oxidant in the standards mixture. At the lowest concentration of the five oxidant systems, a pronounced loss of phenolics was observed for both wine and standards. However, increasing the concentration of oxidant thereafter had little effect. In addition, the phenolic compounds showed a similar ranking order when challenged with H_2O_2, Fe^{3+}, and Cu^{2+}. Over 0-120 minutes incubation, the loss of phenolics also exhibited little change in activity.

Figures 28 and 29 show the anti-oxidant activities of the red wine and phenolic standards in the presence of H_2O_2 at 0, 60, and 120 minutes incubation. The anti-oxidant activities of the wine phenolic compounds decreased in the order: EC > KAEM ≈ SYR ≈ QUE ≈ RES ≈ *p*-CA ≈ MYR > CA > PCA > C > GA. For the phenolic standards, anti-oxidant activities were in the order: RES > KAEM ≈ QUE > SYR ≈ *p*-CA > PCA > MYR > EC > CA > C > GA.

The anti-oxidant activities of the wine phenolics in the presence of Fe^{3+} (Figures 30 and 31) decreased in the order: EC > RES > KAEM > QUE ≈ SYR ≈ *p*-CA ≈ MYR > CA > PCA > C > GA. For the phenolic standards, anti-oxidant activities were in the order: RES > KAEM ≈ QUE ≈ *p*-CA ≈ SYR > PCA ≈ MYR > EC ≈ CA > C > GA. When challenged with Cu^{2+} (Figures 32 and 33), the anti-oxidant activities of the wine phenolics were: EC > KAEM > QUE ≈ RES ≈ SYR ≈ *p*-CA ≈ MYR > PCA > CA > C > GA. For the phenolic standards, anti-oxidant activities were in the order: RES > KAEM ≈ QUE > SYR > *p*-CA > PCA ≈ MYR > EC > CA > C > GA.

The overall activities of both the wine and standards were similar in the presence of H_2O_2, Fe^{3+}, and Cu^{2+} oxidant systems. However, for both Fenton systems, anti-oxidant activities were higher in the wine relative to the standards. Increased peak loss was exhibited for both standards and red wine upon addition of both Fenton systems. Catechin, epicatechin, kaempferol, and resveratrol were found to have the highest anti-oxidant potencies in wine, with 100% peak reduction. Both Fenton systems showed a similar ranking order of phenolic compounds.

The anti-oxidant activities of the red wine and phenolic standards in the presence of Fe^{2+}-H_2O_2 are shown in Figures 34 and 35. The efficiencies of the wine phenolic compounds decreased in the order: EC = C > KAEM > MYR ≈ RES > QUE ≈ SYR ≈ *p*-CA > CA > PCA > GA, whereas for the phenolic standards these were: RES > KAEM ≈ QUE > SYR ≈ *p*-CA ≈ PCA > MYR > EC ≈ CA > C > GA. The anti-oxidant activities of the wine in the Cu^{+}-H_2O_2 system (Figures 36 and 37) also reflected that of the Fe^{2+}-H_2O_2 Fenton system as follows: EC = C = KAEM > RES > MYR > QUE ≈ SYR ≈ *p*-CA > CA > PCA > GA. The anti-oxidant activities of the standards had a similar order as well: RES > KAEM ≈ QUE ≈ SYR ≈ *p*-CA ≈ MYR ≈ PCA > EC ≈ CA > C > GA.

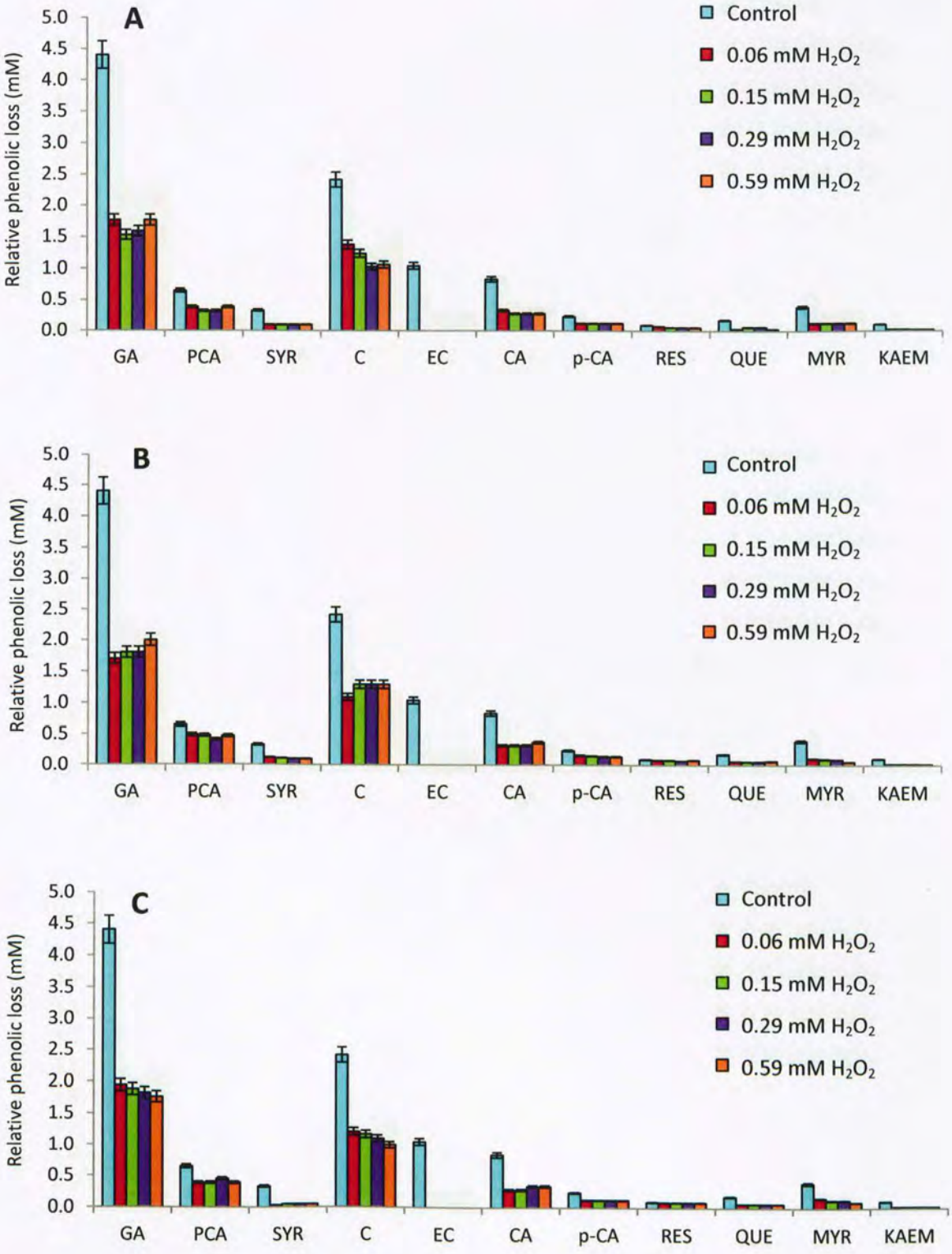


Figure 28: HPLC analysis showing loss of RW phenolic compounds (mM ± SEM) challenged with 0.06-0.59 mM H_2O_2, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

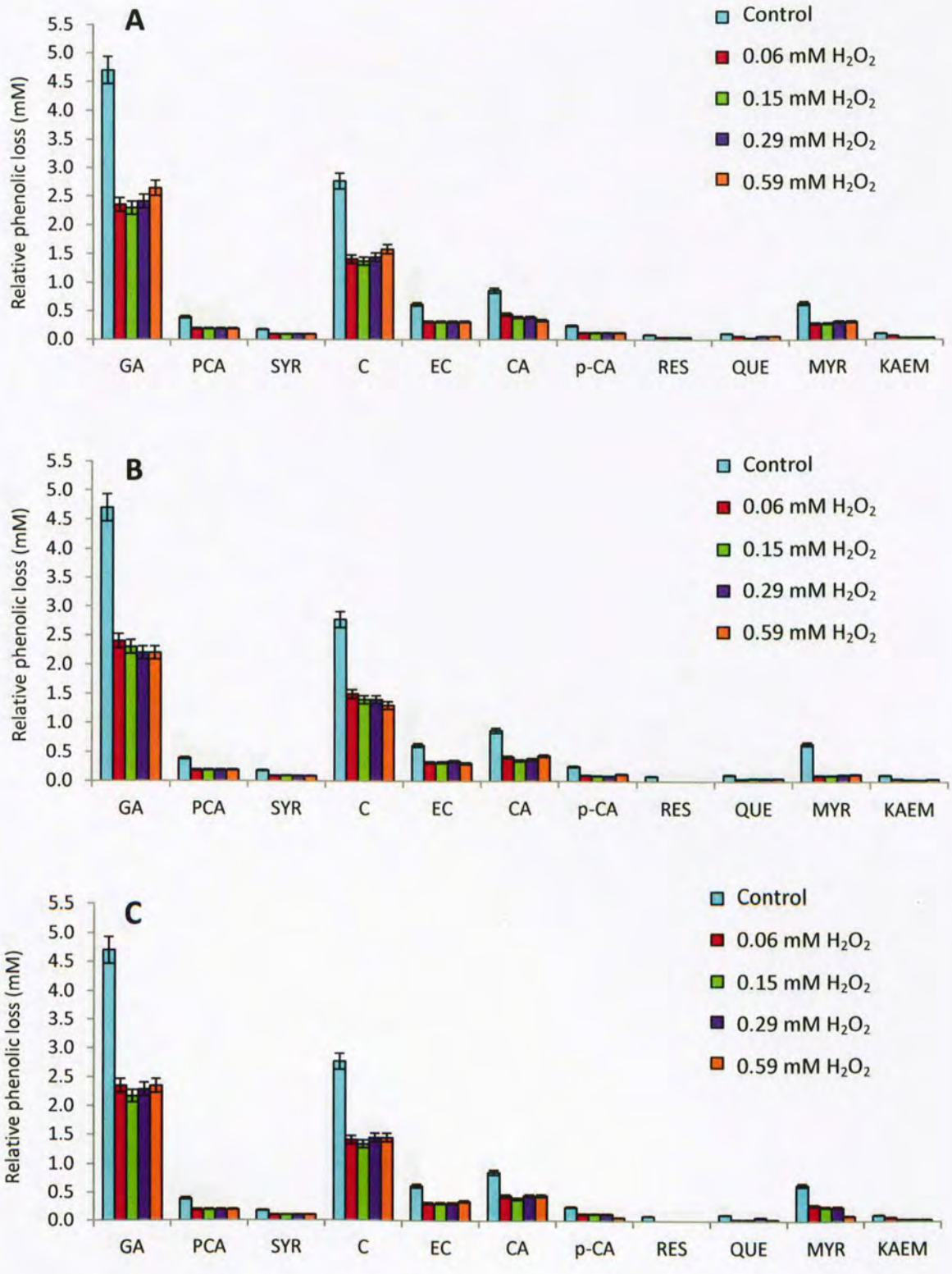


Figure 29: HPLC analysis showing loss of standard phenolic compounds (mM ± SEM) challenged with 0.06-0.59 mM H_2O_2, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

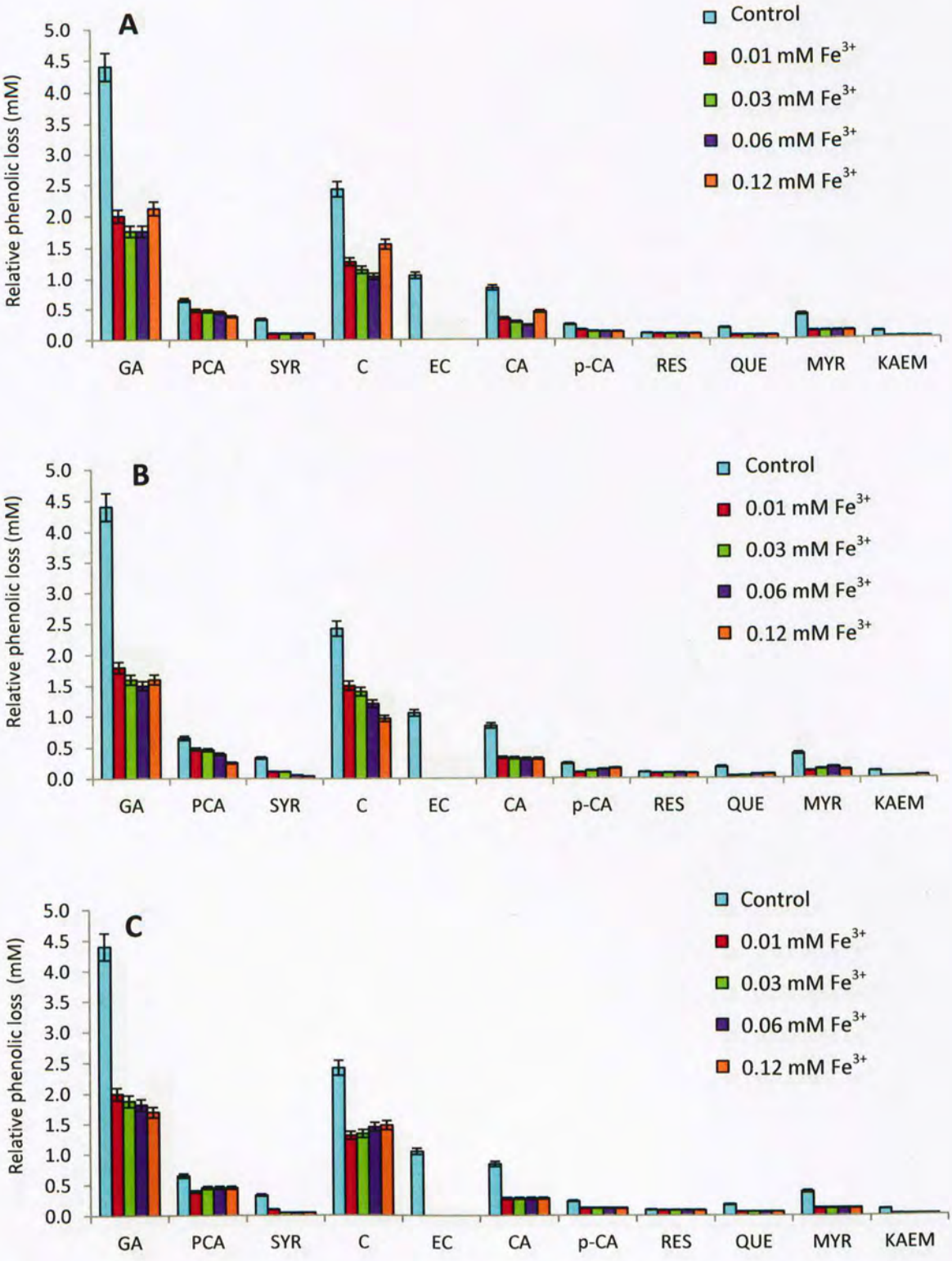


Figure 30: HPLC analysis showing loss of RW phenolic compounds (mM ± SEM) challenged with 0.01-0.12 mM Fe^{3+}, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

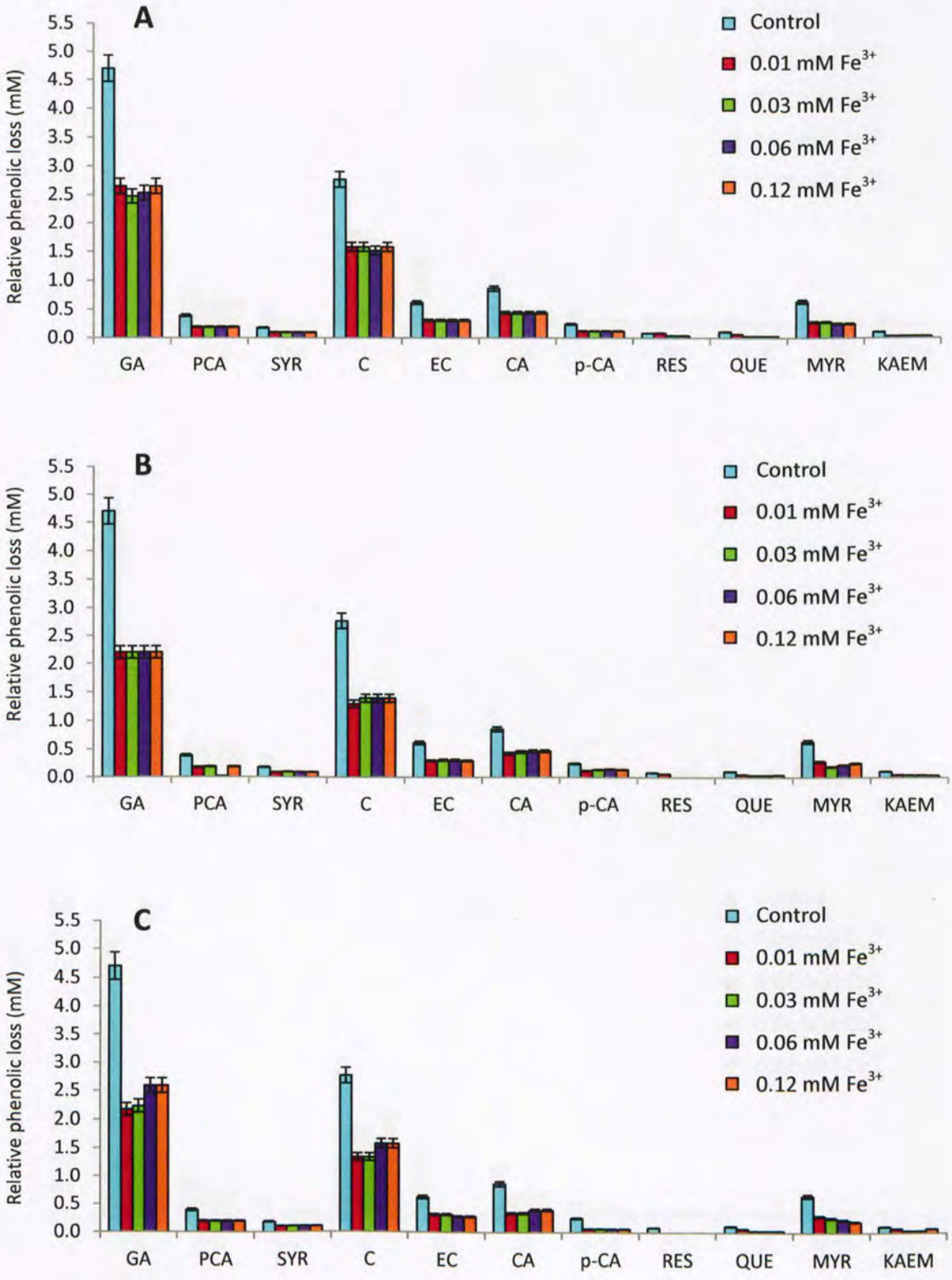


Figure 31: HPLC analysis showing loss of standard phenolic compounds (mM ± SEM) challenged with 0.01-0.12 mM Fe^{3+}, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

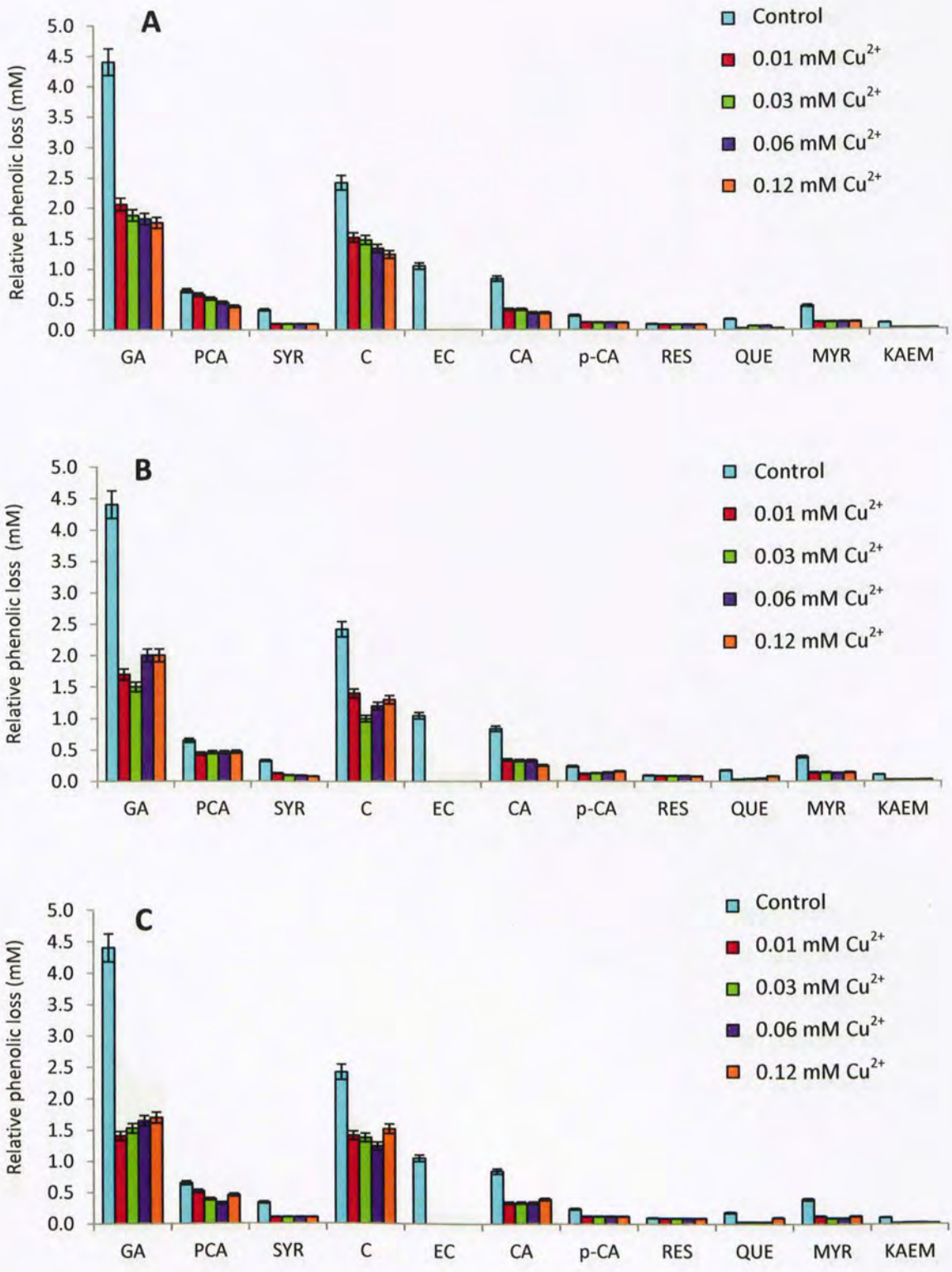


Figure 32: HPLC analysis showing loss of RW phenolic compounds (mM ± SEM) challenged with 0.01-0.12 mM Cu^{2+}, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

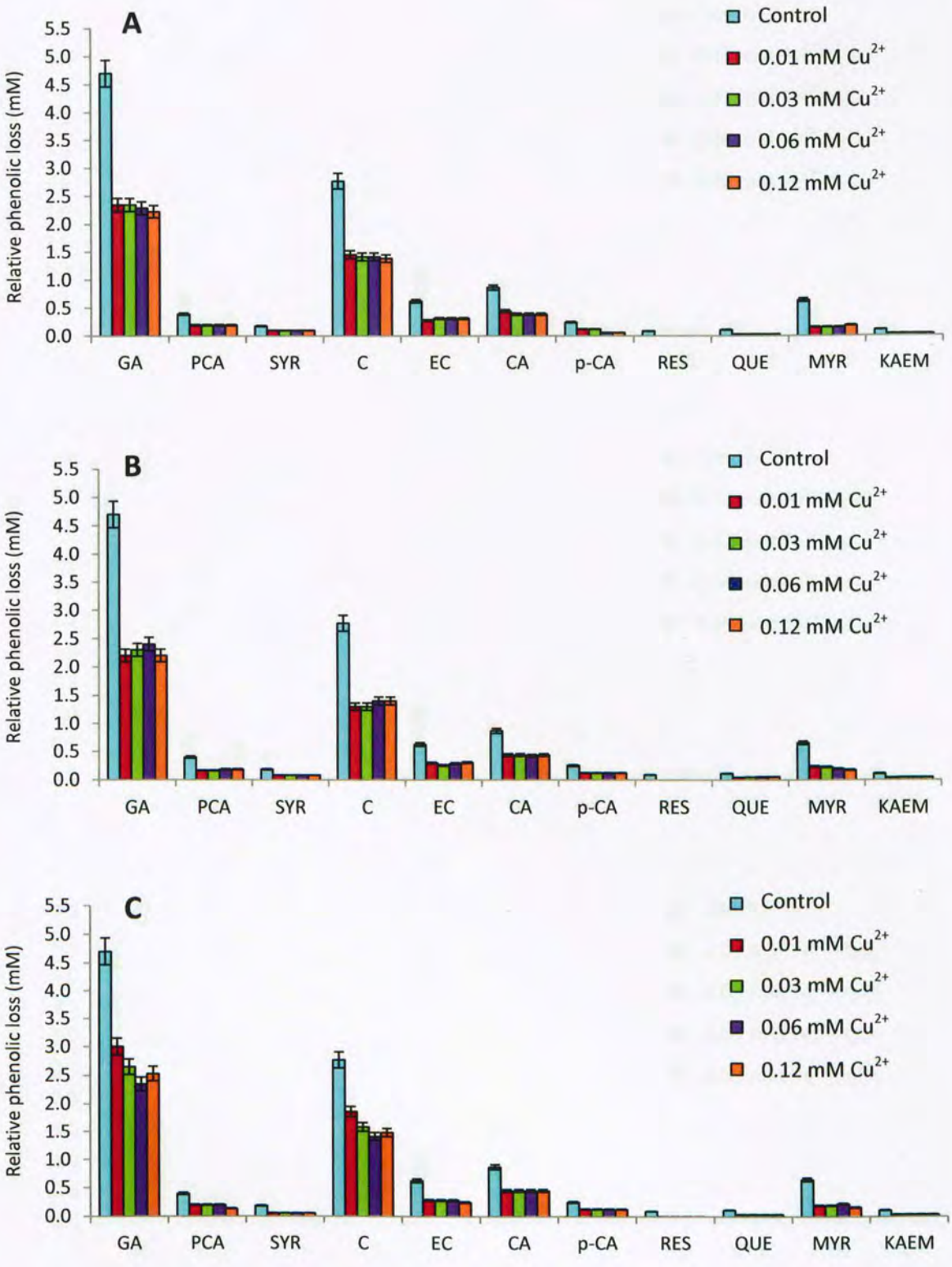


Figure 33: HPLC analysis showing loss of standard phenolic compounds (mM ± SEM) challenged with 0.01-0.12 mM Cu^{2+}, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

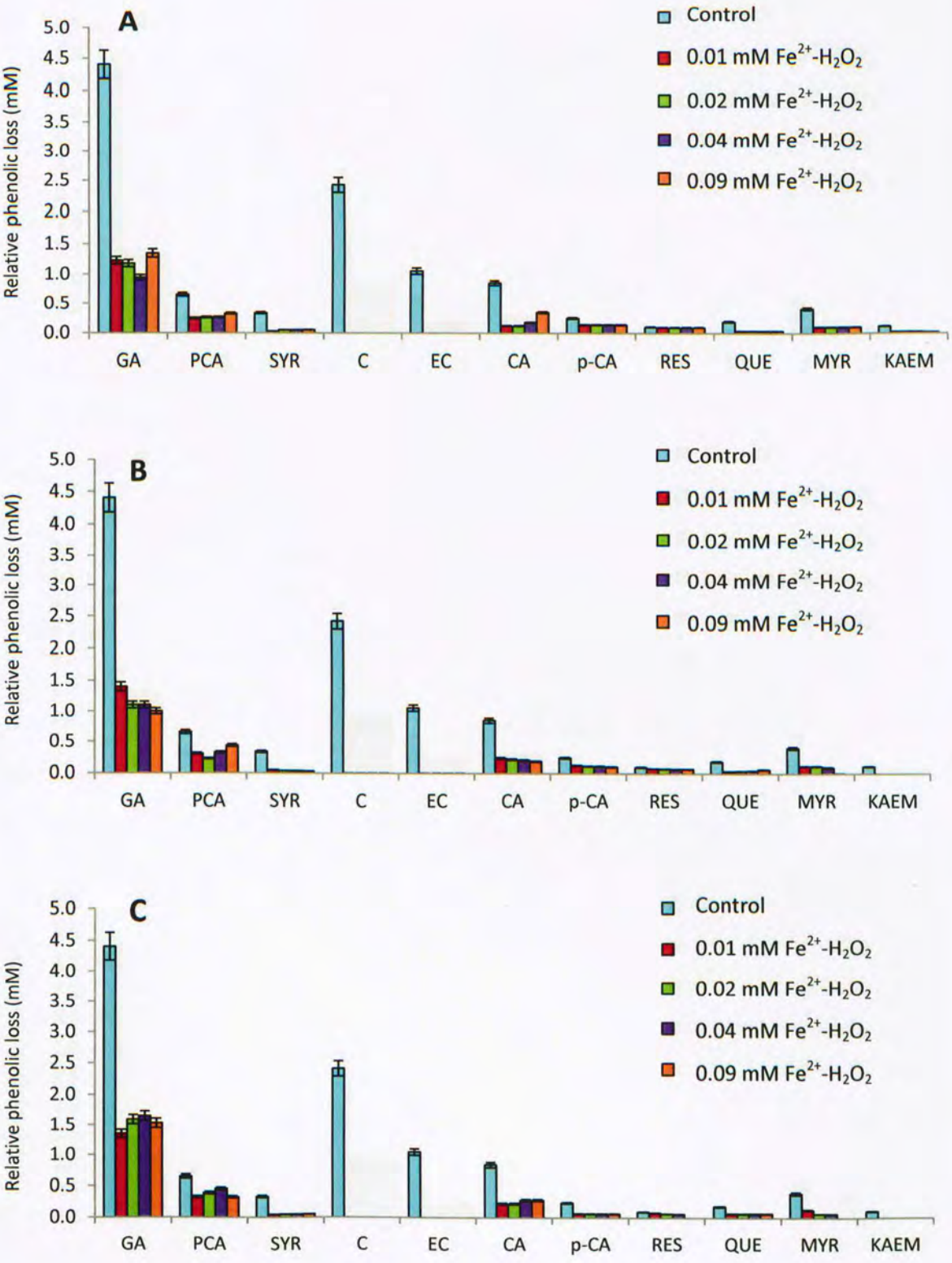


Figure 34: HPLC analysis showing loss of RW phenolic compounds (mM ± SEM) challenged with 0.01-0.09 mM Fe^{2+}-H_2O_2 Fenton reagent, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

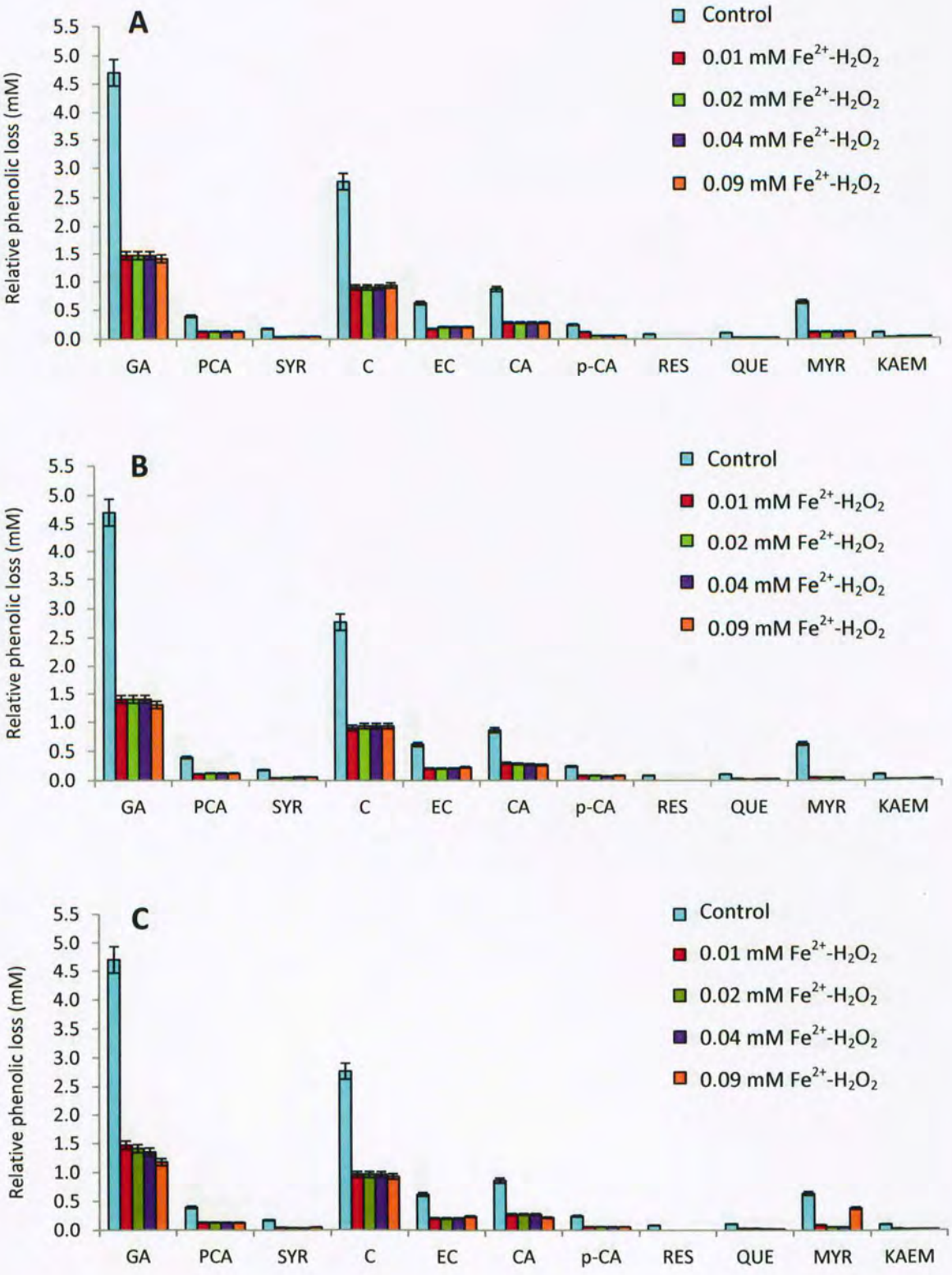


Figure 35: HPLC analysis showing loss of standard phenolic compounds (mM ± SEM) challenged with 0.01-0.09 mM Fe^{2+}-H_2O_2 Fenton reagent, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

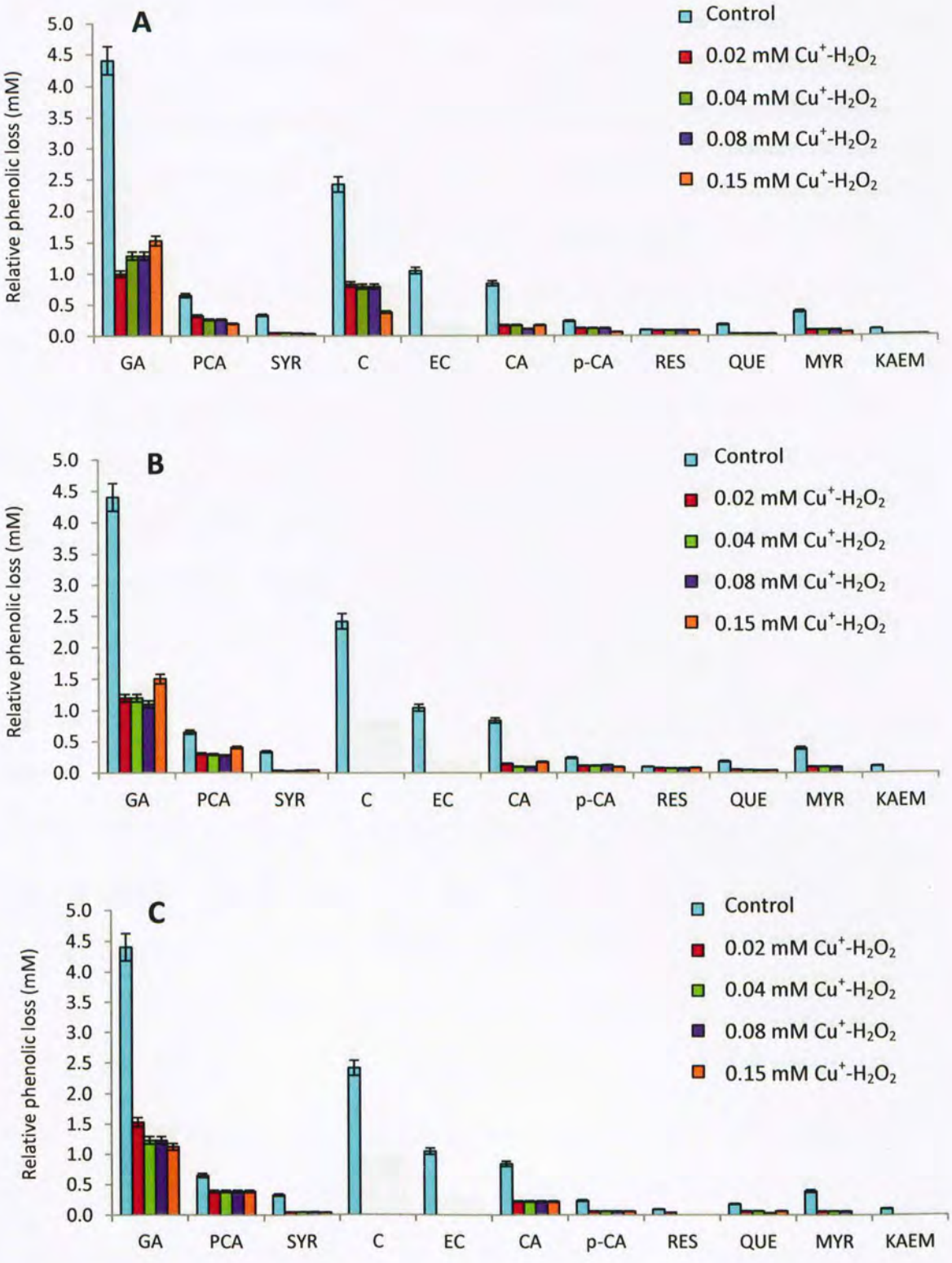


Figure 37: HPLC analysis showing loss of RW phenolic compounds (mM ± SEM) challenged with 0.02-0.15 mM Cu^{+}-H_2O_2 Fenton reagent, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

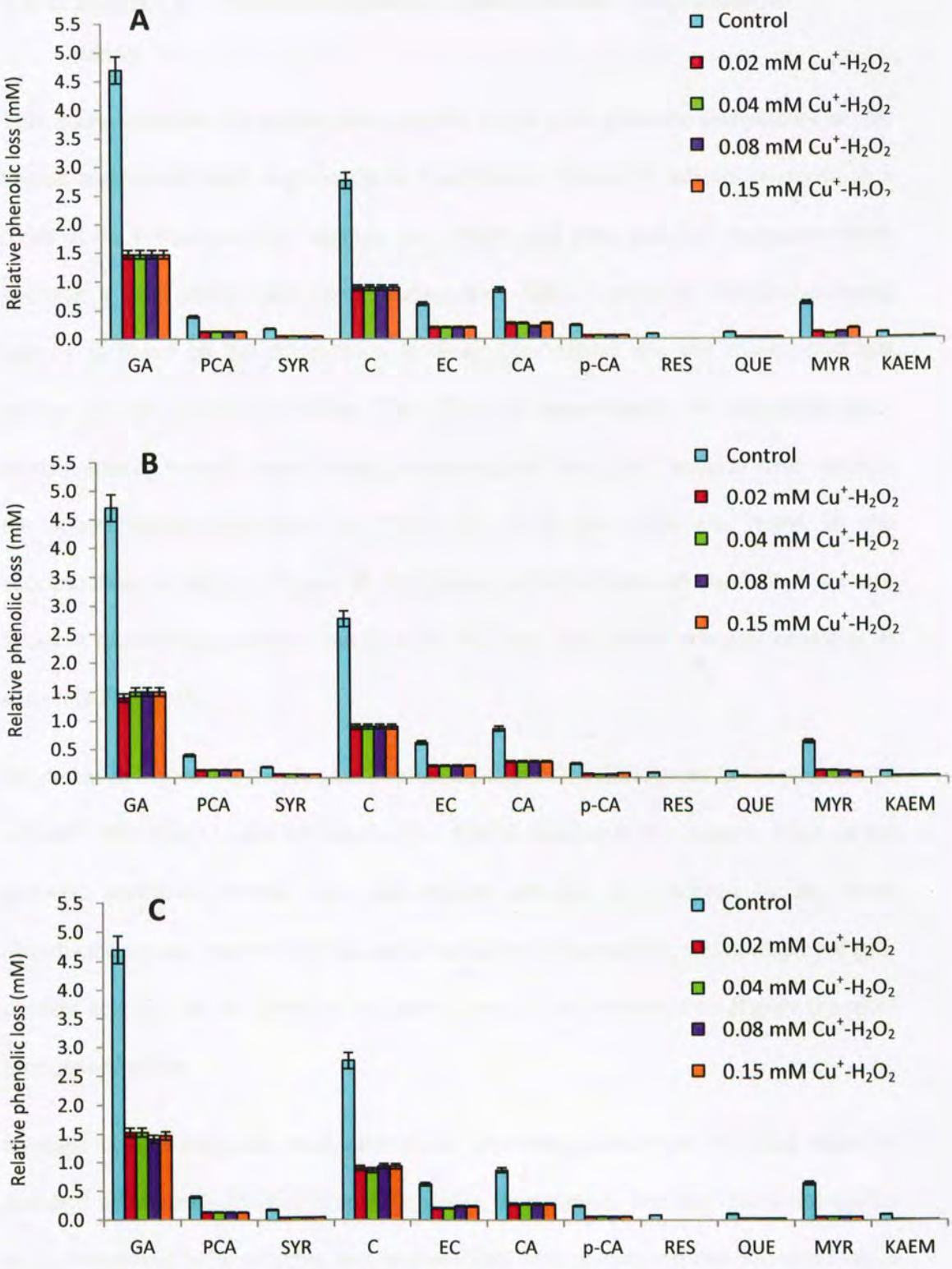


Figure 37: HPLC analysis showing loss of standard phenolic compounds (mM ± SEM) challenged with 0.02-0.15 mM Cu^{+}-H_2O_2 Fenton reagent, at **(A)** 0, **(B)** 60, and **(C)** 120 minutes incubation. A lower value relative to the control indicates anti-oxidant activity.

5.4.3. Hydroxyl radical-mediated deoxyribose degradation assay

This assay measures the anti-oxidant activity of the wine phenolic compounds on the Fenton reaction-initiated degradation of deoxyribose. Hydroxyl radicals produced as a result of the Fenton reaction, degrade deoxyribose and form carbonyl fragments which generate a pink chromogen upon heating with TBA. Hydroxyl radical-scavenging activity is based on the competition between deoxyribose and the anti-oxidant test substance with hydroxyl radicals. The effect of concentration on anti-oxidant/pro-oxidant activity towards deoxyribose was determined. Here, the standards were tested at the concentrations determined in Table 17, whilst the wine was tested at one concentration as shown in Figure 38. In addition, both the wine and standards were also tested at increasing concentrations from 20-640 mg/L in order to compare activities as shown in Figure 39.

As shown in Figure 38, the standards exhibited significant differences in the presence of AA-Fe^{3+}-EDTA-H_2O_2 and Fe^{3+}-H_2O_2 ($p < 0.001$) relative to the control. Most of the phenolic standards showed high anti-oxidant activities, as observed by the lower absorbance values, except for gallic acid, catechin and epicatechin, which displayed pro-oxidant activity. Of the three pro-oxidants, catechin was observed to display the most pronounced effect.

Syringic acid, *p*-coumaric acid, resveratrol, and kaempferol were the most effective standard compounds in inhibiting deoxyribose degradation. For four phenolics (gallic acid, *p*-coumaric acid, catechin and epicatechin), anti-oxidant activity decreased when ascorbic acid and EDTA were omitted from the reaction mixture compared to that of AA-Fe^{3+}-EDTA-H_2O_2. However for five of the phenolic standards (protocatechuic acid,

syringic acid, myricetin, quercetin, and kaempferol) there was no change in anti-oxidant activity between the two oxidant systems. Only caffeic acid and resveratrol showed a slightly higher anti-oxidant potential in the presence of Fe^{3+}-H_2O_2 compared to the AA-Fe^{3+}-EDTA-H_2O_2 system.

Figure 39 shows the effect of increasing concentrations of the wine and standards towards inhibition of deoxyribose. At the tested concentrations, red wine exhibited a decrease in anti-oxidant activity as the concentration increased (as shown by the lower absorbance relative to the control), with the highest concentration (640 mg/L) showing pro-oxidant activity. Omission of EDTA and ascorbic acid led to a further decrease in anti-oxidant activity at all concentrations compared to the other treatment group. Both oxidant systems showed significant differences at all concentrations of red wine relative to the control ($p < 0.01$), however, at 320 mg/L there was no significant difference compared to the control in the presence of AA-Fe^{3+}-EDTA-H_2O_2. For most of the standards, the results also showed that as the concentration of phenolic increased, the anti-oxidant activity decreased for most of the phenolic compounds.

For both Fenton system models, gallic acid, catechin, epicatechin, caffeic acid, quercetin, and myricetin exhibited pro-oxidant effects relative to the control. These phenolics displayed anti-oxidant activities between 20-160 mg/L for both conditions, but showed pro-oxidant effects at concentrations of 320-640 mg/L. Syringic acid, *p*-coumaric acid, resveratrol, and kaempferol all showed strong inhibition of hydroxyl radical-mediated deoxyribose degradation relative to the control. However, syringic acid and *p*-coumaric acid were the only compounds that did not increase in absorbance as the concentration increased, suggesting strong anti-oxidant capacity.

Gallic acid showed significant differences between most concentrations relative to the control ($p < 0.01$). Protocatechuic acid also showed pro-oxidant activity, but only in the presence of ascorbic acid + EDTA. In the reaction without ascorbic acid and EDTA, it showed anti-oxidant potential. Similar to gallic acid, protocatechuic acid also showed significant differences between most concentrations and the control ($p < 0.01$), whereas for syringic acid these differences were significant over all concentrations ($p < 0.01$).

Catechin exhibited the most pronounced pro-oxidant activity. The decrease in anti-oxidant activity was statistically significant ($p < 0.01$) in the AA-Fe^{3+}-EDTA-H_2O_2 group, except between concentrations of 40 and 80 mg/L. When ascorbic acid was omitted, similar results were observed, but concentrations of 40-160 mg/L were not statistically significant. These results were also statistically similar to epicatechin. The decrease in anti-oxidant activity was statistically significant ($p < 0.01$) in both treatment groups for caffeic acid, *p*-coumaric acid, resveratrol, quercetin, and kaempferol. Myricetin also showed similar results, but the results were not significant between 20 and 160 mg/L for both treatment groups.

Overall, the anti-oxidant activity of the red wine and standard phenolic compounds investigated in the hydroxyl radical-mediated deoxyribose degradation assay decreased in the following order: *p*-CA > SYR > KAEM > RES > PCA > RED WINE > CA > QUE > MYR > GA > EC > C.

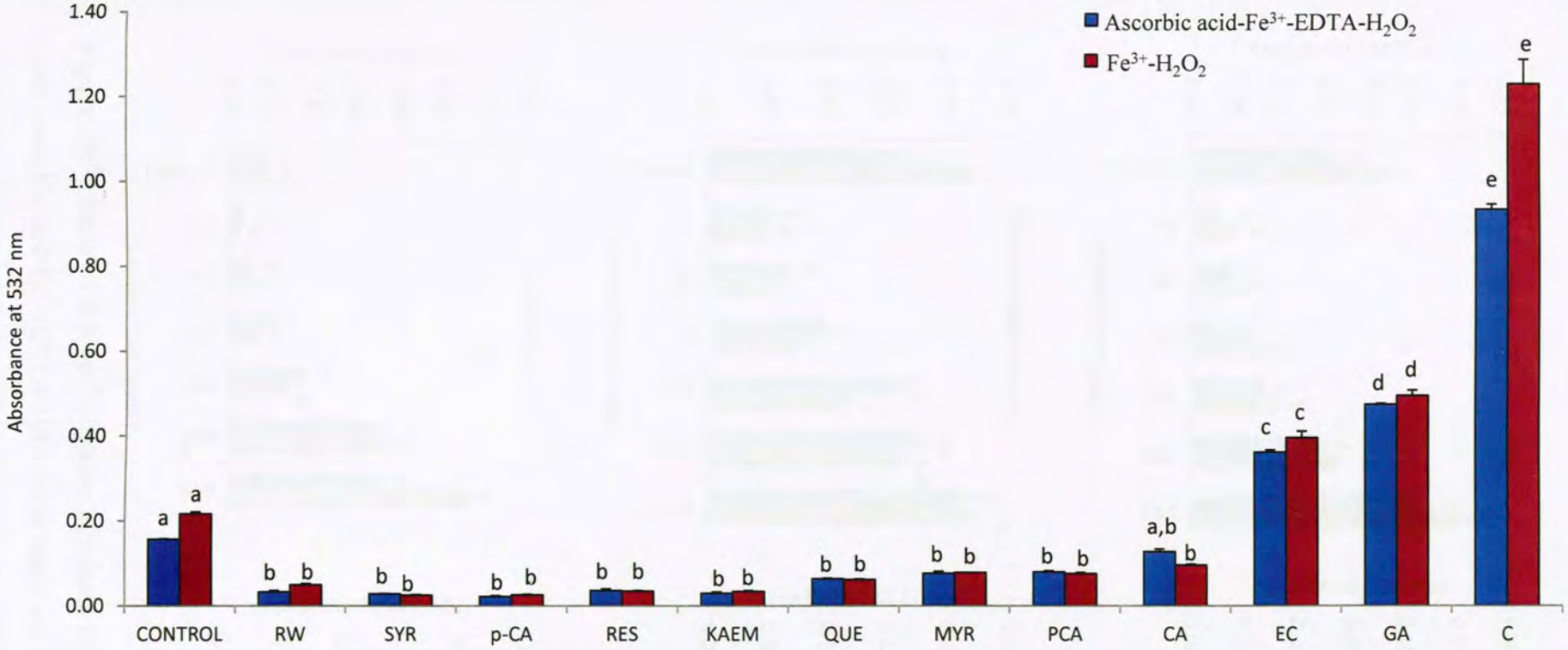


Figure 38: Effects of red wine and phenolic standards on deoxyribose degradation challenged with (i) Ascorbic acid-Fe^{3+}-EDTA-H_2O_2 (Blue bars) and (ii) Fe^{3+}-H_2O_2 (Red bars). Fixed concentrations of standards were used as shown in Table 17, whilst the red wine concentration was 20 mg/L. Data shown as absorbance (532 nm ± SEM). A high absorbance relative to the control indicates pro-oxidant activity. Bars with the same lowercase letter are not significantly different relative to the control ($p > 0.05$).

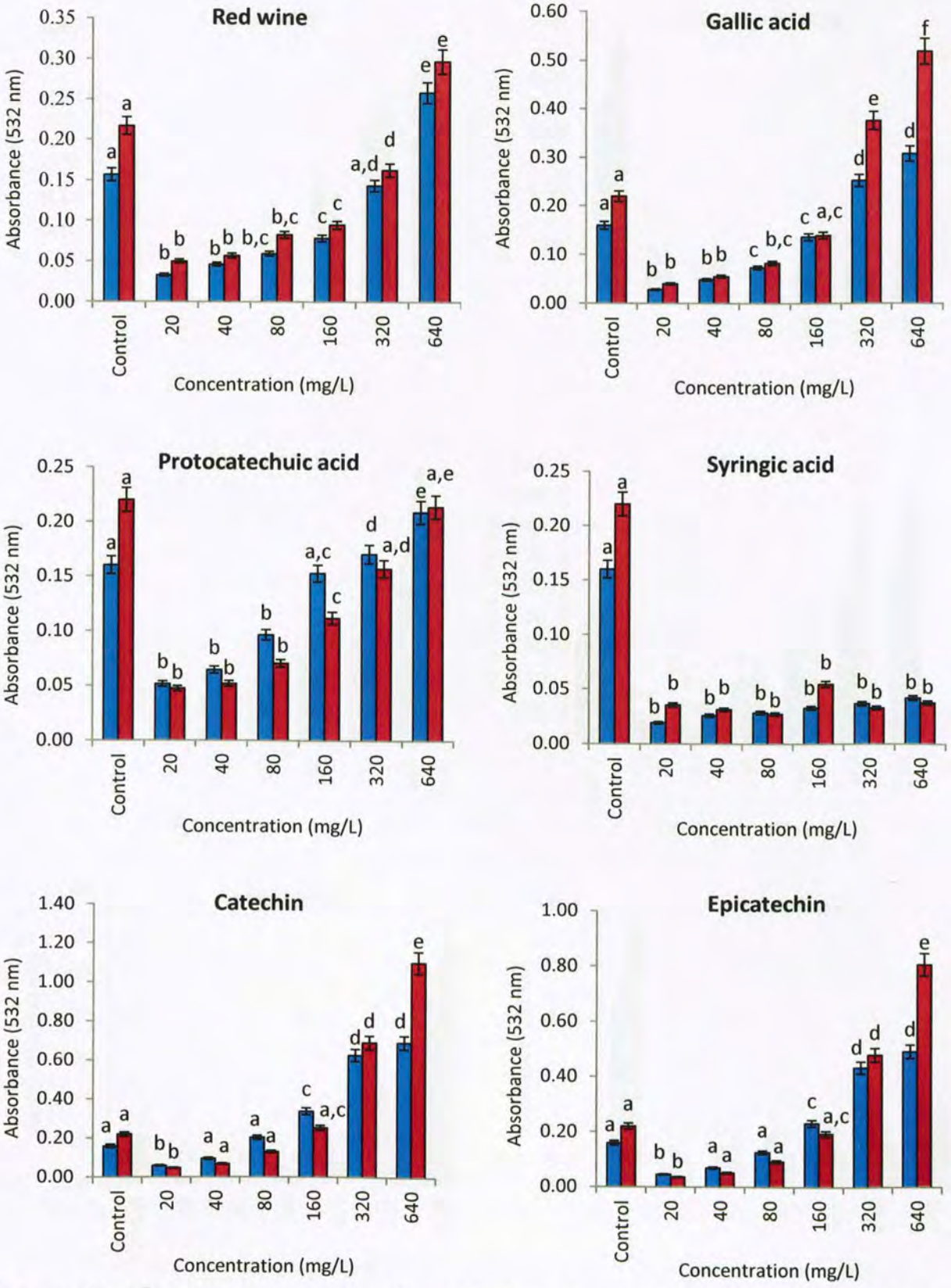


Figure 39: Effects of RW and phenolic standards on deoxyribose degradation challenged with: Ascorbic acid-Fe^{3+}-EDTA-H_2O_2 (Blue bars) and Fe^{3+}-H_2O_2 (Red bars). Concentrations of wine and standards were 20-640 mg/L. Bars with the same lowercase letter are not significantly different relative to the control ($p > 0.05$).

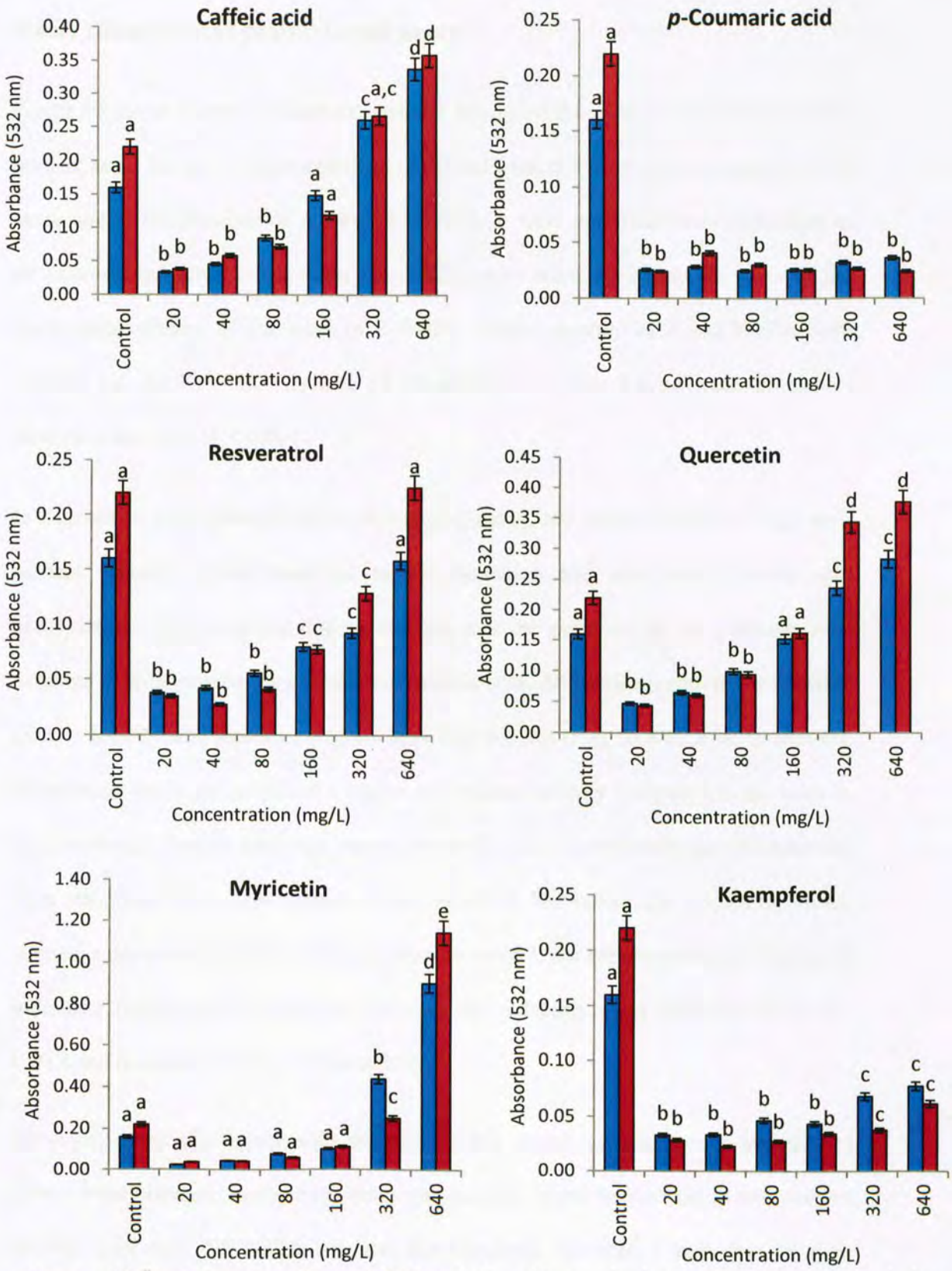


Figure 39 (continued): Effects of RW and phenolic standards on deoxyribose degradation challenged with: Ascorbic acid-Fe^{3+}-EDTA-H_2O_2 (Blue bars) and Fe^{3+}-H_2O_2 (Red bars). Concentrations of wine and standards were 20-640 mg/L. Bars with the same lowercase letter are not significantly different relative to the control ($p > 0.05$).

5.4.4. Linoleic acid peroxidation assay

Figure 40 shows the pro–oxidant/anti-oxidant activity of the wine and standards towards linoleic acid. The anti-oxidant activities of the red wine and most of the standards added alone and in the presence of AA-Fe^{3+}-EDTA-H_2O_2 were not statistically significant at 60 minutes, but after 240 minutes these differences were significant for most of the compounds relative to red wine ($p < 0.001$). When ascorbic acid and EDTA were omitted, i.e. the Fe^{3+}-H_2O_2 system, all the standards exhibited significant differences relative to the wine ($p < 0.001$).

In contrast to the hydroxyl radical scavenging assay, red wine exhibited a high anti-oxidant efficacy against hydroxyl radical formation and subsequent linoleic acid peroxidation. This could be due to the fact that the presence of the phenolic anti-oxidants in wine slowed the oxidation of linoleic acid. Anti-oxidant activity of the wine alone + linoleic acid increased slightly after 240 minutes from 56.4-62.8 %. In contrast, all phenolic standards exhibited a higher anti-oxidant activity compared to the wine in the presence of linoleic acid only, ranging from 65.1-81.8 % at 60 minutes. All activities were enhanced after 240 minutes, from 69.3-87.6 %, except for myricetin, which showed a decrease in activity. Compared to the control, red wine was highly efficient at inhibiting linoleic acid oxidation. Omitting the reducing agent (ascorbic acid) and EDTA led to enhanced anti-oxidant activity.

However, when challenged with the two Fenton model systems, more pronounced effects were observed. Instead red wine was found to show an increase in anti-oxidant activity over time (73.9-82.6 %), and the standards exhibited a reduction in their capacity to inhibit hydroxyl radical-mediated oxidation of linoleic acid (43.6-56.0 %). However, the standards showed a slight increase in anti-oxidant activity after 240

minutes incubation (31.9-62.2 %). Gallic acid was the only phenolic in this oxidant system to show a reduction in anti-oxidant activity after this time. When ascorbic acid and EDTA were omitted from this reaction mixture, there was even greater reduction in activity, with all standards showing pro-oxidant activity after 60 minutes incubation. These activities were statistically significant relative to the wine ($p < 0.001$). Gallic acid showed the greatest pro-oxidant activity (-62.1 %), followed by caffeic acid (-47.0 %), then resveratrol (-41.7 %) at 60 minutes. However, after 240 minutes incubation, further oxidation of linoleic acid was inhibited as shown by an increase in anti-oxidant activity for nearly all standards (-32.8-9.4 %). The three flavonols, quercetin, myricetin, and kaempferol all showed recovery of anti-oxidant activity after this time. The wine showed a slight decrease in anti-oxidant capacity from 60 to 240 minutes, however (75.8-63.2 %). After 240 minutes, syringic acid showed an increase in pro-oxidant activity.

Overall, the anti-oxidant activity of the red wine and standard phenolic compounds investigated in the linoleic acid peroxidation assay decreased in the following order: RED WINE > KAEM > QUE > MYR > C > *p*-CA > PCA > SYR > EC > RES > CA > GA.

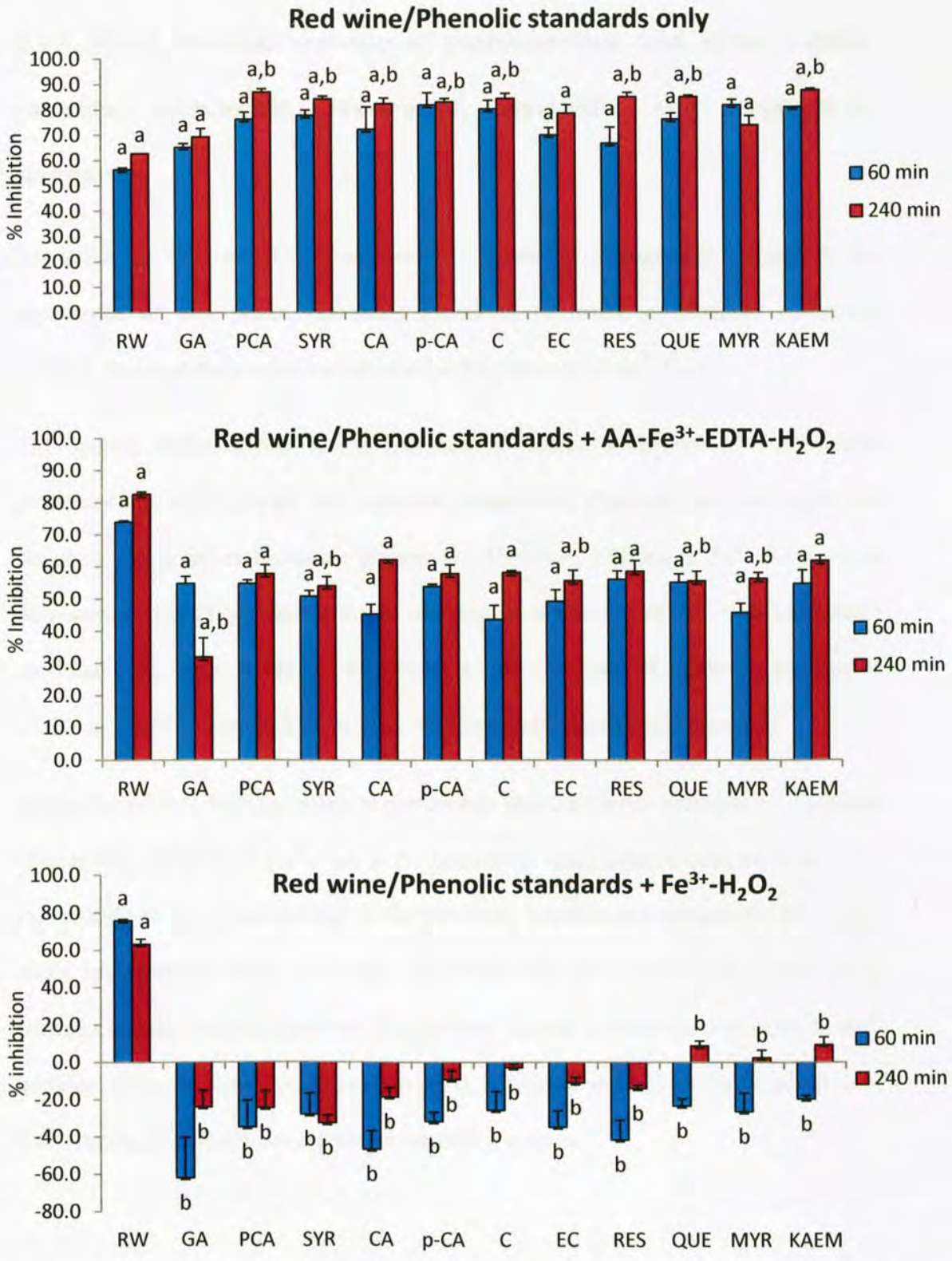


Figure 40: % Inhibition (mean ± SEM) of RW and phenolic standards in the linoleic acid peroxidation system challenged with the two Fenton systems. The concentrations of standards used were those shown in Table 17, while the red wine sample was used undiluted. Negative values indicate pro-oxidant activity. Bars with the same lowercase letter are not significantly different ($p > 0.05$).

5.4.5. Metal chelation activities of protocatechuic acid, syringic acid, catechin, epicatechin, resveratrol, myricetin, and kaempferol standards

Interaction of Fe^{2+} and Cu^{2+} ions with the phenolic anti-oxidants resulted in the appearance of new peaks, indicating formation of metal ion-phenolic compound chelates. Spectral shifts were not observed in the presence of Fe^{3+} ions.

The spectra indicated that kaempferol was an effective chelator of Fe^{2+}, whilst protocatechuic acid, syringic acid, catechin, epicatechin, myricetin, and resveratrol did not show any complex formation (Figure 41). However, addition of Fe^{2+} did result in decreases in absorption maxima for all compounds. Interaction of Fe^{2+} with kaempferol produced a new peak at 405 nm, and a peak at 266 nm (band II). However, addition of EDTA to the Fe^{2+}: kaempferol complex did not restore the original spectrum.

Interaction of Cu^{2+} with the phenolic compounds showed similar results as Fe^{2+} addition (Figure 42). Addition of Cu^{2+} ions to protocatechuic acid, syringic acid, myricetin, and resveratrol resulted in no spectral shifts. However, catechin and epicatechin did exhibit slight bathochromic shifts at 300 nm. Treatment with EDTA led to the spectra being returned to their original positions. Kaempferol showed similar spectral shifts to Fe^{2+} addition. However, band I split into two peaks at 338 nm and 405 nm in the presence of Cu^{2+}. Again, EDTA did not restore the original spectrum.

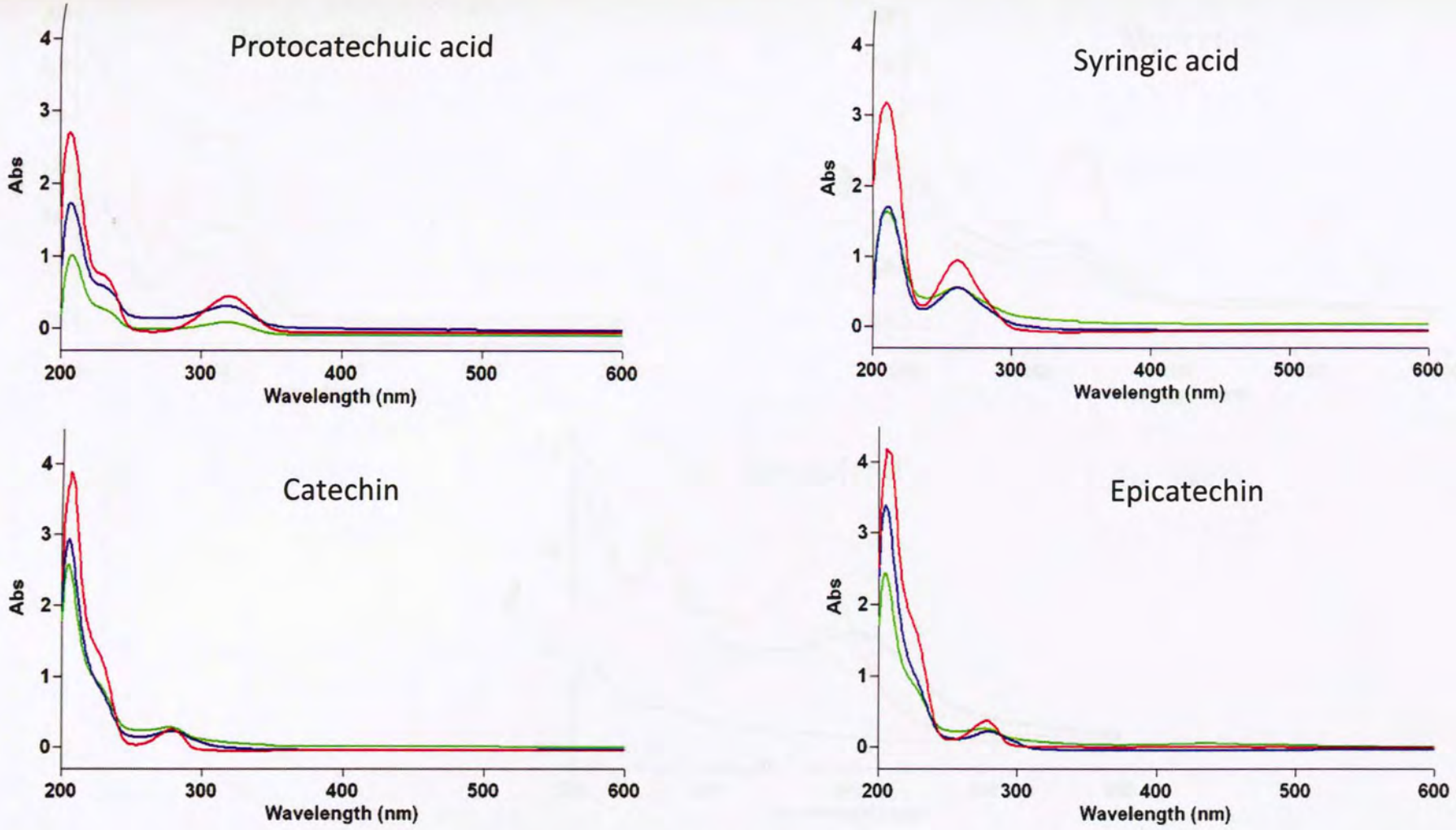


Figure 41: Absorption spectra of the standard phenolic compounds in the presence of Fe^{2+} ions. Red line = Phenolic compound only, Blue line = Phenolic compound + Fe^{2+}, Green line = Phenolic compound + Fe^{2+} + EDTA.

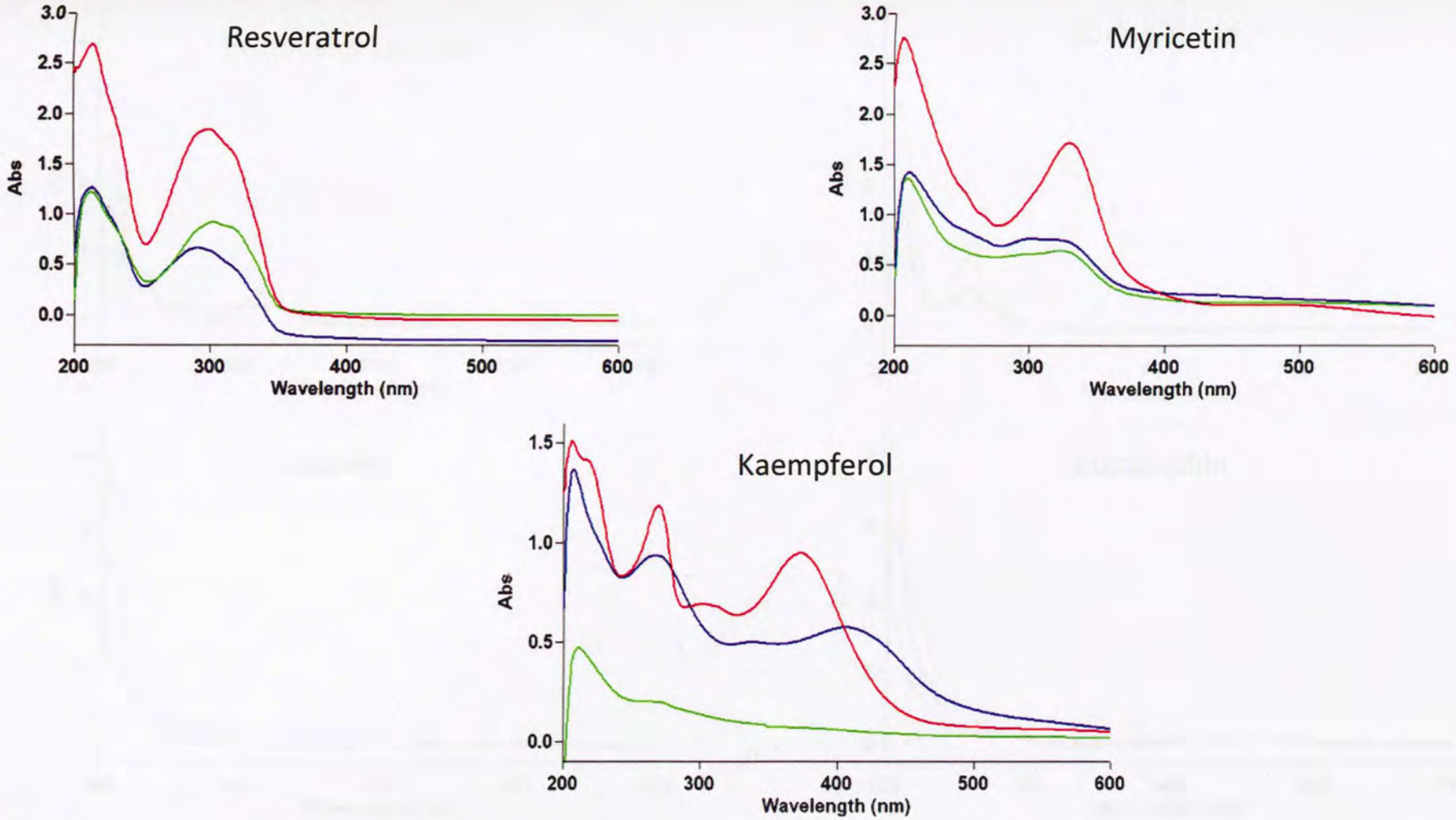


Figure 41 (continued): Absorption spectra of the standard phenolic compounds in the presence of Fe^{2+} ions. Red line = Phenolic compound only, Blue line = Phenolic compound + Fe^{2+}, Green line = Phenolic compound + Fe^{2+} + EDTA.

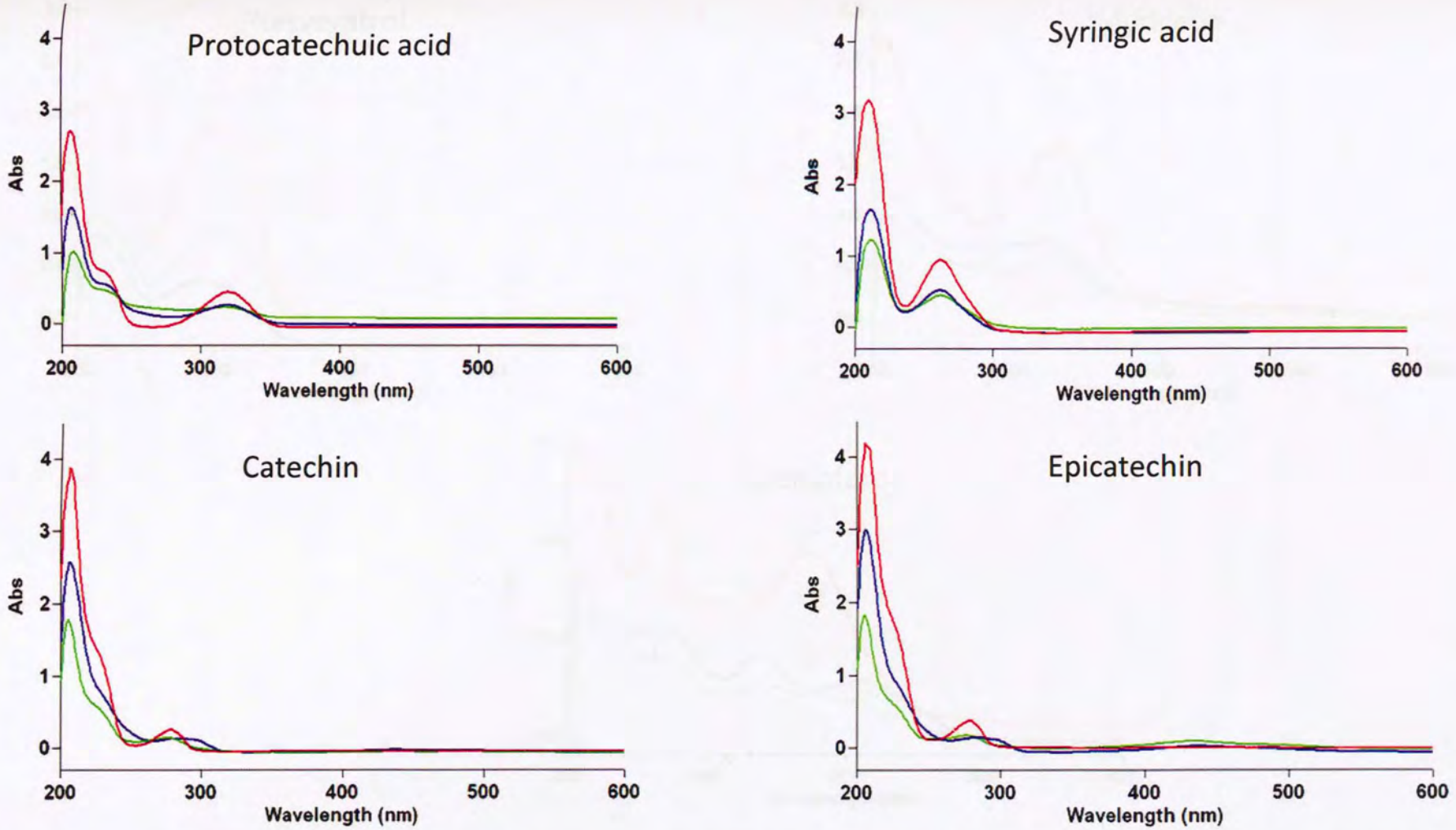


Figure 42: Absorption spectra of the standard phenolic compounds in the presence of Cu^{2+} ions. Red line = Phenolic compound only, Blue line = Phenolic compound + Cu^{2+}, Green line = Phenolic compound + Cu^{2+} + EDTA.

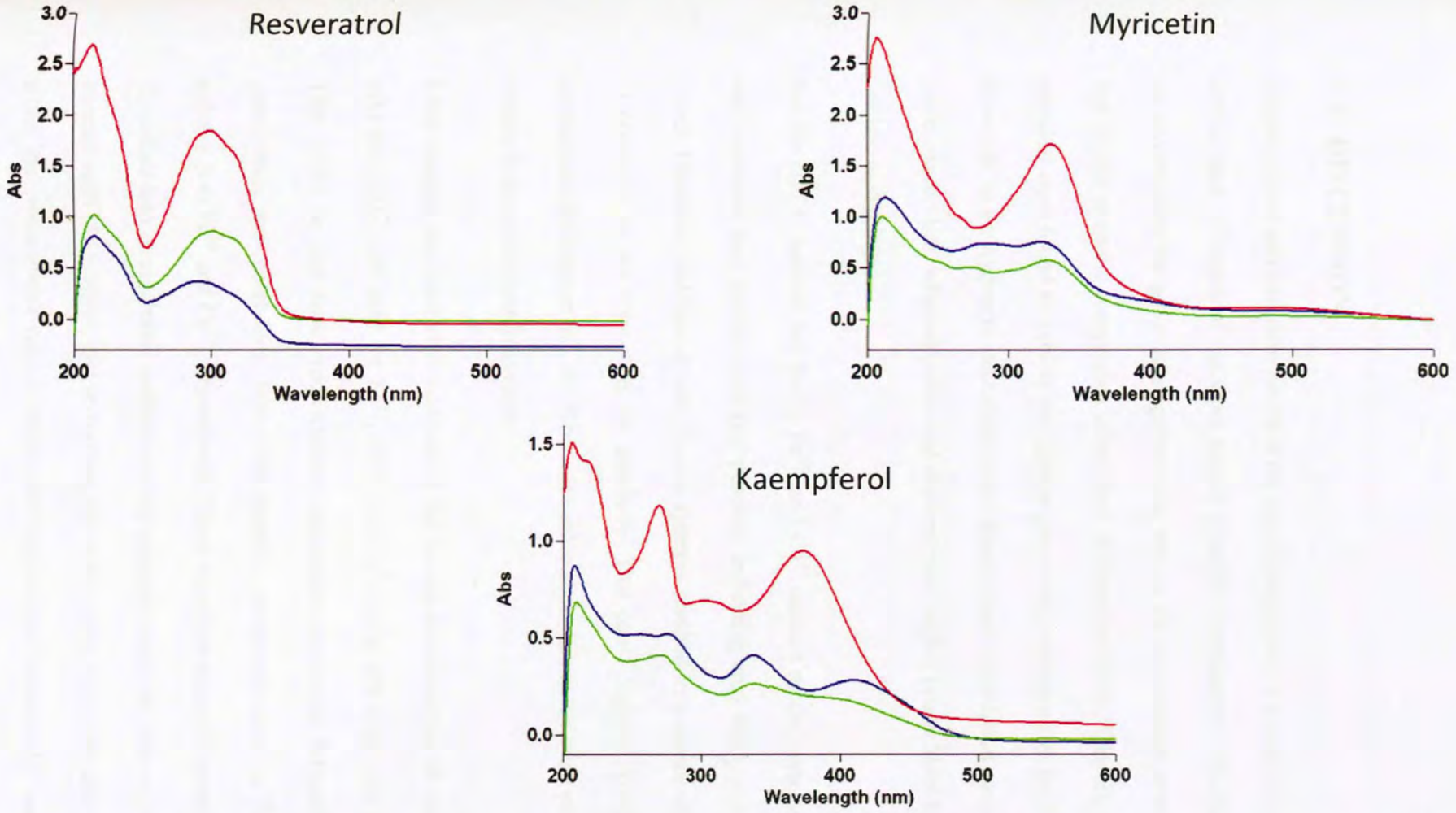


Figure 42 (continued): Absorption spectra of the standard phenolic compounds in the presence of Cu^{2+} ions. Red line = Phenolic compound only, Blue line = Phenolic compound + Cu^{2+}, Green line = Phenolic compound + Cu^{2+} + EDTA.

5.5. DISCUSSION

Chapters three and four demonstrated the use of functional TLC and HPLC to assess the anti-oxidant efficacies of red wine and its phenolic components. This chapter focussed on investigating the pro-oxidant potential as well as the anti-oxidant activity of red wine and eleven phenolic compounds using three different methods. Overall, gallic acid and catechin were found to exhibit the highest pro-oxidant potentials in the HPLC analysis. However, in the hydroxyl radical-mediated deoxyribose degradation assay, catechin was more pro-oxidant, whereas gallic acid demonstrated higher pro-oxidant behaviour in the linoleic acid assay.

For the HPLC method, the H_2O_2, Fe^{3+}, and Cu^{2+} oxidant model systems showed wine and standards had similar activities possibly indicating that the matrix had minimal effect. However, addition of both Fenton systems resulted in a comparably greater loss of phenolics in the wine than the standards. This could suggest possible synergistic interactions (Hidalgo et al., 2010) between other phenolics in the wine which may act to reduce hydroxyl radical formation.

Little change was seen after addition of the lowest concentration of oxidant (i.e. 0.06 mM for H_2O_2, 0.01 mM for Fe^{3+}, Cu^{2+}, and Fe^{2+}-H_2O_2, and 0.02 mM for Cu^{+}-H_2O_2). This could be due to a redox-cycling mechanism occurring between the phenolic compounds and metal ions. When the phenolic compound binds to Fe^{3+} or Cu^{2+}, it reduces it to Fe^{2+} and Cu^{2+}, respectively. These metal ion-phenolic complexes may then dissociate into semiquinone radicals and the reduced metal ion, thus recycling the metal to react with H_2O_2 again, and re-starting the redox-cycle. H_2O_2 can also be regenerated when the semiquinone radical reacts with O_2 to form superoxide, and subsequently H_2O_2 (Perron et al., 2011). The speed of this reaction can be increased by the phenolic-

metal ion complexes, which can act as catalysts. In addition, the free radicals generated from redox reactions by the metal-phenolic complex, can be scavenged by the ligand complex itself, which could account for the results seen in the Fe^{3+} and Cu^{2+} model systems (Fernandez et al., 2002). Similar results were also reported by Chobot, (2010), who found that quercetin and juglone entered complex redox reactions depending upon the components in an assay system, and concentration of phenolic compound. There was also little change observed over 120 minutes incubation.

Although gallic acid and catechin were present at the highest concentrations in wine, they displayed the lowest overall anti-oxidant activities using HPLC analysis. However, epicatechin was also present at a high concentration, but was the strongest anti-oxidant in the wine in all oxidant systems, as shown by the 100 % peak loss on the chromatogram.

The presence of three hydroxyl groups in gallic acid should confer strong anti-oxidant activity compared to the other benzoic acids (Rice-Evans et al., 1996). However, it was the least efficient not only among the other benzoic acids, but also of the other phenolic compounds in all the tested methods reflecting the possible pro-oxidant potential of this compound. The high pro-oxidant potential of gallic acid could suggest that it was less efficient than the other compounds at reducing/chelating Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{2+} metal ions or scavenging H_2O_2. The greater anti-oxidant activity of syringic acid could be due to its comparatively lower concentration than gallic acid, in addition to the presence of a methoxy group in its structure which might confer enhanced radical scavenging and hydrogen-donating capacity (Cai et al., 2006). The diphenolic structure of protocatechuic acid was also a more efficient anti-oxidant than gallic acid, and again could be attributed to the lower concentration of protocatechuic acid. In addition, the

monophenolic structure of *p*-coumaric acid was more effective than the diphenolic caffeic acid, though this could be attributed to the lower concentration of the former compared to the latter compound.

The red wine phenolic, catechin, had one of the highest anti-oxidant activities in the presence of both Fenton systems. However, for the pure catechin standard, the anti-oxidant efficiency was considerably reduced in both Fenton systems, suggesting possible flavonoid-flavonoid interactions within the red wine matrix accounting for a higher anti-oxidant activity. For epicatechin, although it showed the strongest anti-oxidant potency in the wine in all systems, the epicatechin standard displayed low anti-oxidant activity in the Fenton systems. This could suggest that the standard compound was less efficient than catechin in chelating/reducing the Fe^{3+} and Cu^{2+} metal ions. These results were reflected in the deoxyribose assay, which also showed the low efficacy of these compounds in inhibiting hydroxyl radical-mediated deoxyribose degradation. Soobrattee et al., (2005) also found that epicatechin was more efficient than catechin in the TEAC and FRAP assays.

Despite the fact that kaempferol possesses one less hydroxyl substitution than quercetin, it was still found to be efficient in the HPLC and deoxyribose assay. As kaempferol had the second lowest content in the wine, these results could suggest that it was oxidised more readily. Like kaempferol, the strong anti-oxidant activity of resveratrol in both the wine and especially the standards mixture was probably due to the fact that it had the lowest concentration, compared to the other phenolic compounds in the wine. The resveratrol structure also possesses three hydroxyl groups, which could account for its considerable anti-oxidant efficiency (Cai et al., 2006).

Although the flavan-3-ols, catechin and epicatechin, contain the structural properties required for effective radical scavenging and chelating ability, they lack the double bond in the C ring, thus conferring flavonols with a higher anti-oxidant potential (Rice-Evans et al., 1996). However, epicatechin was the most efficient anti-oxidant in the wine. In addition, catechin was also more efficient than the flavonols in both Fenton systems. Although the number and position of hydroxyl groups of quercetin is equal to those of the catechin structure, it was more efficient than catechin in all systems, except in the Fenton systems (wine only). This could be attributed to the fact that upon 1-e oxidation, a stable aryoxyl radical is formed which delocalises the radical, and subsequently stabilised by conjugation. In the catechin structure, however, the A and B rings of the flavonoid structure are perpendicular to each other (Beker et al., 2011; Rice-Evans et al., 1996).

The ability of phenolic compounds to reduce Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}, respectively, can contribute to formation of radicals (Aruoma, 1996; Heim et al., 2002; Procházková et al., 2011). In the presence of H_2O_2, these catalytic transition metal ions can promote highly reactive hydroxyl radical ions via the Fenton reaction. Addition of ascorbic acid to the Fenton reaction can greatly increase $^{\cdot}OH$ generation (Caillet et al., 2007). Both the deoxyribose and linoleic acid assays showed that the Fe^{3+}-H_2O_2 system had a higher oxidising power than the AA-Fe^{3+}-EDTA-H_2O_2 system. However, greater pro-oxidant effects were observed in the linoleic acid assay when EDTA and AA were omitted.

At fixed concentrations of red wine and standards, the wine was an efficient inhibitor of deoxyribose degradation at a concentration of 20 mg/L relative to the control. For the standards, gallic acid, catechin, and epicatechin exhibited pro-oxidant behaviour. These

compounds also showed low anti-oxidant potential using HPLC (except for catechin in the Fenton systems). The pro-oxidant activity of gallic acid, catechin, and epicatechin standards can be attributed to the higher concentrations of these compounds compared to the other standards. This relatively weak Fe^{3+} and Cu^{2+} reduction/chelation activity of these three phenolic standards was also observed using HPLC.

When increasing concentrations of wine and standards were tested (both at the same concentrations), there was a concentration-dependent increase in pro-oxidant activity for the red wine and eight of the eleven standard phenolic compounds, i.e. gallic acid, protocatechuic acid, catechin, epicatechin, caffeic acid, reseveratrol, myricetin, and quercetin in the hydroxyl radical-mediated deoxyribose degradation assay. Wine was a weak inhibitor of deoxyribose degradation, and a pro-oxidant effect was seen at 640 mg/L wine. Further diminished effectiveness was observed when ascorbic acid and EDTA were omitted from the reaction. These results demonstrated the diminishing anti-oxidant activity of wine towards inhibiting hydroxyl radicals and subsequent deoxyribose degradation. This is in agreement with work by Hötzer et al., (2005) and Laggner et al. (2005), also demonstrating pro-oxidant effects of red wine. However, a study by Li et al., (2009), found that red wines had a high anti-oxidant capacity towards scavenging of hydroxyl radicals, although in this study the authors used dilutions of 1:50 and 1:100 of wine.

Although epicatechin and resveratrol were found to exhibit pro-oxidant behaviour, they were the most efficient anti-oxidants using HPLC analysis. This could demonstrate the different test conditions used in both methods. In general, most of the phenolics demonstrated slightly higher anti-oxidant activity (as shown by a lower absorbance) when challenged with the Fe^{3+}-H_2O_2 system up to a certain concentration. However, at

the highest concentrations, i.e. 320-640 mg/L, this activity decreased, with some compounds showing a significant reduction. This weak hydroxyl radical scavenging ability is possibly due to these phenolic compounds (reducing agents) recycling ferric ions back to ferrous ions, thereby continually driving the Fenton reaction (Caillet et al., 2007).

In contrast to the hydroxyl radical scavenging assay, red wine exhibited a high anti-oxidant efficacy against linoleic acid peroxidation. This could be due to the fact that the presence of the phenolic anti-oxidants in wine slowed the oxidation of linoleic acid. However, the standards were less effective than the wine in the presence of the Fenton systems. A study by Sánchez-Moreno et al., (1999) also found that red wine exhibited a high anti-oxidant activity in inhibiting linoelic acid oxidation.

As shown for the results obtained for the HPLC analysis discussed in chapter four, the matrix could account for the high anti-oxidant activity observed in the wine in the linoleic acid assay in the presence of both Fenton reagents, by acting to reduce hydroxyl radical formation and subsequent damage to the substrate. Another explanation could be the formation of redox-active phenolic-Fe^{3+} complexes that mimic superoxide dismutase activity, which maintains the complex in a continuous Fe^{3+}-Fe^{2+}-Fe^{3+} conversion. This prevents subsequent superoxide radicals and Fe^{2+} formation, and could also account for the enhanced activity of wine in the presence of the Fenton reagents in the HPLC analysis (Pardo-Andreu et al., 2006).

The pro-oxidant behaviour of all phenolic compounds in the linoleic acid assay, and eight compounds in the deoxyribose assay in the absence of EDTA, suggests that Fe^{3+} was more easily reduced in the phenolic compound complex than in the EDTA complex (Chobot, 2010). Interestingly, a recovery of anti-oxidant activity was observed for the

three flavonols which was not seen for the other compounds. This could be due to the structural properties of these flavonoids, which possess the characteristics required for radical scavenging. Andueza et al., (2009) found that caffeic acid exerted pro-oxidant activity upon heat treatment. However, a partial recovery in anti-oxidant activity was observed possibly due to polymerisation reactions between phenols.

The capacity of the various phenolic compounds to form complexes with Fe^{2+} and Cu^{2+} metal ions, monitored spectrophotometrically, showed varying results. As shown in chapter four, gallic acid, caffeic acid, and quercetin were found to be good chelators of these metal ions. *p*-Coumaric acid, however, showed no complex formation.

In this chapter, of the seven phenolic compounds, kaempferol was the most efficient at chelating both metal ions, but was not able to restore these spectra upon addition of EDTA. A summary of the metal binding/EDTA efficiencies of the seven standard phenolics is given in Table 18. Brown et al., (1998) also found that addition of Cu^{2+} to kaempferol led to major structural changes, with EDTA being unable to regenerate the original spectrum. This suggests that oxidation of the kaempferol molecule occurred, perhaps at the 3-hydroxy and the 4'-hydroxy groups, as shown for quercetin in chapter four. This observation could account for the high anti-oxidant efficiency of kaempferol in all three test models relative to the other phenolics.

Protocatechuic acid, which has a catechol moiety, did not show complex formation. Andjelković et al., (2006) also found that protocatechuic acid had a weaker chelating ability compared to gallic acid, which bears a galloyl moiety. However, the trihydroxy-benzoic structure of gallic acid was also found to exhibit a lower iron binding capacity compared to some other dihydroxy compounds. Andjelković et al., (2006) also found that syringic acid, which possesses a methoxy group, but lacks galloyl and catechol

groups, was unable to form Fe^{2+} metal ion chelates. However, although this compound showed poor chelation abilities, the methoxy group is able to stabilise phenoxyl radicals, which could account for its efficient anti-oxidant capacity in this study.

Catechin and epicatechin were found to show some complex formation in the presence of Cu^{2+}, but not Fe^{2+}. Fernandez et al., (2002) found that the redox reactions of some flavonoids were higher with Cu^{2+} than with Fe^{2+}, which could be due to the lower redox potential of Cu^{2+}. The lack of the double bond between positions 2 and 3 in the C ring could account for the lower binding ability of catechin and epicatechin. Therefore, the structural property of the catechin molecule responsible for Cu^{2+} binding is likely to be the *ortho*-catechol group in positions 3' and 4' in the B ring (Fernandez et al., 2002). Resveratrol and myricetin, however, did not show complex formation of either of the two metal ions. This is in contrast to Fernandez et al., (2002) who found that myricetin was able to chelate Fe^{2+}.

Table 18: Summary of the metal binding/EDTA efficiencies of the seven standard phenolics: protocatechuic acid, syringic acid, catechin, epicatechin, resveratrol, myricetin, and kaempferol.

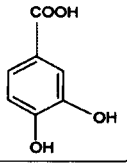
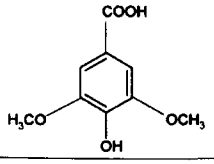
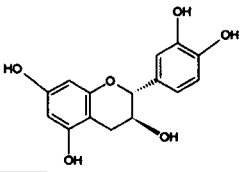
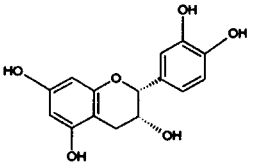
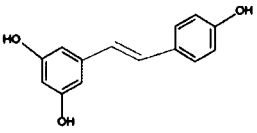
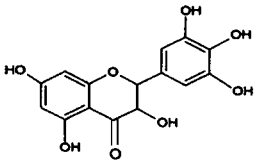
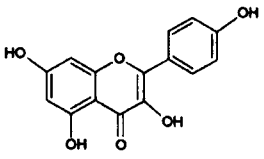
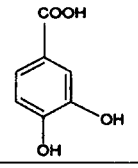
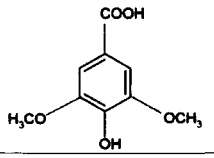
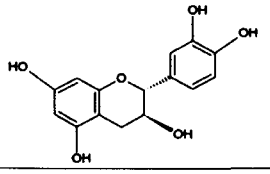
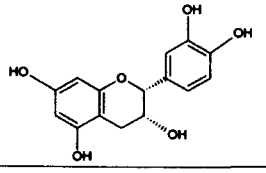
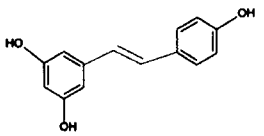
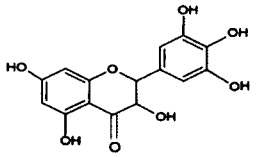
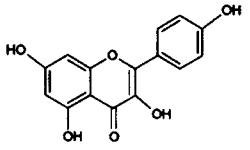
	Chemical structure	Metal binders	Spectra restored upon EDTA addition?
Fe^{3+} ions			
Protocatechuic acid		X	-
Syringic acid		X	-
Catechin		X	-
Epicatechin		X	-
Resveratrol		X	-
Myricetin		√	-
Kaempferol		√	-

Table 18 (continued): Summary of the metal binding/EDTA efficiencies of the seven standard phenolics: protocatechuic acid, syringic acid, catechin, epicatechin, resveratrol, myricetin, and kaempferol.

Cu^{2+} ions			
Protocatechuic acid		X	-
Syringic acid		X	-
Catechin		√	+
Epicatechin		√	+
Resveratrol		X	-
Myricetin		X	-
Kaempferol		√	-

√: Chelator
+: Spectra restored upon addition of EDTA
X: Non chelator
-: Spectra not restored upon addition of EDTA

5.6. CONCLUSION

The results showed that little change in anti-oxidant activity was exerted by both the red wine and standards at increasing concentrations of oxidants in the HPLC analysis, i.e. the H_2O_2, Fe^{3+}, and Cu^{2+} oxidant model systems added independently showed wine and standards had similar activities. This could possibly indicate that the matrix had minimal effect. However, addition of both Fenton systems resulted in a comparably greater loss of phenolics in the wine than the standards, suggesting possible interactions between other phenolics in the wine, such as tannins and anthocyanins or the formation of catalytic anti-oxidant enzyme mimetics. Similar results were observed in chapters three and four.

The hydroxyl radical-mediated deoxyribose degradation assay revealed a concentration-dependent decrease in anti-oxidant activity for some phenolic standards, with red wine promoting oxidation of deoxyribose at increasing concentrations of wine. However, red wine inhibited linoleic acid peroxidation, whilst all the standards exhibited a greater reduction in anti-oxidant activity in the presence of Fe^{3+}-H_2O_2. These results showed that the standards exhibited pro-oxidant activity in both assays, whilst red wine demonstrated potential pro-oxidant activity in the deoxyribose degradation assay.

The results demonstrated that under the conditions of this experiment, the investigated red wine and phenolic compounds exhibited potential pro-oxidant activity. Further studies are required to investigate the chemical mechanisms of the observed pro-oxidant activity.

Chapter 6

THE EFFECT OF Fe^{3+} AND Cu^{2+} METAL ION ADDITION ON THE ANTI-OXIDANT POTENTIAL OF WINES AND GRAPE JUICES USING MODIFIED $ABTS^{\cdot+}$ AND DPPH ASSAYS

6.1. INTRODUCTION

Chapters three, four, and five demonstrated the use of chromatographic methods to measure the anti-oxidant activity of wine. The commonest methods of measuring anti-oxidant activity include anti-oxidant assays to measure radical-scavenging ability. These measure the bulk anti-oxidant activity and phenolic composition of different wines such as: $ABTS^{\cdot+}$, DPPH, CUPRAC, ORAC, HRSA, SRSA, FRAP, and lipid peroxidation inhibition (linoleic acid/β-carotene-coupled oxidation assays (Alén-Ruiz et al., 2009; Cimino et al., 2007; Di Majo et al., 2008; Fernández-Pachón et al., 2004; Li et al., 2009; Lucena et al., 2010; Minussi et al., 2003).

The phenolic composition of red and white wines differs due to the different phenolic components present in both red and white grapes and in wine processing techniques. These differences are attributed to the fact that red winemaking undergoes maceration whereas white winemaking does not (Pretorius and Høj, 2005). This is thought to account for the relatively lower phenol content, in addition to the lower anti-oxidant activity of white wine. Similarly, red or purple grape juice has been found to possess a higher phenolic content and anti-oxidant activity compared to white grape juice. Again,

this could be due to the procedures used in the manufacture of these fruit juices (Sánchez-Moreno et al., 1999).

Villaño et al., (2004) used the TEAC assay to measure the anti-oxidant activity of wines, and found that red wine had significantly high radical scavenging ability, ten times higher than that of white and sherry wines. This was correlated with the higher phenolic content in red wine than white wine. In another study by Kondrashov et al., (2009), Cabernet Sauvignon wines were found to have a higher anti-oxidant capacity and phenolic content compared to Merlot. A strong positive correlation was found between the total anti-oxidant capacity and the phenolic content in both types of wines tested.

Similarly, anti-oxidant activity was evaluated in two types of red wine (Mencía and Brancellao) grown in NW Spain using the DPPH and β-carotene/linoleic acid assays (Alén-Ruiz et al., 2009). The DPPH assay revealed Mencía wines had a higher ant-oxidant activity than Brancellao wines. Whereas the β-carotene/linoleic acid assays showed that anti-oxidant capacity was higher in Brancellao wines. This discrepancy in results could be due to the different solubilities of wine anti-oxidants in the media used in the assays, i.e. DPPH uses an aqueous medium, whereas β-carotene/linoleic acid uses an emulsion.

The anti-oxidant and anti-inflammatory activity of two Greek wine extracts – red (Cabernet Sauvignon) and white (Robola) were studied using the DPPH, linoleic acid peroxidation, and lipoxygenase inhibition assays (Xanthopoulou et al., 2010). The results showed that Cabernet Sauvignon extracts had a higher radical-scavenging activity and were more potent inhibitors of lipid peroxidation than Robola extracts. Although many studies report a correlation between total phenolic content and anti-

radical efficiency such as Kondrashov et al., (2009), others such as Xanthopoulou et al., (2010) found that this may not be necessarily true. It was argued that the molecular structure of phenolics should be taken into account rather than their amount when evaluating anti-oxidant activity.

Hydrogen atom transfer methods are also commonly used to measure the anti-oxidant activity of wine. These methods, including the ORAC, TRAP, and LDL oxidation assays are more specific, in that they directly measure the capacity of an anti-oxidant to quench free radicals (Prior and Cao, 1999). However, the electron transfer methods, such as $ABTS^{\cdot+}$, DPPH, CUPRAC, and FRAP, are simpler to measure, rapid, and inexpensive compared to other test models. In addition, the TEAC assay can be used to study both hydrophilic and lipophilic anti-oxidants. Although these studies highlight the anti-oxidant activities of different wines using standard assays, data on the effects of various food matrices challenged with redox-active metal ions is scarce. This is needed in order to determine the potential pro-oxidant effects these foodstuffs can exert. This chapter was therefore concerned with measuring the effects of adding Fe^{3+} and Cu^{2+} metal ions to both wines and grape juices, and comparing and contrasting their anti-oxidant activities. The DPPH and $ABTS^{\cdot+}$ assays were selected due to the reasons outlined above, and modified by addition of metal ions to the reaction mixture.

6.2. AIMS

The aims of this chapter are:

- To measure the total phenol content of red wine, white wine, rosé wine, red grape juice, and white grape juice.

- Use a modified DPPH assay to measure the anti-oxidant activity of each of the samples in the absence and presence of Fe^{3+} and Cu^{2+} metal ions.
- Use a modified $ABTS^{\cdot+}$ assay to measure the anti-oxidant activity of each of the samples in the absence and presence of Fe^{3+} and Cu^{2+} metal ions.

6.3. METHODS

The methods are given in chapter two. Briefly:

- The total phenolic content of the wines and grape juice samples was measured using the Folin Ciocalteau method.
- DPPH and $ABTS^{\cdot+}$ assays were used to determine the effect of adding increasing concentrations of Fe^{3+} and Cu^{2+} to the wines and grape juice samples. A concentration range of 0.01-0.05 mM metal ions was prepared. These metal ion solutions were incubated with the various samples at a 1:1 ratio, and the absorbances measured after specific time intervals.

6.4. RESULTS

6.4.1. Measurement of total phenolic content using the Folin-Ciocalteau assay

The phenolic composition of the wines and grape juices is shown in Figure 43. Red grape juice was found to have the highest total phenolic content. The overall ranking of the samples was: red grape juice (3372.50 GAE) > red wine (1497.50 GAE) > white grape juice (1435.00 GAE) > rose wine (1247.50 GAE) > white wine (935.00 GAE).

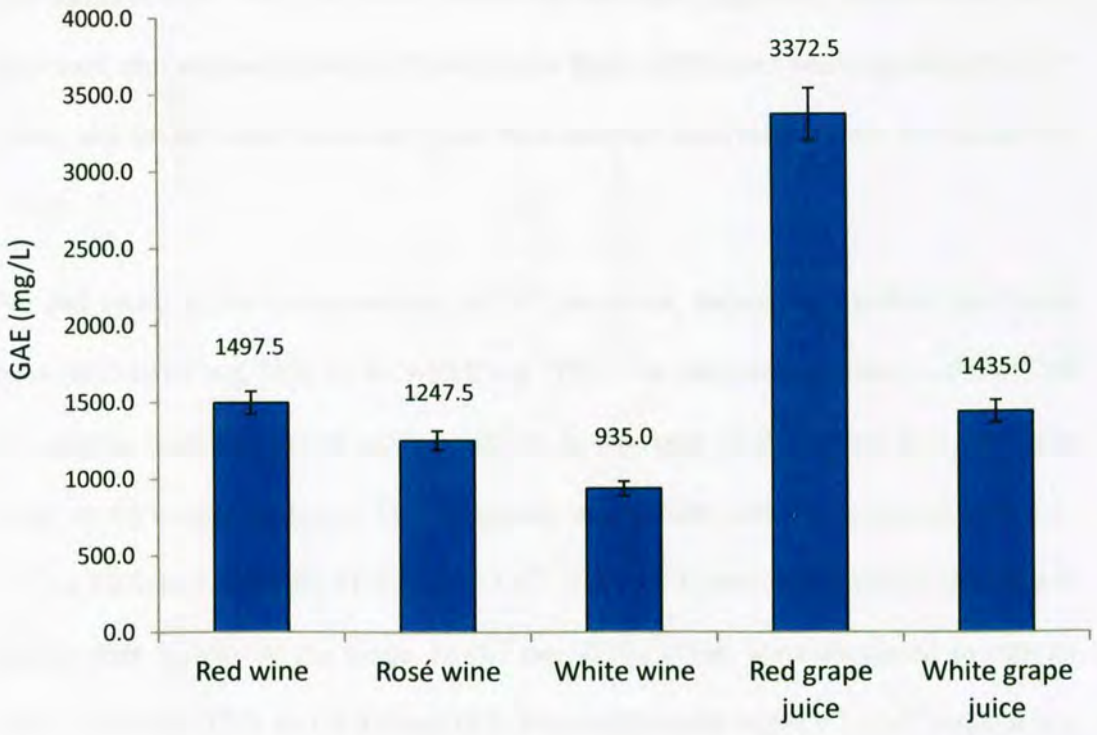


Figure 43: Total phenolic content of red wine, white wine, rosé wine, red grape juice, and white grape juice. Data presented as gallic acid equivalents (GAE ± SEM).

6.4.2. DPPH radical-scavenging assay

A modified version of the DPPH assay was used to determine the effect of the addition of Fe^{3+} and Cu^{2+} metal ions on anti-oxidant activity of wine and grape juice. As shown in Figure 44, red wine was found to exhibit the highest anti-oxidant activity, with white wine being the least efficient anti-oxidant. The order of anti-oxidant activity measured over 30 minutes was: red wine (86.8-103.9 mg TE/L) > red grape juice (12.1-14.8 mg TE/L) > white grape juice (4.7-5.7 mg TE/L) > rosé wine (4.3-5.5 mg TE/L) > white wine (2.7-3.5 mg TE/L).

The addition of Fe^{3+} and Cu^{2+} metal ions to the wine and grape juice samples resulted in decreased anti-oxidant capacity. For red wine these differences were significant at $p < 0.001$, and for the other wines and grape juice samples these results were significant at $p < 0.01$.

For red wine, as the concentrations of Fe^{3+} increased, anti-oxidant activity decreased from 86.8-103.9 mg TE/L to 42.3-55.8 mg TE/L. The same concentrations of Cu^{2+} led to a similar reduction in anti-oxidant activity in the range 43.0-56.6 mg TE/L. For rosé wine, as the concentrations of Fe^{3+} increased, anti-oxidant activity decreased from 4.3-5.5 mg TE/L to 3.2-4.0 mg TE/L. When Cu^{2+} was added, there was a similar decrease in anti-oxidant activity in the range 3.1-4.1 mg TE/L. White wine decreased in activity from 2.7-3.5 mg TE/L to 1.9-2.6 mg TE/L when challenged with Fe^{3+}. Cu^{2+} resulted in a similar reduction in anti-oxidant activity in the range 1.8-2.4 mg TE/L.

Red grape juice decreased in activity from 12.1-14.8 mg TE/L to 8.7-11.5 mg TE/L over 30 minutes in the presence of Fe^{3+}. Cu^{2+} addition, however, was slightly more oxidising than Fe^{3+}, and resulted in a decrease in activity in the range 9.1-12.0 mg TE/L. White grape juice decreased in activity from 4.7-5.7 mg TE/L to 3.2-4.6 mg TE/L. Cu^{2+} resulted in a similar decrease in anti-oxidant activity in the range 3.4-4.7 mg TE/L.

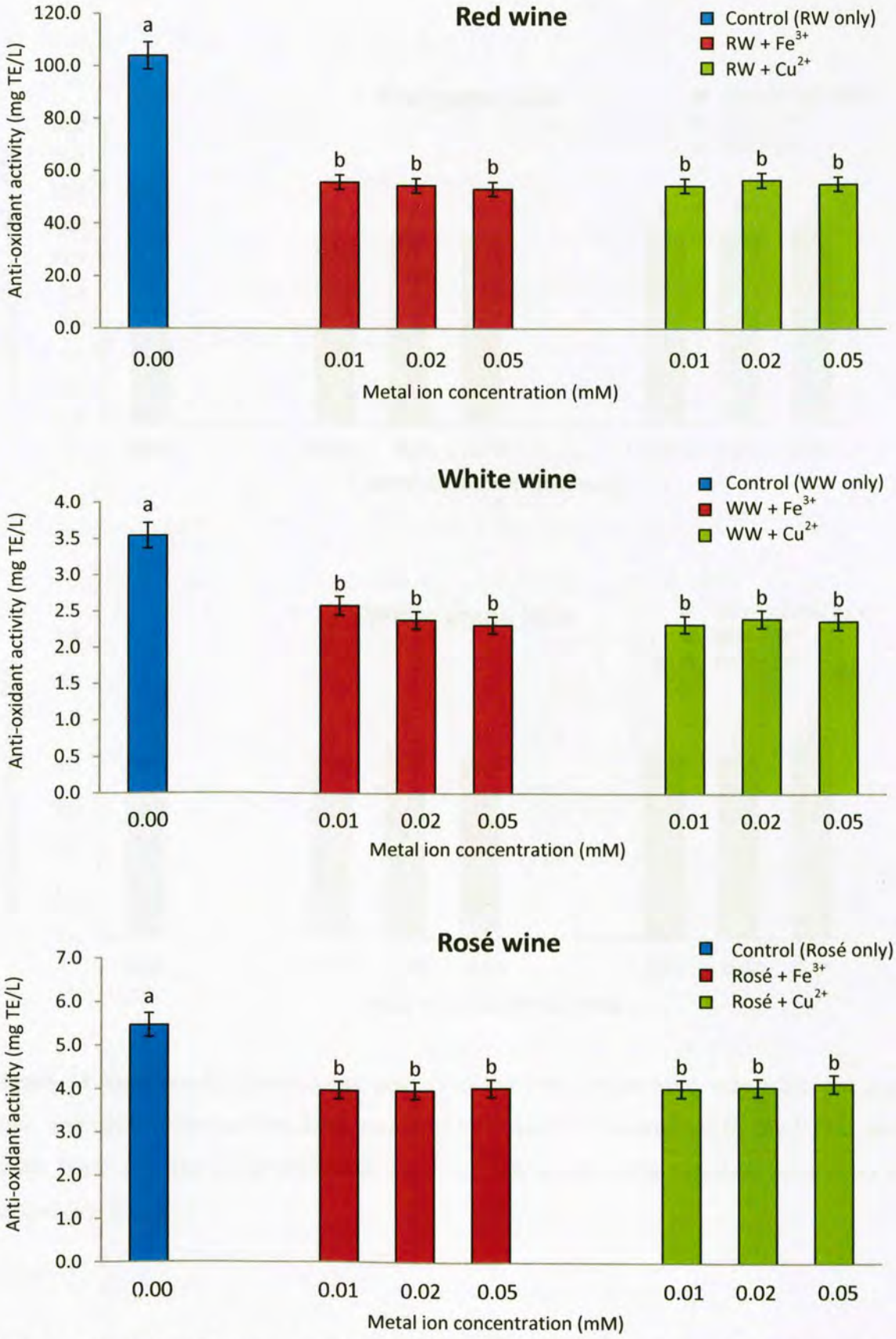


Figure 44: Anti-oxidant activity of red wine, white wine, rosé wine, red grape juice, and white grape juice in the presence of Fe^{3+} and Cu^{2+} determined by the DPPH assay. Bars with the same lowercase letter are not significantly different relative to the control ($p > 0.05$).

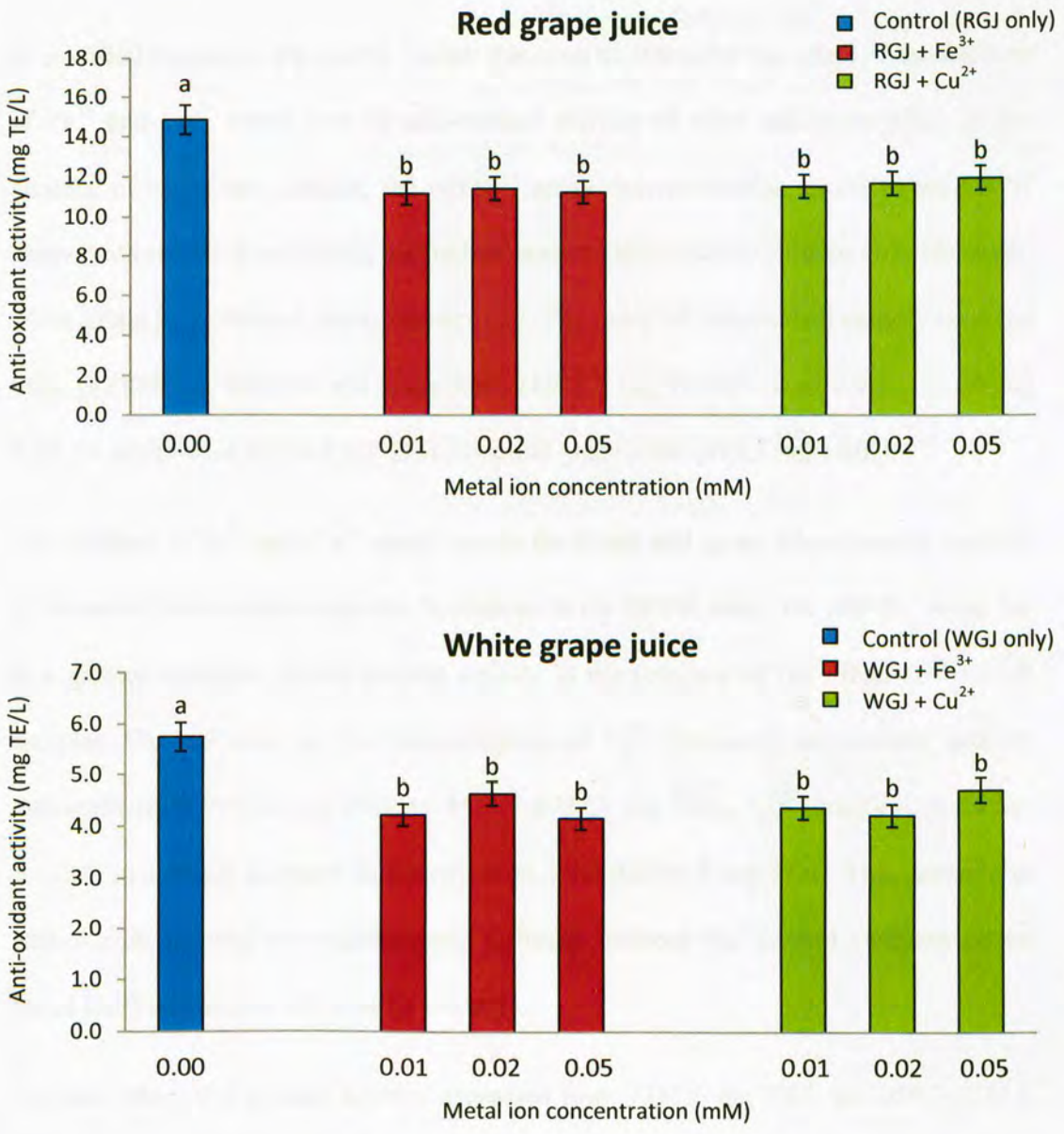


Figure 44 (continued): Anti-oxidant activity of red wine, white wine, rosé wine, red grape juice, and white grape juice in the presence of Fe^{3+} and Cu^{2+} determined by the DPPH assay assay. Bars with the same lowercase letter are not significantly different relative to the control ($p > 0.05$).

6.4.3. $ABTS^{\cdot+}$ radical-scavenging assay

A modified version of the $ABTS^{\cdot+}$ assay was used to determine the effect of the addition of Fe^{3+} and Cu^{2+} metal ions on anti-oxidant activity of wine and grape juice. In the absence of metal ion addition, the $ABTS^{\cdot+}$ assay showed similar results to the DPPH assay, with red wine exhibiting the highest anti-oxidant capacity (Figure 45). However, white grape juice showed the lowest activity. The order of anti-oxidant activity was: red wine (7032.8 mg TE/L) > red grape juice (1533.7 mg TE/L) > rosé wine (1311.6 mg TE/L) > white wine (1136.1 mg TE/L) > white grape juice (895.2 mg TE/L).

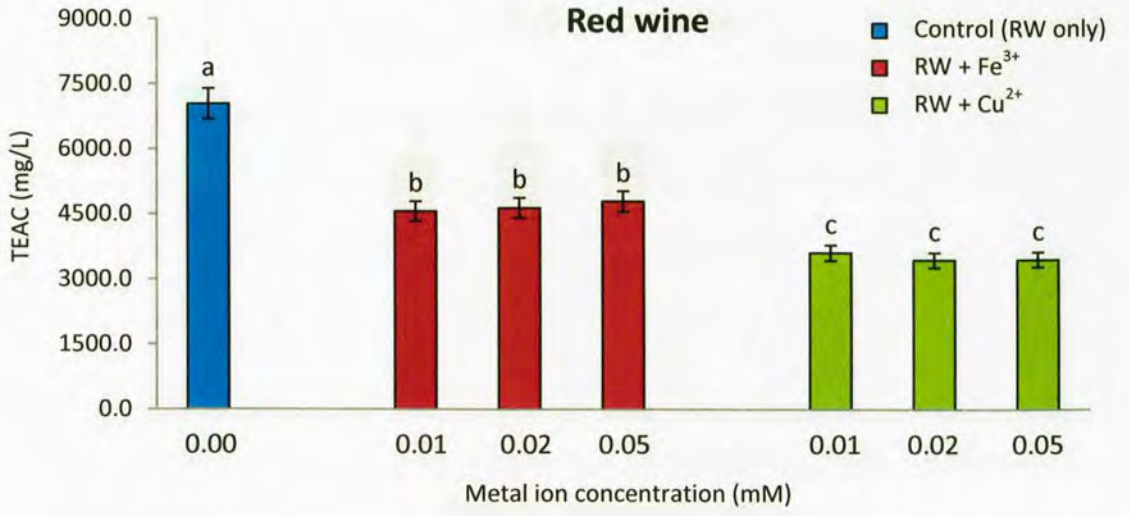
The addition of Fe^{3+} and Cu^{2+} metal ions to the wines and grape juice samples resulted in decreased anti-oxidant capacity. In contrast to the DPPH assay, the $ABTS^{\cdot+}$ assay led to a greater reduction in anti-oxidant activity in the presence of Cu^{2+} than Fe^{3+} for all samples. For red wine, as the concentrations of Fe^{3+} increased, anti-oxidant activity decreased from 7032.8 mg TE/L to 4556.7-4782.1 mg TE/L. Cu^{2+} addition, however, resulted in a steady decrease in activity from 3600.0-3444.8 mg TE/L. This decrease in anti-oxidant activity was significantly different between the control (without added metal ions) and treated red wine ($p < 0.001$).

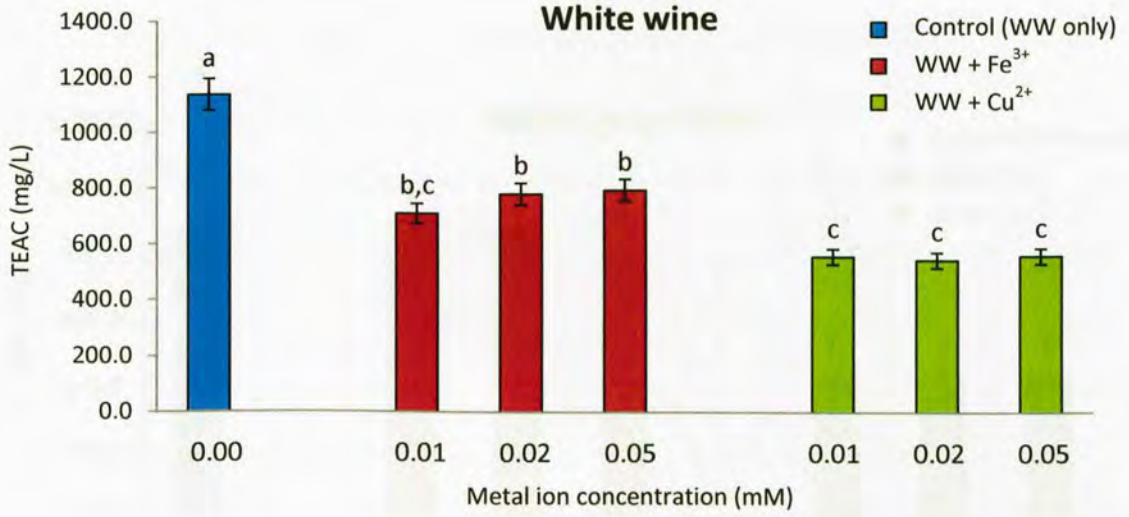
For rosé wine, anti-oxidant activity decreased from 1311.6 mg TE/L to 1059.7-1234.6 mg TE/L in the presence of Fe^{3+}. This decrease was not significantly different however. Cu^{2+} addition resulted in a decrease in activity in the range 548.1-660.9 mg TE/L. This decrease in activity, however, was significantly different ($p < 0.001$).

White wine exhibited a decrease in activity from 1136.1 mg TE/L to 710.5-794.9 mg TE/L when challenged with Fe^{3+}. This activity further decreased to 556.4-558.8 mg TE/L when subjected to Cu^{2+} treatment. The addition of both metal ions relative to the control showed they were all significantly different ($p < 0.001$).

Red grape juice exhibited a decrease in activity from 1533.7 mg TE/L to 1196.4-1260.0 mg TE/L in the presence of Fe^{3+}. A further decrease of between 696.4-774.9 mg TE/L was observed when Cu^{2+} was added. The addition of both metal ions compared to the control showed they were all significantly different ($p < 0.001$).

Unlike the other samples, white grape juice showed a slight increase in anti-oxidant activity in the presence of Fe^{3+} from 895.2 mg TE/L to 921.2 mg TE/L. This increase was not significantly different relative to the control however. In contrast, increasing Cu^{2+} concentrations led to a reduction of anti-oxidant activity in the range 428.4-440.6 mg TE/L. This decrease in activity was significantly different ($p < 0.001$).





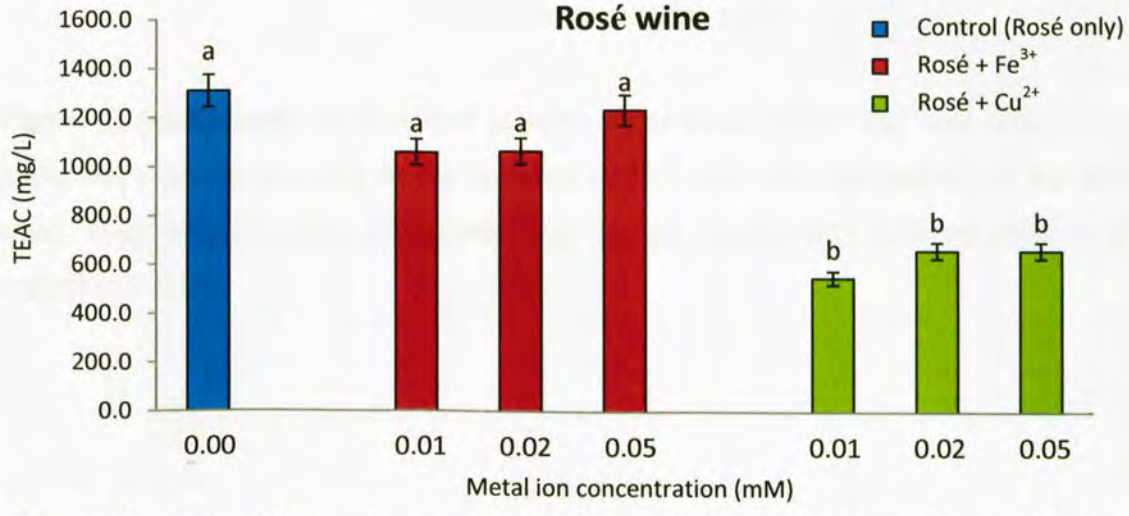
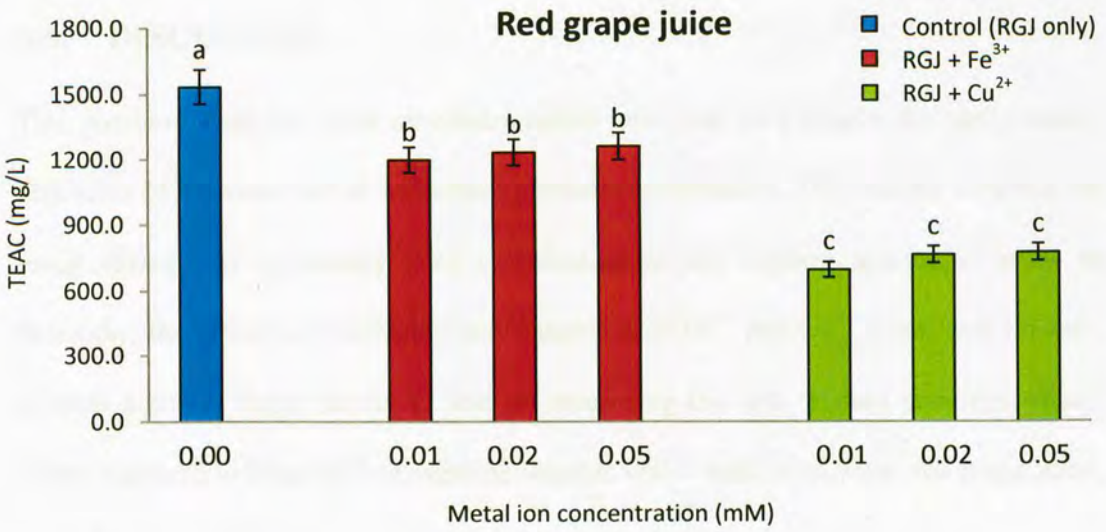


Figure 45: Anti-oxidant activity of red wine, white wine, rosé wine, red grape juice, and white grape juice in the presence of Fe^{3+} and Cu^{2+} determined by the $ABTS^{\bullet+}$ assay. Bars with the same lowercase letter are not significantly different relative to the control ($p > 0.05$).



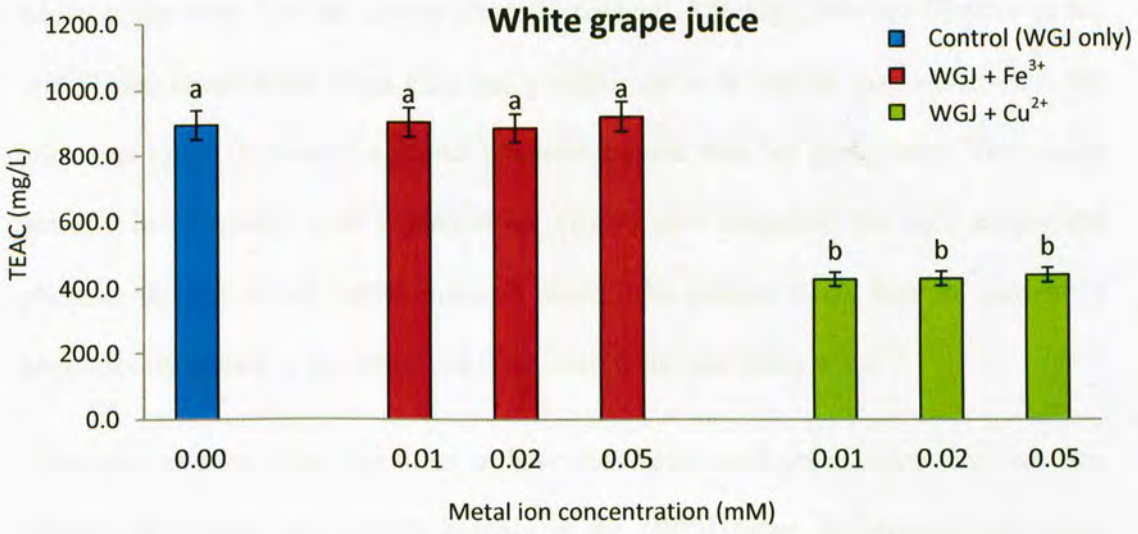


Figure 45 (continued): Anti-oxidant activity of red wine, white wine, rosé wine, red grape juice, and white grape juice in the presence of Fe^{3+} and Cu^{2+} determined by the $ABTS^{\cdot+}$ assay. Bars with the same lowercase letter are not significantly different relative to the control ($p > 0.05$).

6.5. DISCUSSION

The previous chapters used chromatographic methods to evaluate the anti-oxidant efficacies of red wine and its individual phenolic components. This chapter focussed on using variants of commonly used chemical-based anti-oxidant assays, in order to determine the effects of increasing concentrations of Fe^{3+} and Cu^{2+} metal ions on anti-oxidant activity. Here, the focus was on measuring the anti-oxidant activities of red wine compared to those of four other beverages: white wine, rosé wine, red grape juice, and white grape juice.

Red grape juice was found to have the highest phenolic content, followed by red wine, whilst white wine had the lowest phenolic content. Although Sánchez-Moreno et al., (1999) also found white grape juice had a higher phenolic content than white wine, red wine was found to possess a greater phenolic content than red grape juice. The results are also in agreement with Paixão et al., (2007) who compared the anti-oxidant and phenolic content of red, white and rosé wines. The authors found that the content of phenolics decreased in the order: red wine, rosé wine, and white wine.

Although red grape juice was found to have the highest total phenolic content, red wine showed the highest anti-oxidant activity in the DPPH assay, followed by red grape juice. White grape juice had the next highest DPPH scavenging activity, followed by rosé wine, then white wine. The same ranking order was observed for the total phenol assay, possibly indicating the effect of phenolic compounds on anti-oxidant activity, which has been reported in many studies (Porgali and Büyüktuncel, 2012).

For the $ABTS^{\cdot+}$ assay, red wine again showed the greatest anti-oxidant activity relative to the other wines and grape juices. A similar ranking order was observed as the DPPH assay. However, white grape juice was found to exhibit the lowest anti-oxidant activity

in the $ABTS^{\cdot+}$ assay. This could possibly be due to methodological differences between the two assays, and highlights the importance of carrying out multiple assays to assess total anti-oxidant activity. Interestingly, unlike the DPPH assay, which showed no significant differences between both Fe^{3+} and Cu^{2+} treatment groups, the $ABTS^{\cdot+}$ assay showed significant differences between the two metal ion groups. Cu^{2+} was found to give a more pronounced reduction in anti-oxidant activity than Fe^{3+}, which could indicate that the samples showed a higher potential pro-oxidant activity in the presence of Cu^{2+}.

Overall, both the $ABTS^{\cdot+}$ and DPPH assays demonstrated reduced anti-oxidant activity in the presence of both metal ions, which could suggest potential pro-oxidant effects. In addition, there was minimal change in activity as the concentration of metal ion increased. This is in agreement with Espinoza et al., (2009), who found that the presence of increasing concentrations of iron and copper metal ions resulted in a reduction in DPPH free radical-scavenging capacity for all the wines studied. Dowling et al., (2010) also used the DPPH assay to demonstrate the effect of metal ions on the anti-oxidant activity of isoflavones. It was found that iron isoflavone chelates exhibited pro-oxidant activity compared to the free isoflavones. However, copper isoflavone chelates demonstrated higher anti-oxidant activity compared to the free isoflavones.

6.6. CONCLUSION

Overall, both the $ABTS^{\cdot+}$ and DPPH assays demonstrated reduced anti-oxidant activity when challenged with both Fe^{3+} and Cu^{2+} metal ions, which could suggest potential pro-oxidant effects. In addition, there were minimal changes in activity as the concentration of metal ion increased. The results suggested that, overall, the radical-scavenging

activity of the wines and grape juices decreases in the presence of metal ions. In the DPPH assay, Fe^{3+} and Cu^{2+} had similar activities. However, in the $ABTS^{\cdot+}$ assay, Cu^{2+} was shown to have the more reducing/oxidising effect compared to Fe^{3+}.

Chapter 7

CONCLUSIONS AND FUTURE WORK

The anti-oxidant activities of red wine using various chemical-based anti-oxidant assays have been extensively reported. In addition, *in vivo* clinical studies have demonstrated the benefits of red wine in protecting against cardiovascular disease. However, assessing the anti-oxidant efficacy of red wine and its phenolic components using techniques such as TLC and HPLC has remained relatively unexplored. Many studies use HPLC to quantify the phenolic compounds in wine, rather than using this technique to evaluate anti-oxidant activity.

There are far more studies on the anti-oxidant activity of plant extracts and phenolic compounds than pro-oxidant activity. Research on the potential pro-oxidant effects of red wine is also limited. Therefore, this research aimed to contribute to existing knowledge by assessing the anti-oxidant and pro-oxidant profiles of red wine and selected phenolic compounds, using TLC, HPLC, and variants of the commonly used anti-oxidant assays.

Chapter three used TLC to separate the components of complex mixtures, namely white wine, rosé wine, red grape juice, white grape juice, and red and green grapes. Initial TLC analysis of these test samples showed that the selected mobile phases proved unsatisfactory in the separation of components, and were thus not analysed further. However, the white and rosé wines as well as the grape juices did show the presence of one unidentified zone. Red wine showed clear resolution of compounds, and was therefore used for anti-oxidant testing.

Functional TLC was applied to monitor the loss of red wine anti-oxidants in the presence of different model oxidant systems involving hydrogen peroxide and Fe^{3+}/ Cu^{2+} metal ions. The developed TLC method was able to demonstrate the anti-oxidant efficacies of the individual phenolic components. Of the five phenolics identified, quercetin and caffeic acid were found to be the most potent anti-oxidants, followed by gallic acid, which showed moderate anti-oxidant potential. Chlorogenic acid and *p*-coumaric acid were found to be the least efficient anti-oxidants. The five oxidant model systems also differed in terms of oxidising power, with more compounds being lost in the presence of Fenton both reagents (metal ions + H_2O_2), than when each oxidant was added independently. The matrix appeared to have little effect on anti-oxidant activity, with the standards exerting higher activity even at a lower concentration to wine.

Further work could focus on using other methods other than conventional extraction methods, such as solid phase or liquid-liquid extraction, in order to extract the compounds of interest in those samples that did not show separation. Some authors have noted higher efficiency of polyphenol extraction from red grape pomace and skin using pressurised liquid extraction (Wijngaard et al., 2012). This technique uses high pressures, usually ranging from 4 to 20 MPa, and elevated temperatures above boiling point. Other extraction techniques include pulsed electric fields, ultrasound waves, and microwave assisted extraction (Wijngaard et al., 2012). However, the drawback with these different treatments is that they can result in degradation of flavonoids.

Other work could involve using different solvents and ratios of mobile phases to establish the key natural products in these mixtures using TLC. These purified components could then be tested further by investigating their anti-oxidant and pro-oxidant profiles using both TLC and a range of standard *in vitro* assays.

Functional TLC could also be used to rank the efficacies of the components of other types of wines and grape juices in the presence of these different oxidant systems. Comparisons of oxidant profiles can then be evaluated. Another technique that could be used to give more sensitive, reliable, precise, and reproducible quantitative results is HPTLC. Hyphenation with other methods such as HPLC and MS can generate further information about compounds of interest (Morlock and Schwack, 2010).

In chapter four, a new quantitative approach was developed to assess the relative anti-oxidant activities of these red wine phenolics using HPLC. The results demonstrated the effectiveness of applying this approach in assessing the relative anti-oxidant efficacies of these individual phenolics. Like the TLC study, quercetin and caffeic acid were found to exhibit the highest anti-oxidant activities, whilst gallic acid and *p*-coumaric acid were less effective anti-oxidants. All oxidant systems led to considerable peak reductions for all phenolics, but when challenged with the Fenton systems, even greater peak reduction was observed. Metal binding studies reinforced the results obtained for the HPLC analysis, by showing the formation of metal ion–phenolic complexes for quercetin, caffeic acid, and gallic acid. These complexes were not apparent in *p*-coumaric acid however.

Both functional TLC and HPLC studies demonstrated that an understanding of red wine phenolic anti-oxidant interactions with redox-active transition metal ions is required in order to determine potential pro-oxidant effects. Therefore, in order to extend these previous observations, further studies were conducted to examine the potential pro-oxidant effects of red wine. These findings were presented in chapter five using HPLC and two assays to measure pro-oxidant capacity-the hydroxyl radical-mediated deoxyribose degradation assay and the linoleic peroxidation assay. Of the eleven phenolic compounds studied, gallic acid was found to exhibit the highest pro-oxidant

potential in all the tested methods. Red wine was found to exhibit pro-oxidant activity towards deoxyribose, but was a strong inhibitor of linoleic acid peroxidation. The two Fenton systems showed differing activity, with Fe^{3+}-H_2O_2 having a greater oxidising power than AA-Fe^{3+}-EDTA-H_2O_2. Similar to the previous chapters, HPLC analysis showed that the matrix had no effect when the three oxidants were added separately, as both the wine and standards displayed similar results. Increasing the concentration of each oxidant also had minimal effect on phenolic loss after addition of the lowest concentration.

Future work could involve measuring the possible synergistic and/or antagonistic interactions occurring between phenolic compounds in the wine and standards which could affect anti-oxidant/pro-oxidant behaviour. This could be achieved by using different assays such as the DPPH or FRAP (Hidalgo et al., 2010). Potential pro-oxidant species in wine could be isolated using HPTLC-HPLC, and the structures determined using methods such as MS and NMR. Although the effect of dose on the deoxyribose assay was measured, only fixed concentrations of anti-oxidants were tested in the linoleic acid assay. Therefore, the effect of increasing concentrations of anti-oxidant on linoleic acid peroxidation could also have been measured.

In chapter six, the anti-oxidant potential of the three wines and two grape juices in the presence of both metal ions was measured. Both the modified DPPH and $ABTS^{\cdot+}$ assays revealed that Fe^{3+} and Cu^{2+} ions led to a decrease in anti-oxidant activity over time. This decrease was more apparent for Cu^{2+} addition compared to Fe^{3+} in the $ABTS^{\cdot+}$ assay. This technique was a useful method in characterising anti-oxidant activity of phenolic compound-metal chelates, and potential pro-oxidant effects.

Future work could involve using other anti-oxidant assays, such as SOD, FRAP, and ORAC to measure the anti-oxidant activities of these and other varieties of wine and grape juices, in order to gain a more comprehensive view of anti-oxidant activity. Other food extracts could also be tested in order to compare their individual oxidant profiles. In addition, on-line HPLC-coupled anti-oxidant assays, usually DPPH or $ABTS^{\cdot+}$, is another technique which could also be used as a fast, effective, and sensitive alternative to the standard chemical assays (Niederländer et al., 2008). The advantage of this method is that it allows rapid identification of anti-oxidant constituents. Although many studies have used various plant and food extracts using online anti-oxidant activity assays, studies on the use of this technique are relatively few. Nuengchamnong and Ingkaninan, (2010) used an online HPLC-MS-DPPH assay to analyse the anti-oxidant activity of *Antidesma thwaitesianum* Muell. fruit wine. The authors found this assay to be a powerful technique for screening anti-oxidant activity, and characterising the structures of the various phenolic components.

Elemental analyses on the rosé wine, white wine, and grape juices were not performed. Further work could establish the elemental profiles of these samples using ICP-OES in order to determine the native concentration of metals. Other work could investigate the effect of adding other redox-active transition metal ions such as cobalt, chromium, nickel, and cadmium to phenolic compounds, and comparing anti-oxidant activity to redox-inert metals such as zinc. In addition, for the assays ($ABTS^{\cdot+}$, DPPH, hydroxyl radical-mediated deoxyribose degradation, and linoleic acid peroxidation), reaction kinetics of the red wine and phenolic components could have been carried out to determine the mechanism of action of these anti-oxidants, and rate of scavenging of radicals.

Studies such as Iacopini et al., (2008) have highlighted the importance of measuring possible synergistic or antagonistic interactions of different phenolic anti-oxidants which can affect total anti-oxidant capacity. The authors found that quercetin, rutin, and resveratrol exhibited synergistic effects towards $ONOO^-$. However combinations of catechin, epicatechin, quercetin, rutin, and resveratrol generated antagonistic interactions towards DPPH. Knowledge of these interactions could generate information about the effect of matrix on anti-oxidant activity, and could explain the results observed between the red wine and individual standards. Generally, more studies are necessary in order to establish phenolic anti-oxidant interactions.

As with any study using *in vitro* anti-oxidant tests to measure the activity of plant extracts and natural products, it is important to take into consideration how these results translate *in vivo*. Other techniques such as cell culture could be used to further examine the potential anti-inflammatory effects of wine in comparison with other food products. However, although the literature abounds with studies on the effects of anti-oxidants on cells in culture, these results need to be interpreted with caution, as the observed anti-oxidant activity is often the result of artifacts (Halliwell, 2008; Halliwell, 2011). The risk/benefit ratio of all dietary anti-oxidants still warrants further research. In addition, bio-kinetics modelling should be utilised in order to facilitate the development of standardised dosages. This is key, due to contradictory data on the anti-oxidant activity of high-dose and low-dose mixtures (Ndhlala et al., 2010).

This study reinforces the potential health benefits afforded through the anti-oxidants in red wine, but caution must be exerted when extrapolating these observations to complex biosystems. Although this study used millimolar levels of red wine phenolic standards, it is important to note that bioavailability and resulting concentrations of phenolic compounds post ingestion are found at the nanomolar to low micromolar range in

human plasma (Del Rio et al., 2010; Halliwell, 2008). In addition, this study used millimolar concentrations of iron and copper oxidants, which are found at low micromolar levels in plasma. Thus, it is questionable whether such low concentrations of phenolics and metal ions can exert anti-oxidant or pro-oxidant effects *in vivo* (Halliwell, 2009). Further work is needed to determine how phenolic compounds and metal ions interact both *in vitro* and *in vivo*. In addition, the metabolites of these phenolic compounds should be studied further to understand their bioactive effects. In this regard, the development of a screening tool to assess the relative anti-oxidant:pro-oxidant capacities of different foods could be further explored.

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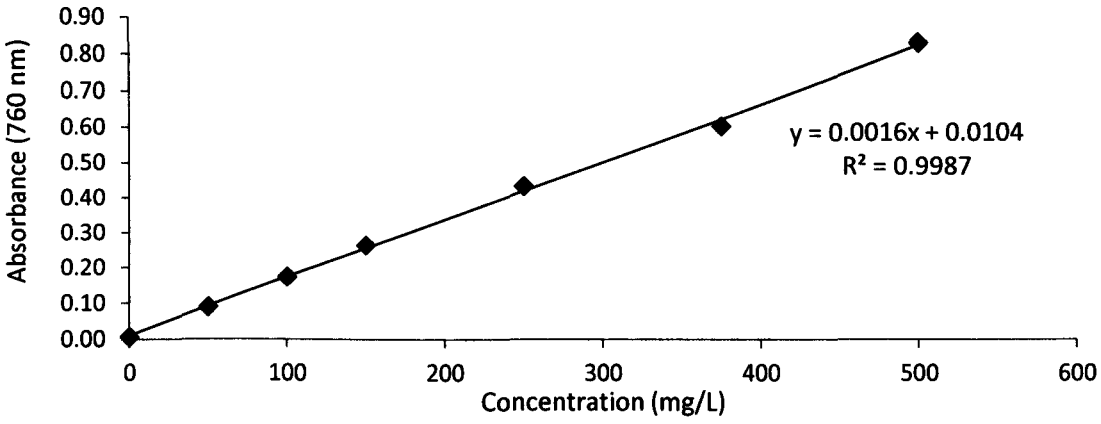
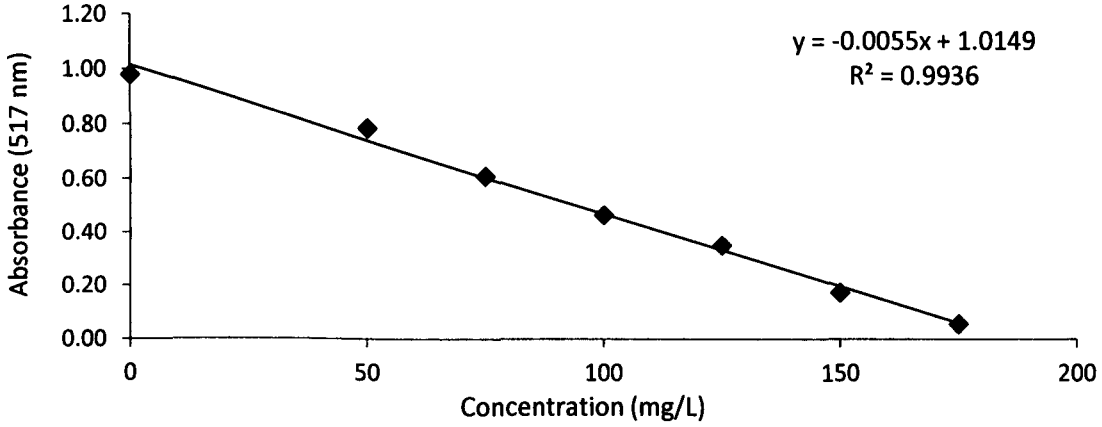
APPENDIX 1

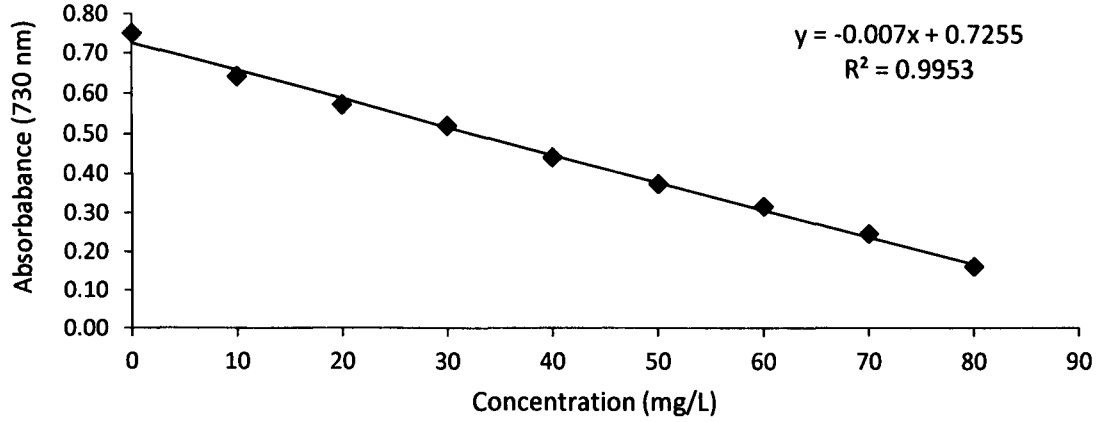
Standard curves for the total phenol, ABTS, and DPPH assays (Chapter 6):



Calibration curve of gallic acid standard in the Folin-Ciocalteau phenol assay.

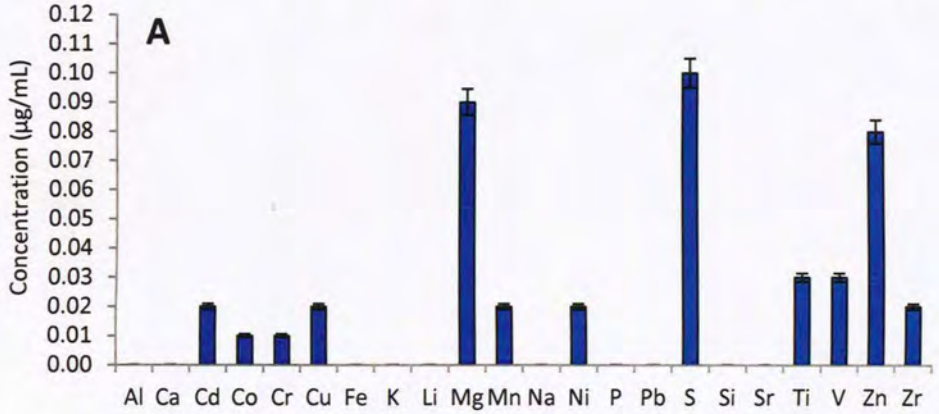


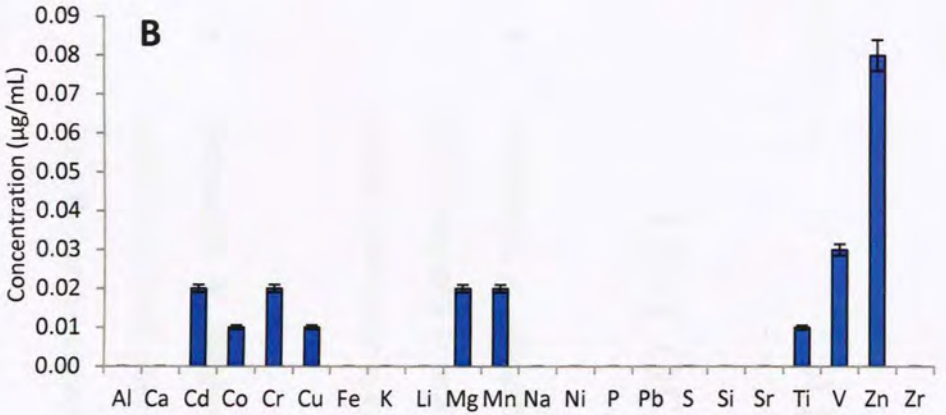
Calibration curve of trolox standard in the DPPH assay.

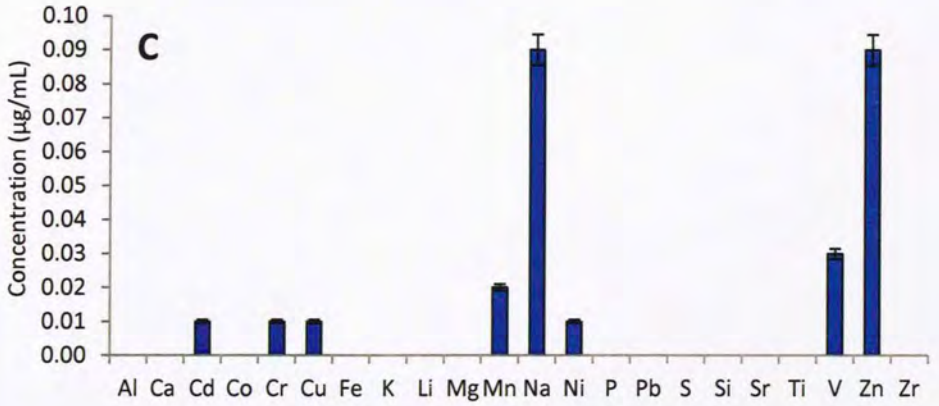


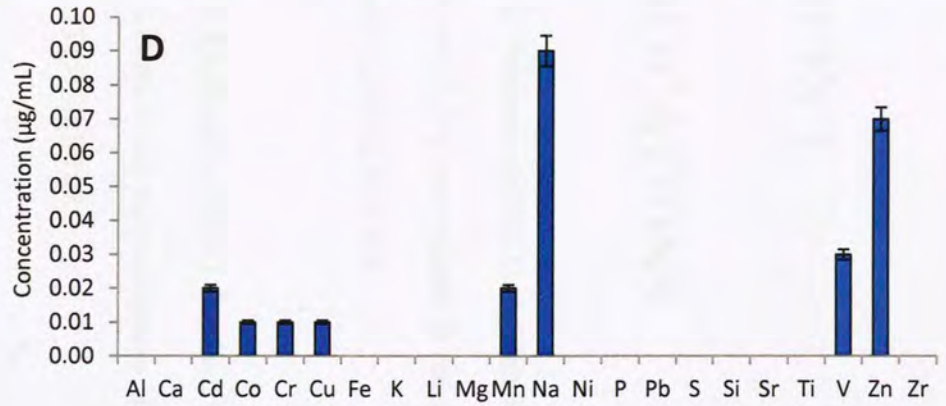
Calibration curve of trolox standard in the ABTS assay.

APPENDIX 2









Quantification of the major and minor elements present in the four standards using ICP-OES showing: (**A**) Gallic acid, (**B**) Caffeic acid, (**C**) *p*-Coumaric acid, and (**D**) Quercetin (Chapter 5).

APPENDIX 3

LIST OF PUBLICATIONS

- Seemungal, A., Naughton, D.P., & Petróczi, A. (2011). Application of thin-layer chromatography to rank the efficacies of five antioxidants in red wine. *Journal of Planar Chromatography-Modern TLC*, 24 (4), 320-324.

- Seemungal, A., Naughton, D.P., & Petróczi, A. (2011). Ranking the efficacies of selected red wine phenolic anti-oxidants using reversed-phase HPLC. *European Food Research and Technology*, 233 (5), 781-789.

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