AGED GARLIC EXTRACT AS AN ANTIOXIDANT IN CARDIOVASCULAR DISEASE

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ABSTRACT

The oxidative modification of LDL by ROS/RNS is recognised as playing an important role in the development and progression of atherosclerosis. Adequate intake of dietary antioxidants such as the antioxidant vitamins, carotenoids, and plant antioxidants such as the polyphenols are important in reducing the risk of development of diseases where the pathogenesis involves free radical-mediated damage to proteins, lipids, and nucleic acids. Antioxidants act to prevent oxidative damage by multiple mechanisms. The non-antioxidant beneficial properties of garlic in preventing cardiovascular disease are well documented and include lowering of blood lipids, reducing platelet aggregation and subsequent thrombus formation, and reducing blood pressure. In this study the antioxidant properties of a commercial aged garlic extract (AGE, Kyolic®) were investigated. AGE displayed the ability to scavenge superoxide in cell free assays, and reduced both 15-lipoxygenase-mediated lipid peroxidation and Cu^{2+}-mediated lipid peroxidation and protein oxidation of isolated human LDL. Two different mechanisms were identified that may be responsible for the observed reduction in Cu^{2+}-mediated oxidation of LDL in the presence of AGE. The first mechanism is the chelation of Cu^{2+} and the second mechanism is the partial preservation of the endogenous LDL antioxidant β-carotene. Chromatographic and colourimetric analyses suggested that AGE is a rich source of phenolic compounds and that they may bind or become incorporated within the LDL particle and this may help to explain the efficacy of AGE in preventing LDL oxidation by preserving β-carotene rather than α-tocopherol levels. Smoking is a human syndrome of increased oxidative stress and an important risk factor for the development of atherosclerotic heart disease. Thus the antioxidant properties of AGE were also identified *in vivo* using non-smoking and smoking human volunteers. A reliable and sensitive novel marker of increased oxidative stress and lipid peroxidation *in vivo* (8-iso-$PGF_{2\alpha}$) and the antioxidant capacity of plasma to scavenge superoxide were used to assess the antioxidant effects of AGE. Smokers had elevated plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ and a decreased antioxidant capacity of plasma to scavenge superoxide when compared to non-smokers. Dietary supplementation with AGE significantly decreased plasma and urinary 8-iso-$PGF_{2\alpha}$ levels in smokers and non-smokers and increased the antioxidant capacity of plasma to scavenge superoxide in smokers. These studies demonstrated that AGE is an efficient antioxidant plant extract *in vitro* and *in vivo* and may be beneficial in reducing oxidative damage to lipid and proteins and thus prevent or reduce the development and progression of free-radical-mediated diseases such as atherosclerosis.

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ABBREVIATIONS

ADP:	Adenosine Diphosphate
AGE:	Aged Garlic Extract
Apo B:	Apolipoprotein B-100
AU:	Arbitrary Units
BMI:	Body Mass Index
COX:	Cyclooxygenase
DADS:	Diallyl Disulphide
DAS:	Diallyl Sulphide
EDTA:	Ethylenediamine Tetraacetic Acid
ELISA:	Enzyme-Linked Immunoassay
Ex:	A Diethyl Ether Extract of AGE
GSH:	Glutathione
GSSG:	Glutathione Disulphide
HDL:	High-Density Lipoprotein
HMG CoA:	β-Hydroxy-β-Methylglutaryl Coenzyme A
HPLC:	High-Performance Liquid-Chromatography
HSA:	Human Serum Albumin
ICAM-1:	Intercellular Cell Adhesion Molecule-1
iNOS:	Inducible Nitric Oxide Synthase
LDH:	Lactate Dehydrogenase
LDL:	Low-Density Lipoprotein
Lp(a):	Lipoprotein (a)
M-CSF:	Macrophage-Colony Stimulating Factor
MDA:	Malondialdehyde
MCP-1:	Monocyte Chemoattractant Protein-1
MTT:	Methylthizol Tetrazoium
NO:	Nitric Oxide
PAF:	Platelet Activating Factor
PBS:	Phosphate Buffered Saline
PG:	Prostaglandin
PMSF:	Phenylmethyl-Sulphonyl Fluoride

PUFA:	Polyunsaturated Fatty Acid
RNS:	Reactive Nitrogen Species
ROS:	Reactive Oxygen Species
SAC:	*S*-allylcysteine
SAMC:	*S*-allylmercaptocysteine
SEM:	Standard Error of the Mean
SOD:	Superoxide Dismutase
SR:	Scavenger Receptor
TBA:	Thiobarbituric Acid
TBARS:	Thiobarbituric Acid Reactive Substances
THF:	Tetrahydrofuran
TLC:	Thin-Layer Chromatography
TXA_2:	Thromboxane-A_2
TXB_2:	Thromboxane-B_2
VCAM-1:	Vascular Cell Adhesion Molecule-1
VLDL:	Very Low-Density Lipoprotein
X-XO:	Xanthine-Xanthine Oxidase

PUBLICATIONS ARISING FROM THIS WORK

Publications arising from the work presented in this thesis. Copies of currently published work are to be found in the Appendices as indicated.

Papers:

Stephanie A. Dillon, Gordon M. Lowe, David Billington and Khalid Rahman. (2002) Dietary Supplementation with Aged Garlic Extract Reduces Plasma and Urine Concentrations of 8-iso-Prostaglandin F2α in Smoking and Non-Smoking Men and Women. *Journal of Nutrition*, 132: 168-171. (Appendix XIII).

Stephanie A. Dillon, Rajpal S. Burmi, Gordon M. Lowe, David Billington, and Khalid Rahman. (2002) Antioxidant Properties of Aged Garlic Extract – An *In Vitro* Study Incorporating Human Low Density Lipoprotein. *Life Sciences*, Submitted.

Abstracts:

Dillon SA., Lowe GM., Billington DA. and Rahman K. (1999) Preliminary evidence to suggest that antioxidant phenolics in aged garlic extract protects against *in vitro* copper oxidation of LDL. *Society for Free Radical Research: BioFlavonoids & Polyphenols in Health & Disease*. Dinard, France, 5th December 1999. (Appendix XIV)

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. CARDIOVASCULAR DISEASE AND ATHEROSCLEROSIS

Cardiovascular disease is the chief cause of death in the USA and Europe with most cases of heart attacks (myocardial infarctions) and many cases of ischaemia (stroke) being secondary to the condition of atherosclerosis (Genest & Cohn, 1995). Epidemiological studies have identified a number of risk factors for the development of atherosclerosis which include elevated serum lipids (cholesterol and triglycerides), increased plasma fibrinogen and coagulation factors, increased platelet activation, alterations in glucose metabolism, and smoking (Frishman, 1998, Wood, 2001). The oxidative modification of low-density lipoprotein (LDL) is now considered an important mechanism in the development of atherosclerosis (Keaney, 2000). Atherosclerosis is a disease of arteries that is characterised by a local thickening of the vessel wall. The effects of atherosclerosis are mainly evident in the muscular arteries e.g. coronary, carotid, femoral and iliac arteries as well as in the aorta.

In general, three types of thickening (atherosclerotic lesion) are recognized (Schwartz et. al., 1991): (1) Fatty streaks are characterized by the presence of foam cells. These are lipid-laden cells that arise from both macrophages and smooth muscle cells. Fatty streaks are the precursors for fibrous plaques. (2) Fibrous plaques often slightly obstruct the vascular lumen and consist of a fibrous cap composed mainly of smooth muscle cells, collagen, elastin and proteoglycans covering an area rich in macrophages, smooth muscle cells, and T-lymphocytes. Often there is a deep necrotic core, which contains debris from dead cells, extracellular lipid deposits and cholesterol crystals. (3) Complicated plaques are fibrous plaques that have been altered by necrosis, calcium deposition, bleeding, and thrombosis. Plaques cause disease by limiting blood flow to an organ such as the heart or brain. A stroke or myocardial infarction occurs when the lumen of an essential artery becomes completely occluded, usually by a thrombus forming at the site of a plaque. Thrombus formation is often triggered by plaque rupture. Plaque disruption occurs most frequently where the fibrous cap is thinnest and most heavily infiltrated by foam cells.

1.1.1. The Oxidative Modification Hypothesis of Atherosclerosis

Evidence implicating oxidative stress in the pathogenesis of atherosclerosis was first reported in 1979 when Brown and Goldstein observed that unlike native LDL chemical modification of LDL in the form of acetylation leads to foam cell formation when incubated with macrophages. The uptake of this chemically modified LDL was shown to take place via saturable, specific receptors termed 'scavenger receptors' (Goldstein et al., 1979). While they could not identify any known endogenous means of LDL acetylation *in vivo*, they did speculate that other modifications of LDL might facilitate recognition of LDL by the scavenger receptor (SR). This prediction was proven as Henriksen and colleagues (Henriksen et al., 1981) found that incubation of LDL with endothelial cells modified LDL in such a way that it served as a ligand for uptake by macrophages and eventual foam cell formation. To date numerous scavenger receptors that share an affinity for oxidised LDL have been identified and characterised which include scavenger receptor class A type I/type II (SR-AI/II), a plasma membrane glycoprotein termed CD36, and scavenger receptor class B type I (SR-BI) (Dhaliwal & Steinbrecher, 1999).

The oxidative hypothesis of atherosclerosis begins initially with an accumulation of LDL in the extracellular sub-endothelial space of arteries. This LDL subsequently becomes progressively oxidised by a number of cell-mediated mechanisms involving reactive species such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), and catalytic metal ions (Fe^{2+} and Cu^{2+}) (Klatt & Esterbauer, 1996). This modified LDL is not subject to normal regulatory mechanisms and is taken up via scavenger receptors expressed on monocyte-derived macrophages present in the sub-endothelial space (Fuhrman et al., 1997a, Henriksen et al., 1981, Steinberg et al, 1990). This unregulated uptake may also occur in smooth muscle cells (Tertov & Orekhov, 1997) and leads to increased cholesterol deposition within these cells, which transform into the foam cells characteristic of the early atherosclerotic lesion (Henriksen et al., 1983, Kritharides et al., 1998).

1.1.2. Structure of LDL

Human LDL is a particle containing both lipid and protein that is isolated by gradient ultracentrifugation between the densities of 1.019-1.063g/ml dependent on the gradient medium utilised (Lowe et al., 1999, Tribble et al., 1992). LDL is spherical with a diameter that ranges between 19 and 25nm, and a molecular weight between 1.8 and 2.8 million. A LDL particle consists of a lipophilic core containing approximately 1600 molecules of cholesteryl ester, and 170 molecules of triglyceride (Esterbauer et al., 1990). The particle surface contains phospholipid, free cholesterol and is embedded with a single apolipoprotein B-100 (apo B). A typical LDL particle contains 2700 fatty acid molecules, about half of these fatty acids are polyunsaturated fatty acids (PUFAs) with the predominant PUFAs being linoleic acid and arachidonic acid, and a small amount of docosahexaenoic acid (Table 1.1).

These PUFAs are highly susceptible to oxidation and are protected by the presence of a variety of lipophilic antioxidants contained in the shell and inner core of LDL (Table 1.1). The major antioxidant is α-tocopherol with approximately 6-8 molecules per LDL particle. All of the other lipid soluble antioxidants are present at amounts <1 molecule per LDL particle. These include γ-tocopherol, ubiquinol-10, β-carotene, lycopene, β-cryptoxanthin, and α-carotene. Since most of these compounds are contained in the diet the content of lipid soluble antioxidants will vary considerably among individuals. More notably, the antioxidative capacity of LDL subfractions markedly differs from each other with the more atherogenic, dense subfractions being more susceptible to oxidation than the less dense ones (Lowe et al., 1999, Tribble et al., 1995).

Table 1.1: Lipid and Antioxidant Composition of Human LDL

Lipid/Antioxidant	nmol/mg LDL protein Mean ± S.D.	mol/mol LDL Mean
<u>Total phospholipids</u>	1300 ± 227	700
Phosphatidylcholine	818 ± 143	450
Lysophosphatidylcholine	145 ± 25	80
Sphingomyelin	336 ± 59	185
<u>Fatty acids</u>		~2700
Linoleic acid	2000 ± 541	1100
Palmitic acid	1260 ± 375	693
Palmitoleic acid	80 ± 44	44
Steric acid	260 ± 118	143
Oleic acid	825 ± 298	454
Arachidonic acid	278 ± 100	153
Docosahexaenoic acid	53 ± 31	29
Free fatty acids	48	26
Triglycerides	304 ± 140	170
Free cholesterol	1130 ± 82	600
Total cholesterol	4090	2200
<u>Antioxidants</u>		
Vitamin E (α+γ-tocopherol)	15.5 ± 2.9	7.95
Ubiquinol-10	0.65 ± 0.28	0.33
β-Carotene	0.53 ± 0.47	0.27
Lycopene	0.41 ± 0.2	0.21
β-Cryptoxanthin	0.25 ± 0.23	0.13
α-Carotene	0.22 ± 0.25	0.11

Adapted from Keaney, 2000

1.1.3. Chemistry of LDL Oxidation

LDL oxidation is principally a free-radical-driven lipid peroxidation chain reaction (Esterbauer et al, 1993, Klatt & Esterbauer, 1996) for which the simplified elementary reactions are shown in Figure 1.1. The oxidation process is started by the attack of an initiating free radical ($X^{\bullet}$) and the abstraction of a hydrogen atom from one of the PUFAs bound to the LDL lipids (LH). This reaction yields a carbon-centred PUFA radical ($L^{\bullet}$) which reacts very quickly with molecular oxygen to yield a lipid peroxyl radical ($LOO^{\bullet}$) which in turn abstracts a hydrogen atom from an adjacent PUFA (LH), yielding a lipid hydroperoxide (LOOH) and a new PUFA radical ($L^{\bullet}$). It is the latter reaction that carries the lipid peroxidation chain. If no chain termination takes place, a single initiating event could convert all LDL PUFAs into lipid hydroperoxides. The actual number of PUFAs oxidised per initiation event depends primarily on the amount of chain-breaking antioxidants contained in the LDL particle. The antioxidants of LDL compete with chain propagation by very efficiently scavenging $LOO^{\bullet}$. This has been demonstrated *in vitro* when isolated LDL is exposed to pro-oxidative conditions (e.g. Cu^{2+}). Before substantial amounts of LOOH are formed LDL becomes depleted of its antioxidants, with α-tocopherol being consumed first and β-carotene last (Esterbauer & Ramos, 1995); this is the lag phase of the process. It is not until the LDL has lost most of its antioxidant compounds that the propagation phase commences and the PUFAs in LDL are rapidly oxidised to LOOH. This is followed by the decomposition phase, when the LOOH breaks down and/or rearranges to form a wide range of products, including aldehydes, ketones, alcohols, epoxides, and F_2-isoprostanes (Bhadra et al., 1991, Brown et al., 1997, Esterbauer et al, 1991a, Moore et al., 1995). As lipid peroxidation proceeds, the lipid radicals and decomposition products attack apo B, which leads to numerous protein modifications such as cross-linking, fragmentation and covalent binding of aldehydes.

Figure 1.1. Elementary Reactions of Lipid Peroxidation

Initiation:	$LH + X^{\bullet}$	$\rightarrow$	$L^{\bullet} + XH$
Oxygen Addition	$L^{\bullet} + O_2$	$\rightarrow$	$LOO^{\bullet}$
Chain Propagation	$LOO^{\bullet} + LH$	$\rightarrow$	$LOOH + L^{\bullet}$
Inhibition by antioxidants	$LOO^{\bullet} + AH$	$\rightarrow$	$LOOH + A^{\bullet}$

LH: lipid containing a PUFA; $L^{\bullet}$: Carbon centred PUFA radical; $X^{\bullet}$: initiating free radical; $LOO^{\bullet}$: lipid peroxyl radical; LOOH: lipid hydroperoxide; AH: antioxidant

Taken from Klatt & Esterbauer, 1996

1.1.4. The F_2-Isoprostanes

The F_2-isoprostanes are isomers of the enzymatically formed prostaglandins. Prostaglandins are formed from arachidonic acid through a cyclooxygenase (COX)-dependent pathway after its release from membrane phospholipids via the action of phospholipases. In contrast, the F_2-isoprostanes are formed *in situ* in membranes following free radical attack on the arachidonic acid and are subsequently released from membrane or LDL phospholipids presumably by the action of phospholipases. They then circulate in the plasma and are subsequently excreted in urine (Morrow et al., 1990a, 1990b, 1992, Pratico et al., 1998). Depending on which of the labile hydrogen atoms is first abstracted by free radical attack up to 64 different F_2-isoprostane isomers, sub-divided into 4 structural classes can be generated (Pratico, 1999). To date the most extensively studied is 8-iso-Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), also known as 8-epi-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III, a class IV member.

8-iso-$PGF_{2\alpha}$ is a biologically active F_2-isoprostane that shares certain properties with conventional prostaglandins. 8-iso-$PGF_{2\alpha}$ causes renal vasoconstriction in rats, potent pulmonary vasoconstriction in rabbits and rats, and constricts coronary arteries with a potency twice that of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Banerjee et al., 1992, Kang et al., 1993, Kromer & Tippins, 1996, Takahashi et al., 1992). 8-iso-$PGF_{2\alpha}$ also causes a dose-dependent irreversible platelet aggregation in the presence of sub-threshold doses of common platelet agonists and increases platelet adhesion by reducing the anti-adhesive and anti-aggregatory effects of NO (Minuz et al., 1998, Pratico et al., 1996). As well as demonstrating potent bioactive properties a large body of evidence has been accumulated indicating that F_2-isoprostanes and more specifically 8-iso-$PGF_{2\alpha}$ can be reliably monitored through non-invasive techniques and yield sensitive and specific markers of lipid peroxidation *in vitro* and *in vivo* (Pratico, 1999).

1.1.5. Proatherogenic Properties of Oxidised LDL

Oxidised LDL elicits numerous pro-atherogenic properties (Figure 1.2) and these include increased smooth muscle proliferation (Auge et al., 1996, Koba et al., 1999, Natarajan, 1995), together with cytotoxicity to vascular endothelial cells, smooth muscle cells, macrophages, and fibroblasts (Cathcart et al., 1985, Clare et al., 1995, Guyton et al., 1995, Sevanian et al., 1995). Oxidised LDL also induces apoptotic cell death in vascular endothelial cells (Claise et al., 1999, Heermeier et al., 2001, Li et al., 1998, Napoli et al., 2000).

The adhesion of monocytes to endothelial cells is mediated by cell adhesion molecules, which include intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Oxidised LDL induces expression of these cell adhesion molecules (Erl et al., 1998, Frostegard et al., 1993). Oxidised LDL is also chemotactic for monocytes via induced expression of monocyte chemoattractant protein-1 (MCP-1) in endothelial cells and prevents monocyte egress from the arterial wall (Cushing et al., 1990, Quinn et al., 1987). Oxidised LDL can stimulate the release of macrophage-colony stimulating factor (M-CSF) from endothelial cells, which then induces monocyte differentiation into macrophages (Liao et al., 1991).

Impairment of endothelium-dependent arterial relaxation is also elicited by oxidised LDL (Deckert et al., 1997, Froese et al., 1999, Murohara et al, 1994). Platelet activating activity has also been demonstrated by oxidised LDL (Heery et al., 1995, Marathe et al., 1999, Takahashi et al., 1996, Vlasova, 2000). These pro-atherogenic properties of LDL are involved in the development and progression of atherosclerotic lesions.

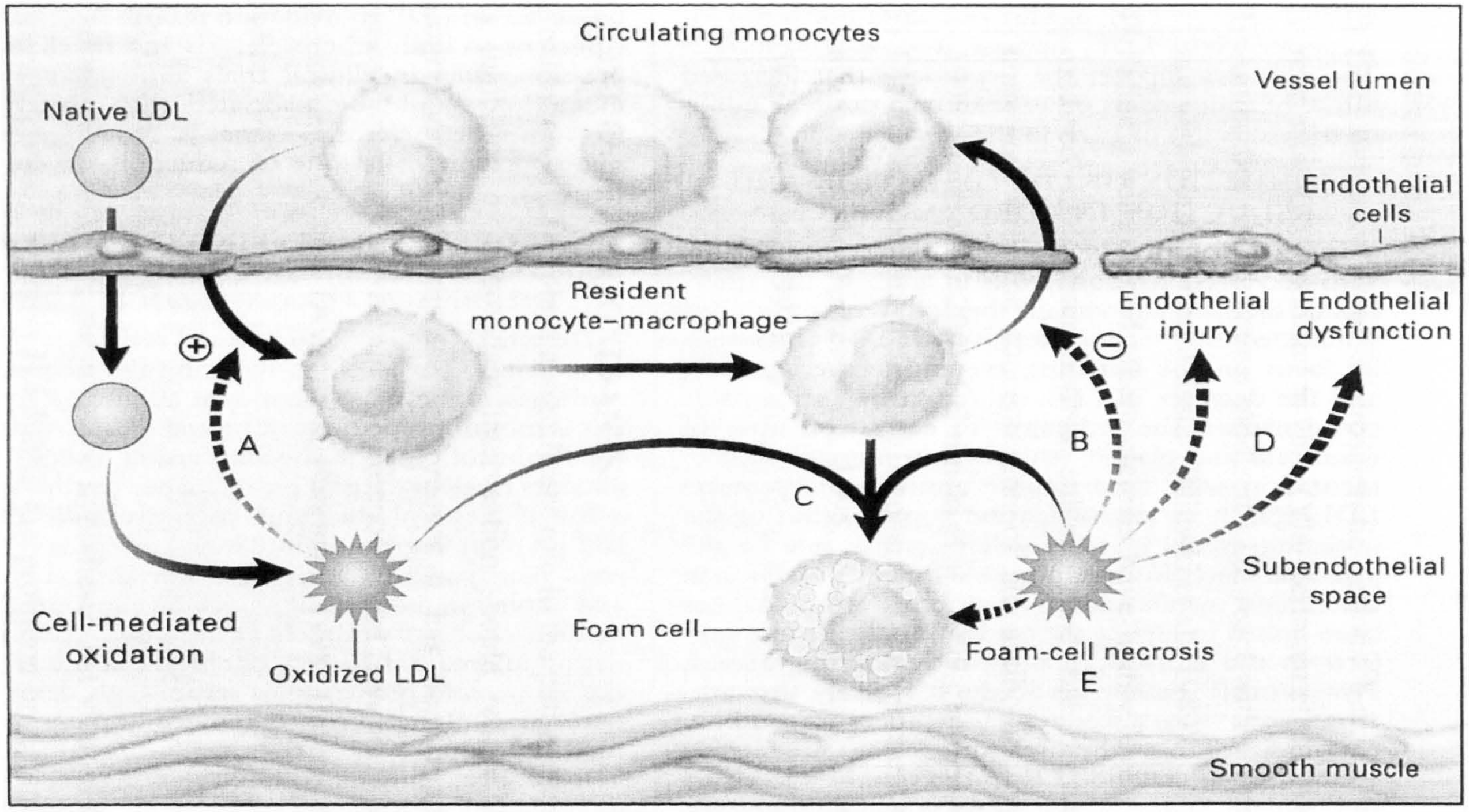


Figure 1.2. Atherogenic Properties of Oxidised LDL

Native LDL becomes trapped in the sub-endothelial space, where it can be oxidised by resident vascular cells such as smooth muscle cells, endothelial cells, and macrophages. Oxidised LDL stimulates (plus sign) monocyte chemotaxis (A) and inhibits (minus sign) monocyte egress from the vascular wall (B). Monocytes differentiate into macrophages that internalise oxidised LDL, leading to foam cell formation (C). Oxidised LDL also causes endothelial cell dysfunction and injury (D) as well as foam cell necrosis (E), resulting in release of lysosomal enzymes and necrotic debris. Taken from Diaz et al, 1997.

1.1.6. Evidence Supporting the 'Oxidative Hypothesis of Atherosclerosis'

There is much evidence consistent with the 'oxidative hypothesis of atherosclerosis'. It includes:

(1) The presence of oxidation products in human and animal atherosclerotic lesions (Breuer et al., 1996, Leeuwenburgh et al., 1997, Oguogho et al., 1999, Suarna et al., 1995).

(2) LDL extracted from atherosclerotic lesions but not plasma-derived LDL resembles LDL oxidised *in vitro* (Yla-Herttuala et al., 1989).

(3) Antibodies raised against oxidised LDL react with atherosclerotic lesions but not with normal arterial tissue (Palinski et al., 1989).

(4) Patients with carotid atherosclerosis have higher levels of autoantibodies to oxidised LDL than normal subjects and the degree of atherosclerosis correlates with the concentration of autoantibodies present (Cheisa et al., 1998, Inoue et al., 2001, Lehtimaki et al., 1999, Salonen et al., 1992).

(5) Dietary antioxidant treatment is consistent with preventing the progression of atherosclerosis (Parker et al., 1995, Sasahara et al., 1994, Sun et al., 1997, Williams et al., 1992).

1.2. DIETARY ANTIOXIDANTS

If oxidation of LDL plays a significant role in the pathogenesis of atherosclerosis, its inhibition by antioxidant intervention should reduce the development and progression of the disease. Moreover it has been reported that nutritional antioxidants such as vitamin E, carotenoids, and flavonoids inhibit LDL oxidation *in vitro* and *ex vivo* (Aviram & Fuhrman, 1998, Bowen et al., 1998, Jialal & Fuller, 1995).

1.2.1. Vitamin E

Considerable evidence supports the hypothesis that vitamin E reduces the risk for atherosclerosis. Vitamin E inhibits the proliferation of smooth muscle cells *in vitro* (Boscoboinik et al., 1991) and increases the resistance of low-density lipoprotein to oxidation when it is added to plasma or administered to humans (Dieber-Rotheneder et al., 1991, Esterbauer et al., 1991b, Fuller et al., 1996, 2000, Mabile et al., 1999). Furthermore, platelets isolated from subjects taking vitamin

E supplements had reduced adhesiveness to collagen and decreased platelet aggregation in response to ADP and arachidonic acid (Calzada et al., 1997, Mabile et al., 1999). Vitamin E supplementation also reduces atherosclerosis in hypercholesterolaemic rabbits (Fruebis et al., 1999).

Plasma levels of vitamin E are significantly lower in patients with coronary artery disease (Delport et al., 1998, Kim et al., 1996) Intake of vitamin E from food or vitamin E supplementation are both inversely related to the risk of and mortality from coronary artery disease (Knekt et al., 1994, Kushi et al., 1996, Rimm et al., 1993, Stampfer et al., 1993) although not all studies have observed a beneficial effect (Ascherio et al., 1999, Klipstein-Grobusch et al., 1999).

1.2.2. Carotenoids

The most abundant carotenoids in human plasma are β-carotene and lycopene. In contrast with other antioxidants, β-carotene and lycopene can inhibit LDL oxidation to a limited degree (Fuhrman et al., 1997b). The protection observed by lycopene was greater than the protection exhibited by β-carotene and both exerted a greater protection when LDL had a high vitamin E content. Dietary supplementation with β-carotene but not lycopene inhibited *in vitro* endothelial cell-mediated oxidation of isolated LDL (Dugas et al., 1999) and β-carotene supplementation normalises the increased susceptibility to oxidation of LDL derived from patients with diabetes mellitus (Levy et al., 2000). It is also important to note that both of these carotenoids exert a hypocholesterolaemic effect by reducing cholesterol synthesis via inhibition of β-hydroxy-β-methylglutaryl coenzyme A (HMG CoA) reductase (Fuhrman et al., 1997c).

Plasma levels of β-carotene are significantly lower in patients with coronary artery disease (Kim et al., 1996). Intake of β-carotene/lycopene from food or β-carotene/lycopene supplementation are both inversely related to the risk of coronary artery disease (Klipstein-Grobusch et al., 1999, Knekt et al., 1994) although, as observed with vitamin E not all studies have observed a beneficial effect (Ascherio et al., 1999, Hennekens et al., 1996, Klipstein-Grobusch et al., 1999).

1.2.3. Plant Polyphenols: Flavonoids

Dietary flavonoids are potent antioxidants and their consumption in the form of red wine or licorice or pomegranate juice by human volunteers for 2 weeks resulted in a significant reduced susceptibility of isolated LDL to lipid peroxidation (Aviram et al., 2000, Fuhrman et al., 1997d, Hayek et al., 1997). These studies also demonstrated that consumption of pomegranate juice, or the red wine polyphenols catechin or quercetin, or the licorice polyphenol glabridin by atherosclerotic apolipoprotein E-deficient mice for 6 weeks resulted in a substantial reduction in the atherosclerotic lesion area, along with a similar reduced susceptibility of their LDL to oxidation. Consumption of flavonoids in tea also reduces the susceptibility of LDL to oxidation (Hodgson et al., 1999b, 2000, Ishikawa et al., 1997).

Intake of flavonoids has been inversely correlated with risk of and mortality from coronary artery disease in the Zutphen Elderly Study and other studies (Geleijnse et al., 1999, Hertog et al., 1993, Knekt et al., 1996). The French Paradox also supports these studies in that despite the pro-atherogenic lifestyle of the Southern French people (high fat diet and smoking tendencies) the incidence of coronary heart disease is relatively low (Renaud & De Lorgeril, 1992). This reduced risk of coronary disease has been attributed to a high consumption of red wine along with a high intake of olive oil and a fresh fruit- and vegetable-rich mediterranean diet. This hypothesis is now controversial as recent evidence suggests that the lower incidence of cardiovascular disease in France is due to a historically lower consumption of animal fat which only recently increased in France but did so decades ago in Britain (Law & Wald, 1999).

It has recently been identified that garlic is also a rich source of antioxidant flavonoids and these include apigenin, myricetin, and quercetin (Miean & Mohamed, 2001). The potent antioxidant activity of a garlic preparation such as aged garlic extract (AGE) may in part be attributed to the presence of a plethora of different antioxidants with differing sites and mechanisms of action (Borek, 2001), which may act alone or in concert with each other. This has been exemplified by lycopene, which synergistically inhibits LDL oxidation in combination with vitamin E, garlic or plant polyphenols (Fuhrman et al., 2000).

This suggests that dietary supplementation with antioxidant plant extracts such as AGE may be more beneficial than supplementation with singular pure antioxidants in reducing the development and progression of multi-risk factor diseases such as atherosclerosis.

Treatment of cardiovascular disease in the western world has involved management of risk factors such as hyperlipidaemia using dietary modification/life-style change and/or drug therapy, and while drug therapy is expensive, compliance with dietary modifications/life-style changes often fails. This has encouraged the current environment, which now promotes self-medication with plant extracts such as garlic, which have numerous beneficial effects on the cardiovascular system (Agarwal, 1996, Rahman, 2001).

1.3. GARLIC: AN HISTORICAL PERSPECTIVE

Garlic (*Allium sativum*) is believed to have originated from Central Asia and to date the genus *Allium* comprises over 600 known species, which include *Allium cepa* (onion), *Allium porrum* (leek), and *Allium schoenoprasum* (chives). The genus *Allium* belongs to the Alliacae family. Garlic is widely cultivated in many countries with the most notable producers being the U.S., Argentina, Mexico, and China and is used universally as a flavouring agent, traditional medicine, and a functional food to enhance physical and mental health. The beneficial effects of garlic consumption in treating a wide variety of human diseases and disorders have been recorded and passed down by many ancient civilisations (Hahn, 1996).

One of the earliest recorded uses of garlic as a spice, condiment, and medicine was provided by the Sumarians and dates back to 2600-2100 BC. Sanskrit texts dated around 500 AD records the use of garlic to treat a wide variety of ailments such as skin diseases, eating disorders, rheumatic conditions, spleen enlargement and haemorrhoids. The Egyptians also new the value of garlic and ancient papyri uncovered during excavations also detailed the seasoning and medicinal properties of garlic. Interestingly, the remains of garlic bulbs were found in the burial chambers of Tutankhamun who was buried in 1352 BC. Records similar to these detailing the uses of garlic are available for many other ancient civilisations

such as the Greeks and Babylonians who acquired their knowledge of garlic from the Egyptians and the Sumarians, respectively. The Romans who gained their knowledge of garlic from the Greeks introduced garlic to Europe where it was accepted and appreciated to a greater or lesser extent. This still remains true today especially in Great Britain were garlic and its somewhat odorous properties are still little appreciated.

Throughout the history of civilisation the medicinal properties of garlic have been greatly valued and have been used to treat a wide variety of human ailments. Yet it is only over the past 10 years that interest has arisen in trying to identify the specific medicinal properties of garlic and, its active principles responsible for these therapeutic effects. Garlic is most commonly used to promote a healthy cardiovascular system due its cholesterol lowering, antithrombotic, antiplatelet, and antihypertensive actions (Agarwal, 1996, Rahman, 2001). These therapeutic actions of garlic may extend beyond the cardiovascular system to reducing the risk of development of certain cancers (Fleischauer et. al., 2000).

1.4. THE COMPOSITION OF GARLIC

The general composition of garlic is shown in Table 1.2. 65% of garlic is water, which is low compared to most fruits and vegetable (80-90%). The bulk of the dry weight is composed of fructose-containing carbohydrates, followed by sulphur compounds, protein, fibre, and free amino acids (Lawson, 1996).

Garlic also contains high levels of saponins, phosphorus, potassium, sulphur, zinc, moderate levels of selenium, and vitamins A and C, and low levels of calcium, magnesium, sodium, iron, manganese and B-complex vitamins. Garlic also appears to have a high phenolic content (12.9μmol/g wet weight) (Vinson et al., 1998). Nearly all of these compounds present in garlic are water-soluble (97%) with a small amount of oil-soluble compounds (0.15-0.7%).

Table 1.2. The General Composition of Garlic

Component	Amount (% fresh weight)
Water	62-68
Carbohydrates (mainly fructans)	26-30
Protein	1.5-2.1
Amino acids: common	1-1.5
Amino acids: cysteine sulphoxides	0.6-1.9
γ-Glutamylcysteines	0.5-1.6
Lipids	0.1-0.2
Fibre	1.5
Total sulphur compounds [a]	1.1-3.5
Sulphur	0.23-0.37
Nitrogen	0.6-1.3
Minerals	0.7
Vitamins	0.015
Saponins	0.04-0.11
Total oil-soluble compounds	0.15(whole)-0.7(cut)
Total water-soluble compounds	97

[a] Excluding protein and inorganic sulphate (0.5%)

Taken from Lawson, 1996.

1.4.1. The Sulphur Compounds of Garlic

Garlic contains unique organosulphur compounds, which provide its characteristic flavour and odour and most of its potent biological activity (Block, 1985). In fact, over 90% of investigations on garlic's active principles have focused on the sulphur compounds. About 85% of the sulphur compounds in whole garlic comprise of alliin and two main γ-glutamylcysteines (Table 1.3). Alliin is considered the parent substance of the therapeutically active sulphur components of garlic. When garlic is crushed, cut, or chewed alliin is exposed to the enzyme alliinase, and the thiosulphinate allicin is formed. Allicin is a reactive intermediate species that can be transformed into a variety of compounds depending on environmental conditions and extraction methods. Of the entire sulphur compounds present in garlic only three minor compounds do not contain the amino acid cysteine (methionine, γ-glutamylmethionine, and thiamine).

1.4.1.1. *γ-Glutamylcysteines: Important Storage Compounds*

Although the cysteine sulphoxide alliin is considered to be the parent substance of many of garlic sulphur compounds it has been demonstrated that the γ-glutamylcysteines may be the precursors for the cysteine sulphoxides (Lawson et al., 1991a, 1991b). These studies showed that cool storage of garlic bulbs for a few months resulted in steady decreases of the γ-glutamylcysteines with almost equimolar increases in the cysteine sulphoxides. This transformation process is due to increased activity of the enzyme γ-glutamyltranspeptidase which slowly hydrolyses the γ-glutamylcysteines to *S*-alkylcysteines followed by rapid oxidation to form cysteine sulphoxides (Figure 1.3) and may function to increase the antibiotic capacity of young garlic plants during wintering and sprouting (Lawson, 1996).

The abundance of the γ-glutamylcysteines is indicated in Table 1.3. The main γ-glutamylcysteines found in garlic cloves are γ-glutamyl-*S*-*trans*-1-propenylcysteine and γ-glutamyl-*S*-allylcysteine (Lawson, 1996). Small amounts of γ-glutamyl-*S*-methylcysteine are also usually found and γ-glutamyl-*S*-*cis*-1-propenylcysteine is barely detectable in fresh garlic cloves, but increases slowly

upon clove storage or boiling of cloves indicating it is formed from the *trans*-isomer.

1.4.1.2. *Cysteine Sulphoxides: Precursors of the Thiosulphinates*

Garlic contains three cysteine sulphoxides capable of producing thiosulphinates, *S*-allylcysteine sulphoxide (alliin), *S-trans*-1-propenylcysteine sulphoxide (isoalliin) and *S*-methylcysteine (methiin). Alliin constitutes the major sulphoxide (85%) while isoalliin (5%) and methiin (10%) are considerably less abundant. Garlic also contains a cyclic cysteine sulphoxide cycloalliin, which cannot be cleaved by alliinase and hence produces no thiosulphinates. The function of cycloalliin is unknown. The cysteine sulphoxide content in garlic is indicated in Table 1.3.

The cysteine sulphoxides are rare in nature and are found almost exclusively in the Alliacae family especially among the genus *Allium*. Alliin however has only been found abundantly in a few Allium species one of these being garlic (Lawson et al., 1991b). The cysteine sulphoxides have no known physiological function other than to be enzymatically transformed by alliinase into biologically active thiosulphinates (Figure 1.3) (Lawson, 1996).

Table 1.3. Total Known Sulphur Compounds in Whole and Crushed Garlic

Compound	Whole Garlic (mg/g fresh weight)	Crushed Garlic (mg/g fresh weight)
<u>Cysteine Sulphoxides</u>		
S-Allylcysteine sulphoxide (alliin)	5-14	nd
S-Methylcysteine sulphoxide (methiin)	0.5-2	nd
S-trans-1-Propenyl cysteine sulphoxide (isoalliin)	0.2-1.2	nd
Cycloalliin	0.5-1.5	0.5-1.5
<u>γ-Glutamylcysteines</u>		
γ-Glutamyl-*S-trans*-1-propenylcysteine	3-9	3-9
γ-Glutamyl-*S-cis*-1-propenylcysteine	0.06-0.15	0.06-0.15
γ-Glutamyl-*S*-allylcysteine	2-6	2-6
γ-Glutamyl-*S*-methylcysteine	0.1-0.4	0.1-0.4
<u>Thiosulphinates</u>		
Allyl 2-propenethiosulphinate (allicin)	nd	2-6
Allyl methyl thiosulphinates [a]	nd	0.3-1.5
Allyl trans-1-propenyl thiosulphinates [a]	nd	0.05-1.0
Methyl trans-1-propenyl thiosulphinates [a]	nd	0.02-0.2
Methyl methanethiosulphinate	nd	0.05-0.1
<u>Others</u>		
γ-Glutamyl-methionine	0.02-0.12	0.02-0.12
γ-Glutamyl-cysteine, oxidized	tr	tr
S-2-carboxypropylglutathione	0.09	0.09
γ-Glutamyl-*S*-allylmercaptocysteine	0.01-0.03	0.01-0.03
S-Methylcysteine	tr	tr
S-1-Propenyl cysteine	nd	nd-0.006
S-Allylcysteine	nd-0.026	nd-0.026
Methionine	0.02	0.02
Thiamine	0.002	nd-0.001
Allithiamine	nd	nd-0.001
Scordinins	0.03	<0.03
Sulpholipids	nd-0.01	nd-0.01
Protein, soluble	0.3	0.3
Protein, insoluble	0.6	0.6
Inorganic sulphate	0.5	0.5

Not detectable (nd), Trace (tr)

[a] Indicates two isomers

Adapted from Lawson, 1996.

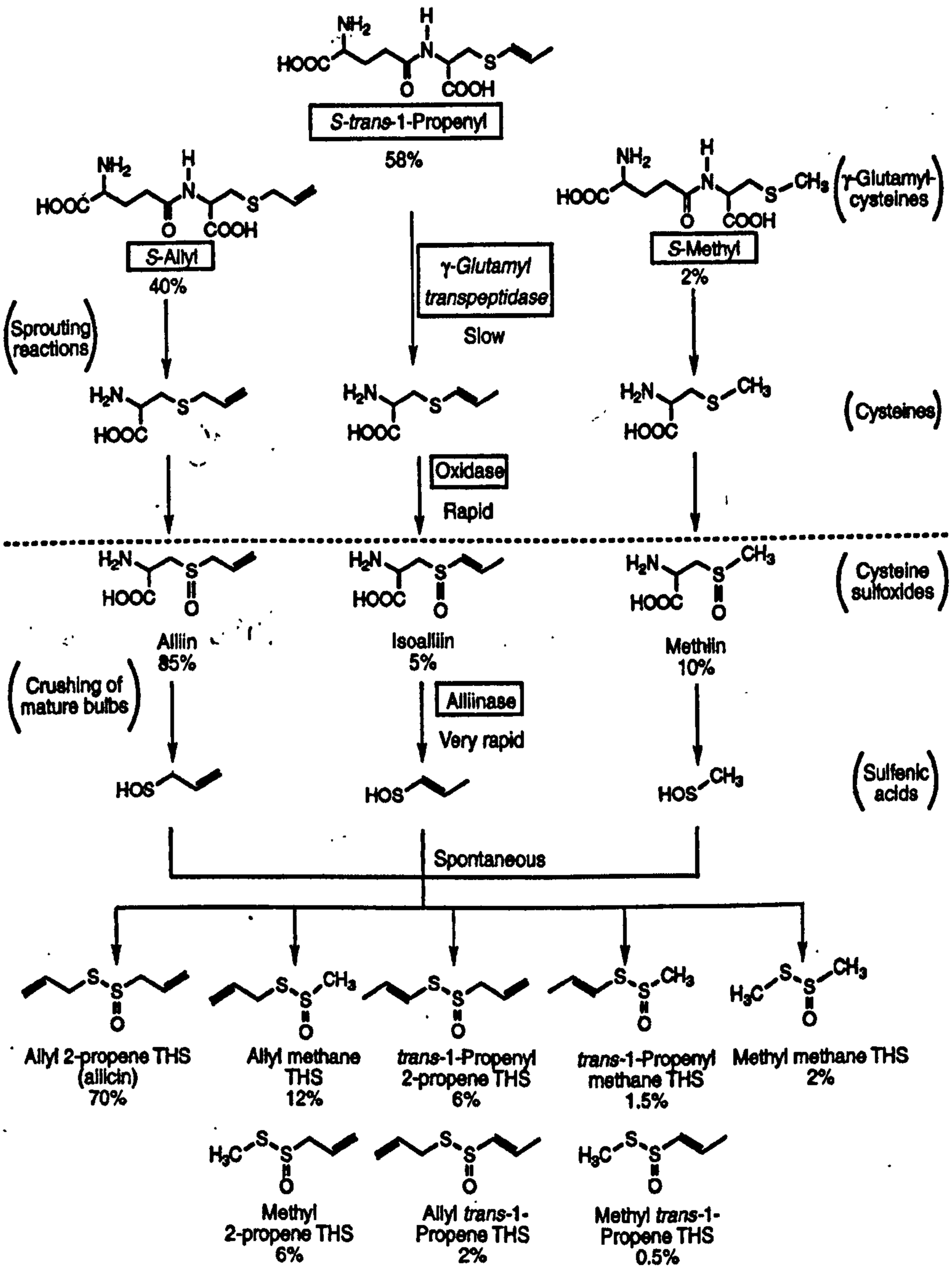


Figure 1.3. Transformation of the γ-Glutamylcysteines and Cysteine Sulphoxides to Thiosulphinates (THS). The γ-glutamylcysteines are slowly hydrolysed to the *S*-alkylcysteines (cysteines) followed by transformation to the cysteine sulphoxides if oxidase activity is present. Sulphenic acids are formed from the cysteine sulphoxides in the presence of alliinase and these spontaneously condense to form the thiosulphinates. Typical weight percentages for compounds found in whole or crushed cloves are shown. Taken from Lawson, 1996.

1.4.1.3. *Allicin and Other Thiosulphinates*

The rapid transformation of the cysteine sulphoxides (alliin, isoalliin, methiin) by allinase in crushed garlic bulbs results in the formation of sulphenic acids, which rapidly condense to form the thiosulphinates (Figure 1.3) (Block, 1985). The abundance of these thiosulphinates in crushed garlic is shown in Table 1.3. Because alliin is much more abundant than the other cysteine sulphoxides, allicin is the main thiosulphinate produced (~70%) while allyl methanethiosulphinate (~12%) is the second most abundant.

Allicin is also thought to be responsible for the usual odour of fresh cut or crushed garlic. The stability of allicin can vary from hours to weeks depending on the storage conditions (Lawson, 1993). Allicin appears to be most stable in water, with a half-life of about 30 days at room temperature and least stable in hexane, with a half-life of 2 hours. Because of this it is assumed that allicin will only be consumed in notable amounts by people who eat raw garlic. Although the pathways of its metabolism in humans are not known in detail and its bioavailability is yet to be proven it is converted to allyl mercaptan and diallyl disulphide (DADS) in the perfused rat liver (Egen-Schwind et al., 1992a) and to allyl mercaptan when incubated with human blood (Lawson & Wang, 1993). The same substances are formed *in vivo* since both are present in human breath after ingestion of raw garlic (Minami et al., 1989, Tamaki & Sonoki, 1999). Allicin decomposes under a variety of conditions and depending on the medium polarity and temperature various transformation products are formed such as the disulphides, vinyldithiins, and mercaptocysteines (Figure 1.4).

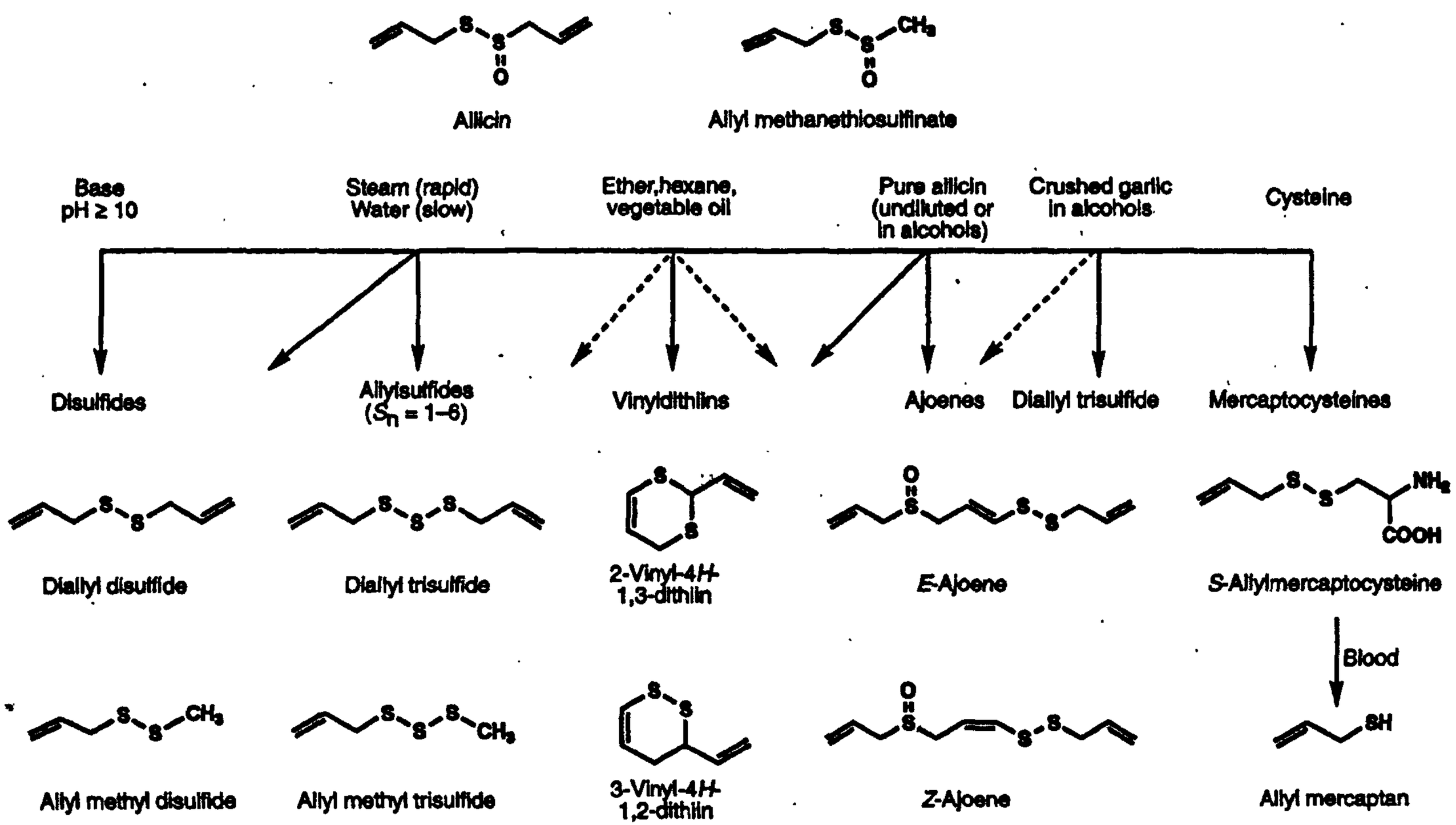


Figure 1.4. Transformation of the Principle Thiosulphinates of Crushed Garlic under Various Conditions. Dashed arrows indicate minor products. Taken from Lawson, 1996.

1.4.1.4. *The S-Alkylcysteines*

Trace amounts of the *S*-alkylcysteines (*S*-allylcysteine, *S*-1-propenylcysteine, and *S*-methylcysteine) have been reported in garlic cloves (Lawson, 1993, Table 1.3). These compounds are thought to be the biosynthetic precursors of the cysteine sulphoxides (Figure 1.3). The content of *S*-alkylcysteines greatly increases when garlic cloves are dried during the manufacture of garlic powders or aged for 3 months or longer in dilute ethanol or water (Lawson, 1993, Lawson & Wang, 1995). These increases are due to the hydrolysis of the γ-glutamylcysteines, which is more efficient and complete when garlic is aged in dilute ethanol or water. This transformation of the γ-glutamylcysteines to *S*-alkylcysteines without further transformation to cysteine sulphoxides is due to the absence of oxidase activity during the ageing process (Lawson & Wang, 1995).

1.4.2. Non-Sulphur Compounds of Garlic

1.4.2.1. *Carbohydrates*

Carbohydrates are the most abundant class of compounds in garlic and account for 26-30% of the wet weight (Lawson, 1996, Table 1.2). The majority of the carbohydrate material in garlic consists of water-soluble fructose polymers called fructans, which constitute approximately 84% of the total carbohydrate material. Besides a carbohydrate storage function, the fructans may play an important role in osmotic regulation and in cold resistance. The known mono- and disaccharides of garlic comprise sucrose (7% dry weight), fructose (1.4%), glucose (1.2%), arabinose, and lactose (both unknown amounts). Raffinose, 1F-fructosylsucrose, and 6G-fructosylsucrose are the three trisaccharides found in garlic. A tetrasaccharide called *scorodose* has been identified in garlic but reports on the content are conflicting.

Cell wall polysaccharides present in garlic comprise galactan, a galacturonan, an arabinan, cellulose, and a xyloglucan. The dry scales of garlic also contain large amounts of the carbohydrate pectin. Pectin from garlic scales is used commercially to make jellies, cosmetics, and pharmaceuticals. The carbohydrate

content of garlic fluctuates throughout the growth of the plant, but reaches its highest levels in the mature bulb.

1.4.2.2. *Enzymes in Garlic*

In addition to the unique abundance of the enzyme *alliinase* which is involved in the transformation of the cysteine sulphoxides to thiosulphinates, and the presence of *γ-glutamyl peptidases* which hydrolyse γ-glutamylcysteines to cysteine sulphoxides other enzymes have been characterised in garlic (Lawson, 1996). These include *adenosine triphosphatase* (ATPase), which is present in all living organisms, *polyphenol oxidase*, which oxidises triphenols and is activated by magnesium and copper, and *phenylalanine-ammonia-lyase* (PAL), which is involved in the synthesis of aromatic compounds in garlic and shows increased activity in the presence of alliin. The antioxidant enzymes *peroxidase*, *catalase*, and *superoxide dismutase* (SOD) are also found in garlic.

In addition, *alcohol dehydrogenase* (ADH), *esterase*, and *phosphoglucose isomerase* have also been identified and their isoenzymes characterised. A whole range of enzymes involved in the synthesis, degradation, and metabolism of carbohydrates has been identified, as well as a species-specific *deoxyribonuclease* and a *lysozyme*.

1.4.2.3. *Protein, Free Amino Acids, and Dipeptides*

The protein content of garlic cloves is 1.5% fresh weight, which represents only 30% of the total nitrogen. The remainder of the total nitrogen is present as free amino acids (25%), cysteine sulphoxides (23%), γ-glutamylcysteines (20%), and γ-glutamylphenylalanine (1.5%) (Lawson, 1996). The free amino acid content of garlic is exceptionally high and is about 65% greater than the total protein. The free amino acids of garlic include nearly all of the common amino acids present in protein (e.g. arginine, glutamic acid, lysine, and serine). The cysteine sulphoxides are equally abundant and the S-alkylcysteines are present in trace amounts.

Garlic also contains a large amount of dipeptides, all of which have been shown to contain glutamic acid with a γ-peptide linkage to the other amino acid. The γ-

glutamylcysteines (Table 1.3) accounts for the majority of these dipeptides; however γ-glutamylphenylalanine, γ-glutamylglycine, and oxidised γ-glutamylcysteine have also been found in garlic bulbs (Lawson, 1996).

1.4.2.4. *Lipids*

Garlic contains only a very small amount of oil-soluble compounds and the lipid content of garlic cloves has been reported to be 1-2mg/g fresh weight (Lawson, 1996). A high proportion of the lipids in garlic are polar (phospholipids and glycolipids). The polar lipids account for 37-60% of the total lipids, and the neutral lipids for 40-63%. The main fatty acid components are linoleic acid (60-65%), palmitic acid (20-30%), oleic acid (3-10%), and α-linolenic acid (3-6%). It is assumed that because of the type of lipids present they are used for structural purposes rather than for energy storage.

1.4.2.5. *Organoselenium Compounds*

The selenium content in fresh garlic is 0.02 mg/100g fresh weight and is drastically less than that of sulphur; however, because selenium belongs to the same family as sulphur it can substitute for sulphur in some organosulphur compounds with a strong preference of selenium binding to the methyl compounds of garlic (Lawson, 1996). Selenium is an important component of endogenous antioxidant systems and recent evidence suggests that low intake/bioavailability contributes to cancers, cardiovascular disease, and infertility (Rayman, 1997). Hence, there is considerable interest in growing garlic in selenium-enriched soils. The resulting increase in selenium concentration in garlic has been shown to provide selenium to selenium-dependant enzymes in animals as well as to enhance the anticancer effects of garlic (Ip et al., 1992, 1994, Ip & Lisk, 1993, 1994)

1.4.2.6. *Polyphenols: Flavonoids and Phenolic Acids*

Considerable interest has arisen in plant polyphenolic flavonoids and phenolic acids due to their antioxidant activity and protective effect against the development of cardiovascular disease and cancer (Rice-Evans, 1995, Visioli et al., 2000a). In spite of this and a recent study which demonstrated that garlic had

a relatively high phenolic content (12.9μmol/g wet weight, Vinson et al., 1998) relatively little work has been reported on the antioxidant activity of these polyphenols in garlic.

Six phenolic acids have been found in peeled garlic cloves whose concentrations total 40μg/g fresh weight and include *p-hydroxybenzoic acid* (13μg/g), *caffeic acid* (10μg/g), *ferulic acid* (7μg/g), *vanillic acid* (6μg/g), *sinapinic acid* (2μg/g), and *p-coumaric acid* (2μg/g) (Lawson, 1996). The scales were found to contain a similar concentration but *p-coumaric acid* was dominant and *p-hydroxybenzoic acid* was absent. *Salicylic acid* has also been found in trace amounts (1μg/g) (Swain et al, 1985). More recently the flavonoids *apigenin, quercetin,* and *myricetin* has been identified and quantified in whole garlic bulbs subjected to acid hydrolysis at concentrations of 217μg/g, 47μg/g, and 693μg/g, respectively (Miean & Mohamed, 2001). *Lignin*, a complex polymer of *p*-propenolphenols is found at 1.6% in garlic scales and serves to provide a rigid wall structure.

1.4.2.7. *Other Non-Sulphur Compounds*

Garlic contains phytosterols that are non-polar compounds with the solubility of lipids. The total sterol content appears to be 18μg/g fresh weight with the dominant sterol being β-sitosterol with lesser amounts of campesterol and cholesterol. Saponins are also present in garlic at 1mg/g. Saponins have strong surfactant (foaming) ability and have a variety of biological activities (Lawson, 1996, Matsuura, 2001).

A wide range of nutritional vitamins and minerals are present in garlic but the amounts are so small they are not important in evaluating the health benefits of garlic consumption. Garlic also contains adenosine, guanosine, and plant hormones such as auxin and gibberellins (Lawson, 1996).

The chemical composition of garlic is extensively varied and interest is clearly focused on the sulphur components of garlic. It is these sulphur compounds and their transformation under different processing conditions that differentiate commercial garlic preparations.

1.5.COMMERCIAL GARLIC PREPARATIONS: PROCESSING AND COMPOSITION

There are many types of commercially processed garlic formulations available. They include powdered dry garlic, oils produced upon treating chopped garlic with steam, vegetable oil, or ether, and aged garlic extracts.

1.5.1. Extract of Garlic Aged in Dilute Alcohol (Aged Garlic Extract)

Aged garlic extract (AGE, Kyolic®) is formulated by soaking sliced raw garlic in 15-20% aqueous ethanol for up to 20 months at room temperature. The extract is filtered and concentrated under reduced pressure at low temperature and is marketed in both dry (tablets and powder capsules) and liquid forms. The liquid form contains 10% (w/v) ethanol.

Whole garlic contains mainly cysteine sulphoxides and γ-glutamylcysteines and the main sulphur constituents of crushed garlic are γ-glutamylcysteines and thiosulphinates (Table 1.3). In comparison, the ageing process described for AGE results in the complete hydrolysis of the main γ-glutamylcysteines; γ-glutamyl-*S*-allylcysteine and γ-glutamyl-*S*-1-propenylcysteine to *S*-allylcysteine (SAC) and *S*-1-propenylcysteine. The oxidation of these S-alkylcysteines to cysteine sulphoxides is prevented due to the absence of oxidase activity during the ageing process (Lawson & Wang, 1995). The SAC content is used to standardise commercial AGE. Two other sulphur compounds that are absent in fresh garlic are also greatly increased: cystine and *S*-allylmercaptocysteine (SAMC). Considerable amounts of alliin are also found and this is thought to be due to complete diffusion of alliin into the medium with little diffusion of the much larger alliinase. Because of this allicin and its oil-soluble transformation products are present only in trace amounts. AGE also contains a Maillard reaction compound; *N*α-(1-deoxy-D-frutos-1-yl)-L-arginine which is formed through a nonenzymatic reaction between reducing sugars and amino acids during the ageing process (Ryu et al., 2001). Numerous steroidal saponins have also been characterised in AGE (Matsuura, 2001).

Whereas other garlic preparations contain mainly the harsh and pungent oil-soluble thiosulphinates and their transformation products, AGE is concentrated in mild and odourless water-soluble sulphur compounds. The thiosulphinate allicin has many unwanted side effects, which include aggravation of the stomach, hepatotoxicity and strong oxidising properties particularly against red blood cells (Egen-Schwind et al., 1992a, 1992b, Freeman et al., 1995, Lawson et al., 1992). Raw garlic and some other garlic preparation have been shown to cause allergic skin reactions, stomach ulcers, other gastrointestinal problems, haemolytic anaemia and other symptoms (Burden et al., 1984, Hoshino et al., 2001, Nakagawa et al., 1980). However, AGE and SAC have not demonstrated such undesirable effects, even at excessive doses (Geng et al., 1997, Nakagawa et al., 1980, Steiner et al., 1996, Steiner & Li, 2001, Yeh & Liu, 2001, Yeh et al., 1995).

1.5.2. Garlic Powder Products

Most commercial garlic powders are prepared by cutting the peeled garlic cloves into small pieces, followed by oven drying at 50-60°C and then pulverization. During this processing of garlic, conversion of some alliin and other cysteine sulphoxides to allicin and other thiosulphinates occur. The more alliin that is converted the greater the odour of the resulting powder. This odour is due to the subsequent conversion of the thiosulfinates to diallyl di- and trisulphides. Even though the sulphides are volatile, significant amounts remain in the powder.

1.5.3. Oil of Steam-Distilled Garlic, Oil of Oil-Macerated Garlic, and Ether-Extracted Oil of Garlic

Oil of steam-distilled garlic is produced upon steam treatment of crushed garlic. This oil contains purely allyl sulphides (allyl, methyl, and 1-propenyl mono- and polysulfides), which are produced from the thiosulphinates during the steam distillation process. Commercial products sold for human consumption are diluted about 200-fold with vegetable oil.

Oil of oil-macerated garlic is formulated by grinding garlic cloves in vegetable oil, followed by isolation of the clear oil. This product contains unique transformation compounds of allicin and other thiosulphinates: the vinyldithiins and the ajoenes, as well as allyl sulphides.

Extraction of the thiosulphinates of crushed garlic with ether followed by evaporation of the ether has been used to produce oil from garlic that has a composition similar to that of oil-macerated garlic preparations.

1.6. THERAPEUTIC EFFECTS OF AGED GARLIC EXTRACT AND OTHER GARLIC PREPARATIONS

1.6.1. Effects on the Cardiovascular System

Epidemiological studies have identified a number of risk factors for the development of cardiovascular disease which include elevated serum lipids (cholesterol and triglycerides), increased plasma fibrinogen and coagulation factors, increased platelet activation, hypertension, alterations in glucose metabolism, and smoking (Frishman, 1998, Wood, 2001). Garlic has been used to treat a variety of cardiovascular problems in many countries for decades and a multitude of studies have been published examining the effect of garlic on the cardiovascular system with a view to preventing or reducing cardiovascular disease.

1.6.1.1. *Cholesterol and Lipid-Lowering Effects*

Blood lipids and lipoproteins circulating in the blood in the form of LDL are decreased in response to treatment with garlic. Various garlic extracts have been shown to reduce diet-induced hypercholesterolaemia in rats and rabbits (Bordia & Verma, 1980, Ismail et al., 1999, Kamanna & Chandrasekhara, 1982, Lata et al., 1991, Slowing et al. 2001). AGE supplementation was effective in lowering plasma cholesterol and triglyceride and LDL-cholesterol in hyperlipidaemic subjects (Lau et al., 1987, Steiner et al., 1996, Yeh et al., 1995). AGE supplementation in normolipidaemic subjects has been reported to have no effect on plasma lipids and lipoproteins (Rahman & Billington, 2000). Supplementation with garlic powder preparations has also been reported to reduce plasma cholesterol and LDL-cholesterol in hypercholesterolaemic subjects (Adler et al., 1997, Jain et al., 1993, Kannar et al., 2001) although similar studies have reported no beneficial effects of garlic powder supplementation (Gardner et al., 2001, Isaacsohn et al., 1998, Simons et al., 1995, Superko & Krauss, 2000). Results for

the effect of dietary supplementation with garlic oil preparations on plasma lipid and lipoprotein levels have also been conflicting (Berthold et al., 1998, Bordia, 1981, Bordia et al., 1998).

Several studies have indicated that the lowering of plasma cholesterol and triglycerides by extracts of garlic and its constituents is due to inhibition of key enzymes involved in cholesterol biosynthesis and fatty acid synthesis in cultured rat hepatocytes and human HepG2 cells (Gebhardt, 1993, Liu & Yeh, 2001, Yeh & Liu, 2001, Yeh & Yeh, 1994). Abuirmeileh et al. (1991) observed lipid-lowering activities of AGE and SAC in conjunction with a reduction in hepatic β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) synthetase and reductase activities in hypercholesterolaemic chickens. Direct measurements of enzyme activity have identified that extract of garlic and various constituents inhibit human squalene monooxygenase and HMG-CoA reductase; enzymes involved in cholesterol biosynthesis (Gebhardt, 1993, Gupta & Porter, 2001). In addition, it has been shown that the more water-soluble sulphur compounds like SAC and S-propenylcysteine present in AGE are less cytotoxic and more efficient in inhibiting cholesterol biosynthesis than the lipid-soluble sulphur compounds like diallyl sulphide (DAS), DADS, and other sulphides (Yeh & Liu, 2001).

1.6.1.2. *Anti-Thrombotic and Anti-Platelet Aggregatory Effects*

Platelet aggregation and subsequent thrombus formation is significantly and efficiently decreased by garlic and its constituents. At low intake (2.4g/d) supplementation with AGE has been shown to increase the threshold levels of collagen- and epinephrine-induced platelet aggregation and platelet adhesion to fibrinogen in healthy individuals (Steiner & Li, 2001). This study also demonstrated that high intake (7.2g/d) of AGE increased the threshold level of ADP-induced platelet aggregation and platelet adhesion to collagen and von Willebrand factor. This inhibition of ADP-induced platelet aggregation after dietary intervention with AGE (5ml/d (equivalent to 1.5g/d)) in normolipidemic subjects has also been observed in another study (Rahman & Billington, 2000). A reduction in collagen- and ADP-induced platelet aggregation was observed in hypercholesterolaemic chickens fed AGE or SAC (Abuirmeileh et al., 1991).

Dietary garlic oil has been shown to inhibit platelet aggregation induced by arachidonic acid, adrenaline, collagen, and calcium and has been shown to decrease the formation of the pro-aggregatory prostaglandin thromboxane-A_2 (TXA_2) in platelets (measured as thromboxane-B_2 (TXB_2)) in coronary artery disease patients (Bordia et al., 1998).

Aqueous extracts of fresh garlic, AGE, and SAC have been shown to reduce cyclooxygenase-mediated TXA_2 (measured as TXB_2) formation by platelets both *in vivo* and *in vitro* animal models of thrombosis and in hypercholesterolaemic chickens (Abuirmeileh et al., 1991, Thomson et al., 2000). Chloroform/acetone extracts of fresh garlic have been shown to inhibit cyclooxygenase activity directly in cell free assays with the acetone extract being more effective (Sendl et al., 1992). In contrast, in this study the chloroform extract of garlic was a more effective inhibitor of ADP- and platelet-activating factor (PAF)- induced platelet aggregation.

1.6.1.3. *Blood Coagulation, Fibrinolysis and Circulatory Effects*

Fibrinolysis is also enhanced by garlic resulting in dissolution of clots and thrombi. Dietary garlic oil has been reported to increase fibrinolytic activity (Bordia & Joshi, 1978, Bordia et al., 1998). Dietary intervention with garlic powder significantly reduces fibrinogen levels and prolongs the prothrombin clotting time of blood in patients with hyperlipoproteinaemia (Harenberg et al., 1988). *In vitro* studies have identified that AGE improves microcirculation and blood properties by preventing lipid peroxidation and haemolysis in oxidised erythrocytes (Moriguchi et al., 2001). In contrast, garlic oil extracts and the allyl sulphides were unable to protect isolated erythrocytes from *t*-butyl hydroperoxide-induced hemolysis (Wu et al., 2001).

1.6.1.4. *Blood Pressure and Vascular Tone*

Dietary supplementation with AGE has been shown to have blood pressure-reducing effects in hypercholesterolaemic subjects (Steiner et al., 1996). A garlic extract has also been shown to modulate the production and function of both endothelium-derived relaxing factor (NO) and constricting factors (endothelin-1) in rat-isolated pulmonary arteries (Kim-Park & Ku, 2000). This observed effect

on NO production has been shown to be selectively regulated where AGE and SAC regulate NO production in macrophages by inhibiting inducible nitric oxide synthetase (iNOS) expression while increasing NO production in endothelial cells (Kim et al., 2001).

1.6.1.5 *Anti-Atherosclerotic Effects*

Oxidation of LDL plays an important role in the initiation and progression of atherosclerotic vascular disease (Keaney, 2000). Oxidised LDL promotes vascular dysfunction by exerting direct cytotoxicity towards endothelial cells, by increasing chemotactic properties for monocytes, by transforming macrophages and smooth muscle cells to lipid-filled foam cells, and by enhancing the proliferation of smooth muscle cells, endothelial cells, and monocytes; all of these events contribute to the development of the atherosclerotic lesion. As well as suppressing oxidation of LDL (Lau, 2001), garlic extracts have been shown reduce many of the pro-atherogenic properties of oxidised LDL.

In a series of studies, Ide & Lau (1999a, 1999b, 2001) assessed the ability of AGE to protect endothelial cells against injury from oxidised LDL. Endothelial cell damage assessed using lactate dehydrogenase (LDH) release as an index of membrane damage, the methylthiazol tetrazoium (MTT) assay for cell viability, and TBARS indicating lipid peroxidation were all prevented by pre-treatment of the endothelial cells with AGE or SAC (Ide & Lau, 1999a, 1999b, 2001). Development of thickened, lipid-filled atherosclerotic lesions in the arteries of rats and rabbits fed a high-cholesterol diet was reduced when the diet was supplemented with AGE, garlic oil, or allicin (Abramovitz et al., 1999, Bordia & Verma, 1980, Campbell et al., 2001, Efendy et al., 1997, Eilat et al., 1995). Campbell et al. (2001) also demonstrated that the presence of AGE inhibited the accumulation of lipids in cultured macrophages and smooth muscle cell proliferation. Garlic powder extracts abolished atherogenic blood serum-induced accumulation of free cholesterol, triglycerides, and cholesteryl esters in smooth muscle cells (Orekhov & Tertov, 1997, Orekhov et. al., 1995). In cells isolated from atherosclerotic plaque, garlic powder extract lowered these lipids and inhibited lipid synthesis in normal cells and cells derived from atherosclerotic lesions. The extract inhibited cholesterol acyltransferase activity hence reducing

cholesterol ester formation and stimulated cholesterol ester hydrolase that degrades cholesterol esters. Garlic powder extract also inhibited the uptake of modified LDL in normal and atherosclerotic foam cells (Orekhov & Tertov, 1997).

1.6.2. Antioxidant Effects

Free radicals and reactive species have been implicated in the pathogenesis of atherosclerotic vascular disease with the oxidation of LDL being an important mechanism (Keaney, 2000). The antioxidative actions of garlic and its constituents have been determined by their ability to scavenge reactive oxygen species (ROS), inhibit lipid peroxide formation and LDL oxidation, and enhance endogenous antioxidant systems.

1.6.2.1. *Scavenging of ROS*

AGE, a water-soluble extract of AGE, and its main constituents SAC, SAMC, and alliin demonstrated the ability to scavenge the ROS H_2O_2 using a cell free system (Ide & Lau, 1999a, Ide et al., 1996). The ability of AGE and SAC to scavenge H_2O_2 may explain the results of an earlier study that observed the presence of AGE and SAC protects vascular endothelial cells from H_2O_2-induced oxidant injury (Yamasaki et al., 1994). AGE and its main constituents have also been observed to scavenge the *t*-butyl hydroperoxide radical and hence prevent lipid peroxidation of liver microsomes (Imai et al., 1994). This study also reported that water extracts of raw and heat-treated garlic enhanced peroxidation of liver microsomes induced by *t*-butyl hydroperoxide. In contrast, a fresh garlic extract (heated or unheated) and allicin has been shown to scavenge the hydroxyl radical ($OH^{\bullet}$) generated by photolysis of H_2O_2 and reduce $OH^{\bullet}$-induced lipid peroxidation in rat liver homogenates (Prasad et al., 1995, 1996). Heat treatment of garlic only slightly reduced its activity. Garlic powder extracts have also demonstrated the ability to scavenge reactive species such as $OH^{\bullet}$ and peroxyl radicals (Aruoma et al., 1997).

1.6.2.2. *Inhibiting Lipid Peroxidation of LDL*

In vitro lipid peroxidation of LDL induced by copper as assessed by the production of TBARS was significantly reduced by the presence of AGE and some of its constituents (SAC, SAMC, alliin, allixin) (Ho et al., 2001, Ide et al., 1997). Dietary supplementation with AGE for seven days has been shown to significantly reduce the susceptibility of subsequently isolated LDL to Cu^{2+}-mediated lipid peroxidation also (Munday et al., 1999, Steiner & Lin, 1998). Interestingly, in this study ingestion of raw garlic had no significant effect on the same perameters. Similar studies have also been performed using garlic powder extracts but results are inconsistent. Supplementation with garlic powder was found to significantly reduce the susceptibility of isolated LDL to oxidation induced by copper (Orekhov et al., 1996, Phelps & Harris, 1993). In contrast, other studies did not observe any effects on the oxidation resistance of LDL after supplementation with garlic powder (Byrne et al., 1999, Simons et al., 1995).

1.6.2.3. *Effects on Endogenous Antioxidant Defences*

In vitro studies have shown that incubation with oxidised LDL depletes intracellular glutathione (GSH) in endothelial cells and macrophages (Ide & Lau, 1999a, 1999b, 2001). These studies demonstrated that pre-treatment of these cells with AGE or SAC prevented GSH depletion. A previous study observed that AGE increases GSH levels in vascular endothelial cells by modulation of the GSH redox cycle specifically increasing glutathione disulphide (GSSG) reductase activity, in addition, an increase in SOD activity was also reported (Geng & Lau, 1997). Garlic supplementation has been shown to prevent the increase in oxidative stress associated with gentamicin-induced nephrotoxicity in rats. Oxidative stress was ameliorated by preserving superoxide dismutase (SOD) and glutathione peroxidase activities (Pedraza-Chaverri et al., 2000). *In vivo* animal studies have been used to investigate the effect of garlic oil supplementation on nicotine-induced lipid peroxidation in rats. Garlic oil supplementation reduced lipid peroxidation as assessed by numerous methods in all the major organs of the rat (heart, lung, liver, and kidney). This decrease in lipid peroxidation was associated with an increase in activity of antioxidant enzymes (catalase, SOD, and glutathione peroxidase) and increased levels of GSH (Helen et al., 1999).

In summary, the overall effects of garlic preparations on the cardiovascular system appear to be profound and varied and include reducing plasma lipids, preventing platelet aggregation and subsequent thrombus formation, and reducing blood pressure. However, the effects observed for commercial garlic powder and oil preparations were inconsistent. The evidence for antioxidant effects of various garlic preparations appears to be less convincing due to lack of *in vivo* human studies demonstrating direct antioxidant activity. *In vitro* and animal studies have demonstrated that AGE possesses substantial antioxidant activity compared with other garlic preparations and intake of AGE by humans is not associated with any undesirable effects as has been observed with other garlic preparations.

1.7. OVERVIEW OF STUDY

In vitro studies have identified the antioxidant properties of AGE with regards to scavenging reactive species, and inhibiting Cu^{2+}-mediated lipid peroxidation of isolated human LDL (Ide et al., 1996, 1997, Ide & Lau, 1999a, Imai et al., 1994). The ability of AGE to scavenge superoxide, inhibit Cu^{2+}-mediated protein oxidation of isolated human LDL, and reduce enzyme-mediated lipid peroxide formation and oxidative modification of LDL has yet to be demonstrated. In this study the ability of AGE to scavenge superoxide and inhibit lipid peroxide formation was investigated using cell-free assays. AGE was then assessed for its ability to inhibit Cu^{2+}- and enzyme-mediated lipid peroxidation and protein oxidation of isolated human LDL. Cu^{2+}-mediated lipid peroxidation of LDL is characterised by a lag phase when endogenous antioxidants (tocopherols and carotenoids) become depleted and a propagation phase when rapid lipid peroxidation occurs (Esterbauer & Ramos, 1995). The ability of AGE to extend this lag phase was investigated and would suggest that preservation of endogenous LDL antioxidants might be occuring.

Other studies have demonstrated that AGE and other plant extracts are able to prevent Cu^{2+}-mediated oxidative modification of LDL, but have failed to address the possibility of the plant extract chelating Cu^{2+} (Fuhrman et al., 1997d, Hodgson et al., 1999b, Ide et al., 1997, Munday et al., 1999). The Cu^{2+} chelating properties of AGE were assessed using a novel approach based on restoration of xanthine

oxidase activity, which is completely inhibited in the presence of Cu^{2+} (Lowe et al., 1998). AGE may protect against Cu^{2+}-induced oxidation of LDL by sparing of endogenous antioxidants such as α-tocopherol and the carotenoids. This hypothesis was investigated by examining α-tocopherol and carotenoid levels during Cu^{2+}-induced oxidation of LDL in the presence and absence of AGE. Plant polyphenols such as flavonoids are potent antioxidants. They have been shown to scavenge reactive species, chelate metal ions, and inhibit enzymes implicated in the pathogenesis of atherosclerosis (Brown et al., 1998, Chang, 1993, Hanasaki et al., 1994, Miller et al., 1996, Robak, 1996). Their consumption by humans significantly reduces the susceptibility of subsequently isolated LDL to lipid peroxidation (Aviram et al., 2000, Belinky et al., 1998, Fuhrman et al., 1997d, Hayek et al., 1997, Hodgson et al., 2000), and the suggested binding of some plant extracts and flavonoids to LDL may explain their effectiveness in inhibiting the oxidation of LDL (Belinky et al., 1998, Grassmann et al., 2001, Hayek et al., 1997, Ivanov et al., 2001, Vinson et al., 1995, 1998). Garlic has also been shown to be a rich source of polyphenols such as flavonoids (Miean & Mohamed, 2001, Vinson et al., 1998). The polyphenolic nature of AGE was investigated and the ability of components of AGE to bind directly to LDL and hence exert a protective effect against Cu^{2+}-mediated lipid peroxidation was assessed.

Human dietary supplementation studies have demonstrated that AGE attenuates many of the risk factors associated with cardiovascular disease and reduces the susceptibility of subsequently isolated LDL to oxidation (Munday et al., 1999, Rahman & Billington, 2000, Steiner et al., 1996, Steiner & Li, 2001). Evidence for direct antioxidant effects of AGE supplementation in humans is lacking. This may be due inadequate methods of assessing lipid peroxidation *in vivo* (Halliwell, 2000, Jackson, 1999). F_2-isoprostanes are a family of compounds formed from arachidonic acid through free radical-mediated lipid peroxidation of membranes and LDL particles (Morrow et al., 1990a, 1990b, Morrow et al., 1992, Pratico et al., 1998). They are increased in human conditions associated with increased oxidative stress and represent reliable and sensitive markers of lipid peroxidation *in vivo* (Davi et al., 1999, Meagher et al., 1999, Pratico, 1999, Reilly et al., 1996,

1998). Due to its potent biological properties the F_2-isoprostane 8-iso-Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) has received much attention (Janssen, 2000, Minuz et al., 1998, Montero et al., 2000). A small clinical trial was constructed using non-smoking and smoking volunteers with the aim of identifying if smokers are subject to increased oxidative stress as evidenced by elevated levels of urinary and plasma 8-iso-$PGF_{2\alpha}$ and a decreased antioxidative capacity of plasma. The effect of AGE supplementation on both of these perameters was then investigated in both non-smokers and smokers.

1.8. AIMS AND OBJECTIVES OF STUDY

The first aim of the study was to investigate the antioxidant potential of AGE *in vitro* using whole AGE and a diethyl ether extract of AGE.

- AGE was assessed for its ability to scavenge superoxide ions generated during the xanthine oxidase mediated breakdown of xanthine to uric acid.
- The effect of AGE on 15-lipoxygenase-mediated lipid peroxidation of linoleic acid was also assessed.
- AGE was subsequently investigated for its ability to prevent oxidation of LDL. Human LDL isolated by density gradient ultracentrifugation was oxidatively modified by both enzymic (xanthine-xanthine oxidase and 15-lipoxygenase) and non-enzymic (Cu^{2+}) methods in the absence and presence of AGE. Measuring thiobarbituric acid reactive substances (TBARS) assessed lipid peroxidation and protein oxidation was assessed by increased electrophoretic mobility on agarose gels.
- In addition, a time course was performed to assess the effect of AGE on the lag phase of Cu^{2+}-mediated lipid peroxidation of LDL.

The second aim of this study was to investigate the properties and mechanisms of AGE using whole AGE and a diethyl ether extract of AGE.

- AGE was assessed for its ability to chelate copper using a novel assay based on the complete inhibition of xanthine oxidase activity by Cu^{2+}.
- In addition, the ability of AGE to preserve endogenous LDL antioxidants (α-tocopherol and carotenoids) during Cu^{2+}-mediated lipid peroxidation was

investigated by extraction of α-tocopherol and carotenoids and subsequent analysis by high-pressure liquid-chromatography (HPLC).

- The polyphenolic nature of AGE was assessed using colorimetric assays and thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC).
- Incubation of whole plasma with AGE prior to LDL isolation and Cu^{2+}-mediated lipid peroxidation was performed to investigate the ability of components of AGE to bind directly to LDL and hence exert a protective effect.

The third aim of this study was to investigate the antioxidant properties of whole AGE *in vivo*.

- A human volunteer supplementation was performed using non-smokers and smokers.
- The aims of this study was to confirm that increased oxidative stress due to cigarette smoking results in elevated levels of plasma and urinary 8-iso-$PGF_{2\alpha}$ and a decreased antioxidative capacity of plasma when compared to non-smokers, and secondly to investigate the antioxidant properties of aged garlic extract *in vivo* by monitoring the effects of AGE supplementation on plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ and the antioxidative capacity of plasma in both smoking and non-smoking individuals.
- Plasma and urinary 8-iso-$PGF_{2\alpha}$ levels were measured using a competitive enzyme-linked immunoassay (ELISA) procedure. The antioxidant capacity of plasma was assessed using a chemiluminescent assay.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals and Enzymes

'Kyolic ®' Aged Garlic Extract (AGE) was supplied by Wakunaga of America Company Ltd (Mission Viejo, CA, USA) and is formulated by soaking sliced raw garlic (*Allium sativum*) in 15-20% aqueous ethanol for up to 20 months at room temperature. The extract is filtered and concentrated under reduced pressure at low temperature. The content of water-soluble compounds is relatively high, while that of oil-soluble compounds is low. The AGE used in this study contained 30.5% extracted solids (305g/L) and S-allylcysteine, the most abundant water-soluble organosulphur compound in AGE, was present at 1.47g/L. Optiprep™, a 60% (w/v) solution of iodixanol with a density of 1.32g/mL, was purchased from Nycomed Pharma, Oslo, Norway. Triglyceride clinical diagnostic kits were purchased from Boehringer Mannheim, East Sussex, UK. Cholesterol and creatinine clinical diagnostic kits were purchased from Sigma-Aldrich Company Ltd., Dorset, UK. Lipoprotein and Lipoprotein (a) gels were purchased from Sebia Hydragels, Issy-les-Maulineaux, France. Optiseal™ polyallomer tubes (11.2mL) were purchased from Beckman Instruments Ltd, Buckinghamshire, UK. Total and free 8-iso-Prostaglandin $F_{2\alpha}$ Enzyme Immunoassay kits were purchased from Assay Design Inc. Ann Arbor, MI48105, USA. Abel® Antioxidant Test kits for Superoxide and other Free Radicals with Pholasin® were purchased from Knight Scientific Ltd., Plymouth, UK. Thin layer chromatography (TLC) plates and all solvents (HPLC grade) were purchased from Merck Ltd, Buckinghamshire, UK. Xanthine oxidase (Grade III from buttermilk), 15-lipoxygenase (Type 1-B from soybean) and all other reagents and chemicals were purchased from Sigma-Aldrich Company Ltd., Dorset, UK.

2.1.2. Preparation of a Diethyl Ether Extract of AGE

Due to the intense dark colouring of AGE, it was necessary to prepare a colourless extract, which did not interfere with some of the spectrophotometric assays. One part AGE was gently mixed with two parts diethyl ether and left to stand at room temperature for 5 minutes. The diethyl ether extract was removed and dried under

a stream of oxygen-free nitrogen gas. The residue was resuspended in phosphate buffered saline (PBS), pH 7.2 to its original volume for all assays unless otherwise stated.

2.2. CELL-FREE ENZYME ANALYSES

2.2.1. Determination of Superoxide Scavenging Ability of AGE

Xanthine oxidase catalyses the oxidation of xanthine which leads to the production of uric acid. The by-products of this reaction are superoxide ions and hydrogen peroxide. Superoxide production and xanthine oxidase activity were measured as cytochrome C reduction and uric acid production, respectively. Xanthine oxidase was prepared to a concentration of 107mU/mL in PBS, pH 7.2, and xanthine was prepared as a 1.6mM solution also in PBS, pH 7.2. Superoxide ions were generated in a reaction volume of 1mL containing 160μM xanthine and 1.25mg cytochrome C. The reaction was initiated by the addition of 10.7mU xanthine oxidase, and superoxide ion production was monitored at 550nm (Edwards et al., 1987). In a series of separate experiments, xanthine oxidase activity was monitored as the production of uric acid at 284nm (Mondal & Mitra, 1994). Generation of superoxide ions was confirmed by the addition of 50U superoxide dismutase (SOD), which inhibited the reduction of cytochrome C without affecting xanthine oxidase activity (Figure 2.1). Whole AGE could not be used in experiments monitoring uric acid production due to excessive interference at 284nm. If the diethyl ether extract of AGE should scavenge superoxide ions then the reduction of cytochrome C would be inhibited without affecting xanthine oxidase activity (i.e. uric acid production). AGE or the diethyl ether extract of AGE was added at 0-10% (v/v). The presence of 3mM ascorbic acid, a known scavenger of superoxide from this source (Nishikimi, 1975), completely inhibited the reduction of cytochrome C. Results for superoxide production are expressed as ΔA550nm/min, whilst, results for uric acid production are expressed as Δ A284nm/min.

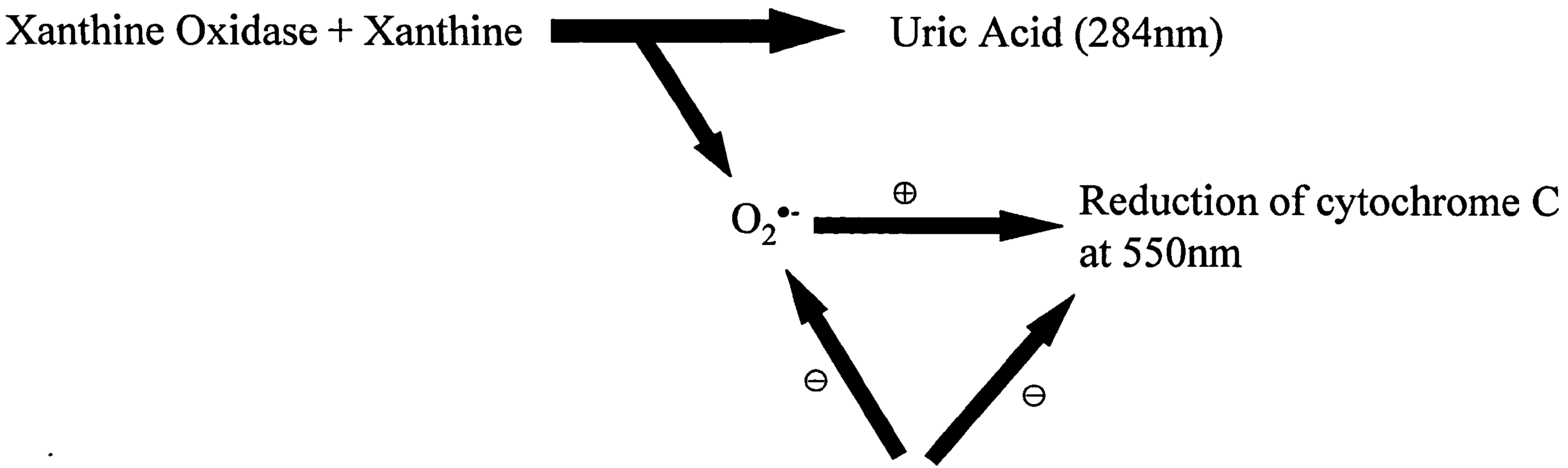


Figure 2.1. Generation of Superoxide during the Xanthine Oxidase Catalysed Breakdown of Xanthine to Uric Acid. Superoxide scavengers such as superoxide dismutase (SOD) would inhibit the reduction of cytochrome C without affecting xanthine oxidase activity monitored as uric acid production.

2.2.2. Determination of Cu^{2+} Chelation by AGE

The Cu^{2+} chelating properties of AGE were assessed using a novel approach based upon restoring the activity of xanthine oxidase. This enzyme is completely inhibited in the presence of 50μM $CuSO_4$ (Lowe et al., 1998). Monitoring either the production of the superoxide ions or the formation of uric acid can assess the activity of xanthine oxidase. If whole AGE or the diethyl ether extract of AGE should chelate Cu^{2+} then xanthine oxidase activity would be restored. AGE or the diethyl ether extract of AGE was added at 0-10% (v/v) along with 50μM $CuSO_4$. Additionally, in separate experiments 60μM EDTA (ethylenediamine tetraacetic acid) was added along with 50 μM $CuSO_4$ as a positive control. Results for superoxide production are expressed as ΔA550nm/min, whilst, results for uric acid production are expressed as ΔA284nm/min.

2.2.3. Assessment of Inhibition of Lipid Hydroperoxide Formation by AGE

15-Lipoxygenase is an intracellular enzyme that adds oxygen to polyunsaturated fatty acids (PUFAs) to yield lipid peroxyl radicals (Yamamoto, 1992). This lipid peroxyl radical abstracts a hydrogen atom from an adjacent PUFA yielding a lipid hydroperoxide and a carbon-centred PUFA radical. This lipid peroxidation process can be monitored via molecular rearrangement of the carbon-centred radical to form a conjugated diene compound exhibiting UV absorbance at 234nm.

15-Lipoxygenase was prepared to a concentration of 10,000U/mL in 0.2M borate buffer (pH 9). Linoleic acid was solubilized in absolute ethanol and prepared as a 0.6μM substrate solution in 0.2M borate buffer (pH 9) with the final ethanol concentration being 0.01% (v/v). The generation of hydroperoxy lipids was performed in a reaction volume of 3mL containing 500U 15-lipoxygenase and 2mL of 0.6μM linoleic acid. The reaction was monitored by the formation of conjugated dienes at 234nm (Esterbauer et al., 1989), and results are expressed as ΔA234nm/min.

Only the diethyl ether extract of AGE was added at 0-10% (v/v). The presence of 1.5mM Trolox®, a more polar analogue of vitamin E, completely inhibited the formation of lipid hydroperoxides.

2.3. *IN VITRO* LDL OXIDATION ASSAYS

2.3.1. Isolation of Human LDL

Ethical approval for the use of human volunteers was obtained from the John Moores University Ethics Committee. Venous blood was drawn from healthy human volunteers and 9 volumes of blood were added into 1 volume of 3.8% (w/v) sodium citrate solution as anticoagulant. Plasma was obtained by centrifugation at 2000g for 20 minutes. LDL was isolated using a method developed by Graham et al (1996) and subsequently modified by Lowe et al (1999).

Plasma obtained was mixed with Optiprep™ (60% (w/v) iodixanol) to give a 12% (v/v) solution of iodixanol; 5mL of this was layered under 5mL of 6% (v/v) solution of iodixanol in saline in an 11.2mL Optiseal™ tube. The tube was topped up with saline and the gradient was ultracentrifuged at 402,000g and 16°C for 3h 10 minutes using a VTi 65.1 rotor. The brightly coloured LDL band was located approximately one third of the way down the tube and was removed with a cannula. LDL was characterised as previously described in Lowe et al (1999) by measuring the amount of protein, triglycerides, and cholesterol present and by measuring the electrophoretic mobility of lipoprotein fractions (Papadopoulos, 1985).

2.3.2. Assessment of the Ability of AGE to Inhibit Oxidation of Human LDL

LDL was used at 200μg protein/ml and was oxidatively modified by both enzymic and non-enzymic methods. LDL was incubated for 5h at 37°C in the presence of 10μM $CuSO_4$, or 16mU xanthine oxidase and 240μM xanthine. In another set of experiments LDL was incubated for 16h at 37°C in the presence of 500U 15-lipoxygenase. These incubations and relevant controls were performed in the presence of 5% and 10% (v/v) whole AGE or 5% and 10% (v/v) diethyl

ether extract of AGE. In addition, a time course over 5h was performed for Cu^{2+}-mediated oxidation of LDL in the presence and absence of the diethyl ether extract of AGE. Samples were removed every 30 minutes and the reaction was terminated by rapid freezing. Samples were stored at –20°C for no longer than 48h prior to analysis for TBARS, or stored at 4°C for no longer than 24h prior to electrophoretic analysis.

2.3.2.1. *Thiobarbituric Acid Reactive Substances (TBARS) Assay*

Malondialdehyde (MDA) is a product of lipid hydroperoxide decomposition and is subsequently used an as index of lipid peroxidation (Esterbauer & Cheeseman, 1990). The MDA concentration of a sample can by measured using the TBARS assay (Mao et al, 1994). This assay is based on a reaction between MDA and thiobarbituric acid (TBA) which produces a pink complex with an absorbance maximum at 532nm.

Protein was precipitated by the addition of 0.5mL of 20% (w/v) trichloroacetic acid (TCA) to 1mL of LDL suspension. Following centrifugation 1mL of 1% (w/v) thiobarbituric acid (TBA) was added to 1mL of the supernatant; samples were then heated to 95°C for 10 minutes, and upon cooling the absorbance was read at 532nm. Hydrolysed tetraethoxypropane was used as the standard. This was prepared as a 10mM solution in 1% (v/v) H_2SO_4; following hydrolysis at room temperature for 2h; the solution was diluted accordingly in PBS and a calibration curve (0-10 nmoles MDA, Appendix I) was constructed using the TBARS assay described. TBARS production could not be used to assess the antioxidant potential of whole AGE as it interfered with this colorimetric assay.

Results are expressed as nmoles of MDA/mg LDL protein. Where appropriate MDA production (nmoles/mg LDL protein) was plotted versus time and lag times were determined from the interception of linear trend lines drawn through the lag phase and propagation phase of Cu^{2+}-mediated lipid peroxidation of LDL. Where there was no observable propagation phase the lag time was taken at the end of the experiment, this being 300 minutes.

2.3.2.2. *Electrophoresis of LDL*

On agarose gels, lipoproteins separate based on overall electronegative charge into the following fractions (in order of increasing mobility and increasing overall electronegative charge, see also figure 2.2):

- The chylomicrons: these are very large lipoproteins with high triglyceride content. They normally remain at the application point.
- The beta lipoproteins or LDL.
- The pre-beta lipoproteins or very low-density lipoproteins (VLDL). They have a molecular weight higher and a density lower than LDL. They are more mobile than LDL.
- The fast pre-beta lipoproteins: this fraction is composed of lipoprotein (a) (Lp(a)) which is similar in size and composition to LDL. Lp(a) when present at a sufficiently high concentration, can be seen migrating between VLDL and HDL.
- The alpha lipoproteins or high-density lipoproteins (HDL): they are the fastest fraction.

The electrophoretic mobility of lipoproteins was in part used to characterise the LDL fraction (Papadopoulos, 1985). Electrophoretic mobility of LDL samples was also used as an indication of protein oxidation. Oxidised LDL has a greater electronegative charge and a greater mobility than native LDL on agarose gels. This is primarily due to oxidative modification of the apo B protein in LDL.

These analyses were performed using Sebia hydragel lipo + Lp (a) gels. Samples (2 or 4μL) were electrophoresed at a constant 27mA/gel for 80 minutes, then oven dried at 85°C and stained with Sudan Black for 15 minutes. The electrophoretic mobility of LDL oxidatively modified in the presence and absence of whole AGE or the diethyl ether extract of AGE was compared to that of native LDL.

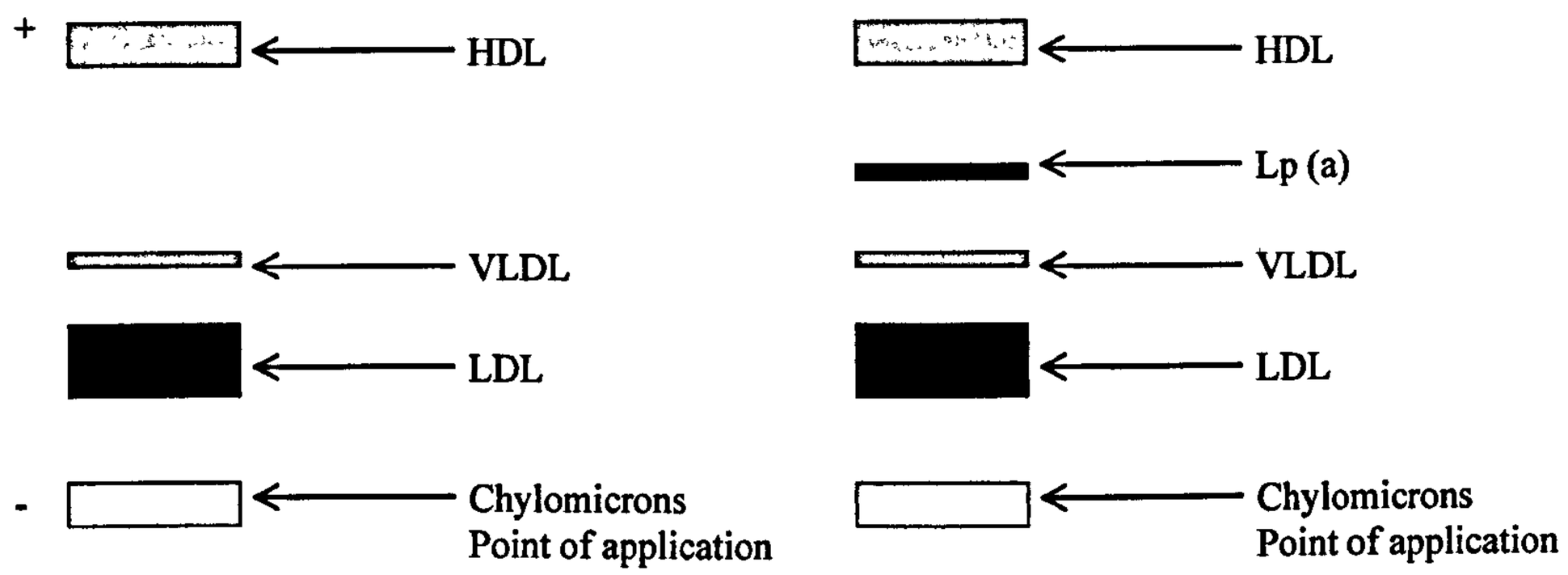


Figure 2.2. The Electrophoretic Profile of Plasma Lipoproteins. Depending on the sample composition, the following lipoprotein bands can be observed. Chylomicrons remain at the point of sample application and would not necessarily be observed if fasting samples were collected. LDL are the slowest fraction after chylomicrons and HDL are the fastest fraction.

2.3.3. Determination of LDL Binding Capacity of AGE

Human plasma was incubated at 37°C for 1h in the presence of 1mM phenylmethyl-sulphonyl fluoride (PMSF) to inhibit any protease activity and whole AGE or the diethyl ether extract of AGE. Whole AGE was included at 1%, 3%, and 5% (v/v) and the diethyl ether extract of AGE was included at 5% and 10% (v/v). Controls contained plasma and PMSF. After 1h incubation plasma samples were removed and LDL was isolated. Isolated LDL used at 200μg protein/mL was subsequently oxidised with 10μM $CuSO_4$. Samples were removed every 30 minutes and the reaction was terminated by rapid freezing. Samples were stored at –20°C for no longer than 48h prior to TBARS and subsequent lag time analysis.

2.3.4. Assessment of Endogenous LDL Antioxidant Preservation by AGE

LDL was isolated as previously described. Whole LDL (0.5mL) was subsequently incubated for 4h at 37°C in the presence of 100μM $CuSO_4$. These incubations and relevant controls were performed in the presence 10% (v/v) diethyl ether extract of AGE (this extract was re-suspended in 250μL PBS). Samples were removed at 0h, 1h, 1.5h 2h, and 4h and the reaction was terminated by rapid freezing. Samples were stored at –20°C for no longer than 48h prior to TBARS analysis or extraction of antioxidants.

2.3.4.1. *Extraction of α-Tocopherol and Carotenoids from LDL*

α-Tocopherol and carotenoids were extracted from LDL as described by Lowe et al. (1999). LDL sample (0.5mL) was thoroughly defrosted followed by addition of 1mL ethanol to precipitate protein. 1.5mL dried diethyl ether was then added, followed by addition of 1.5mL hexane. Samples were briefly vortexed in between addition of solvents. The sample-solvent mixture was then left to settle and the upper layer (slight orange colour) was removed with a glass pasteur pipette. The upper layer was then dried down slowly under a stream of oxygen-free nitrogen gas. Prior to HPLC analysis the residue was either stored at 4°C in the dark or re-suspended in 200μL ethanol: tetrahydrofuran (THF) (9:1, v/v) and vortexed briefly.

2.3.4.2. *Analysis of α-Tocopherol and Carotenoids by HPLC*

HPLC analysis of α-tocopherol and carotenoids was performed as described by Lowe et al (1999). A Rheodyne injection system (model 7125) was used to inject 20μl of sample onto an ODS2 reversed-phase column (Spherisorb 25.0 x 0.46cm, 5μM particles). α-Tocopherol and the carotenoids were eluted using an isocratic solvent system comprising acetontrile:THF:methanol (65:22:13 v/v/v), with 0.33g/L ammonium acetate at 1mL/min using a LKB Pharmacia 2165 pump (LKB Pharmacia, Uppsala, Sweden). A Spectra Physics (Mountain View, CA View, USA) Spectra Focus scanning wavelength detector detected α-tocopherol and carotenoids at 290nm and 460nm, respectively. Integration was performed using Spectra Physics Software. The arbitrary units (AU) determined for α-tocopherol and carotenoids were standardised to LDL protein concentration.

2.4. ANALYSIS OF PHENOLIC COMPOUNDS IN AGE

2.4.1. The Folin-Ciocalteu Phenol Assay

Folin-Ciocalteus' phenol reagent reacts with phenols and non-phenolic reducing substances to form chromagens that can be detected spectrophotometrically (Ohnishi & Barr, 1978). These chromagens exhibit an increased absorbance between 550-750nm.

The dried down diethyl ether extract of AGE was re-suspended in 200μL PBS, pH 7.2 for subsequent analysis. To measure free phenols in whole AGE or the diethyl ether extract of AGE, 0.5mL of either AGE or its extract was added to 5mL 50% (v/v) aqueous methanol. For estimation of total phenols whole AGE or the diethyl ether extract of AGE was subjected to acid hydrolysis with 3.75mL 50% (v/v) aqueous methanol + 1.25mL concentrated hydrochloric acid. Controls contained 0.5mL whole AGE or the diethyl ether extract of AGE added to 5mL distilled water. All samples were then incubated at 90°C for 2h. The extracts were allowed to cool, filtered through a 0.45μM pore, stored at 4°C and used the same day.

The whole AGE filtrate (100μL) was made up to 0.5mL volume with distilled water and the diethyl ether extract of AGE filtrate was used neat for subsequent analysis. The filtrates (0.5mL) of whole AGE and the diethyl ether of AGE were incubated for 10 minutes at room temperature with 5.0mL alkaline reagent (alkaline reagent = 1mL 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) aqueous sodium citrate added to 50mL 2% (w/v) $NaCO_3$ in 0.1M aqueous NaOH). Folin-Ciocalteu reagent was diluted 10-fold with distilled water and 0.5mL was added to the samples. The samples were gently mixed and incubated at room temperature for 30 minutes and the absorbance read at 660nm. Catechin was used as the standard. This was prepared as a 1mg/mL solution and a calibration curve was constructed (Appendix II). The phenolic content of AGE extracts was expressed as mg/mL. To calculate the amount of conjugated phenols present in the AGE extracts the amount of free phenols was subtracted from the amount of total phenols present.

2.4.2. TLC Analysis

Whole AGE was used as supplied and the dried diethyl ether extract of AGE was re-suspended in 200μL methanol. In addition, acid hydrolysed extracts were prepared. Whole AGE was incubated with 2M hydrochloric acid (1:1, v/v) for 1h at 100°C. The extracts were allowed to cool, filtered through a 0.45μM pore, stored at 4°C and used the same day. A diethyl ether extract of this acid-hydrolysed whole AGE was also prepared as previously described and re-suspended in 200μL methanol.

The untreated and acid-hydrolysed AGE extracts were used for TLC analysis. Standard compounds were dissolved in methanol to a concentration of 1mg/mL. Whole AGE (10μL), 30μL diethyl ether extract of AGE, and 10μL standards were loaded onto silica gel 60 F_{254} TLC plates and left to dry. The plates were run for ~1h in an ethylacetate:methanol:water (10:2:1, v/v/v) solvent system. The plates were then dried for 15 minutes at 115°C.

The plates were left to cool then visualised using UV light or ferric chloride spray reagent (2.7% w/v in 2M HCl). Viewing the plates under UV light displays

phenolic compounds with various fluorescence colours, the most common being blue and violet. Fine spraying of the TLC plate with ferric chloride and then heating the plate at 95°C for 15 minutes displays phenolic compounds with brown, pink, and blue colours. In both instances, the distance moved by the solvent front and the sample/standard bands (centre of the band) were measured in millimetres. The distance moved by the sample/standard was divided by the distance moved by the solvent front to obtain the R_f value.

2.4.3. HPLC Analysis

HPLC analysis of phenolics was performed using whole AGE diluted 1/50 with methanol and diethyl ether extract of AGE re-suspended in 1mL methanol. A Waters 2960 Separation Model HPLC (injection system and pump) was used to inject 10µl of sample onto a Waters symmetry C18 column (15.0 x 0.46cm, 3.5µM particles). Column temperature was 30°C. Phenolic compounds were eluted at 1mL/min using an gradient solvent system comprising which started with 5% solution A (acetonitrile + 0.1% (w/v) trifluoroacetic acid) and 95% solution B (water + 0.1% (w/v) trifluoroacetic acid) gradually changing to 80% solution A and 20% solution B over 20 minutes. A Waters 996 photo diode array detector was used to analyse phenolic compounds at 280nm or 200nm-400nm. Integration was performed using Waters Millennium software package.

2.5. *IN VIVO* HUMAN STUDIES

2.5.1. Clinical Trial Design and Subjects

Apparently healthy subjects who were not taking medication for any known disease participated in the study which had the prior approval of the Ethics Committee of Liverpool John Moores University. The information sheet used to recruit volunteers is displayed in Appendix III. They were divided into two groups, non-smokers (n = 10; 5 men, 5 women, mean age 41.6 ± 4.0) and smokers (n =10; 5 men, 5 women, mean age 41.0 ± 4.1). All subjects answered a simple questionnaire about their age, height, weight, average weekly alcohol consumption, and number of cigarettes smoked per day (Appendix IV). Body mass index (BMI) was determined as weight (kg)/height^2 (m^2). On day 0 all

subjects provided 10mL samples of mid-stream urine and venous blood between 10.00h and 12.00h. Subjects then consumed 5mL of AGE daily for 14 days between 0700h and 0900h; otherwise, subjects followed their usual diet and lifestyle, including alcohol intake. On day 14 blood and urine samples were collected in exactly the same way. All subjects then discontinued taking AGE and a final set of blood and urine samples were collected on day 28 of the trial (i.e. 14 days after cessation of AGE supplementation). All blood samples were collected into 100U heparin and plasma was obtained by centrifugation at 2000g for 20 min. Plasma and urine was stored at –70°C as 0.5mL aliquots. Plasma and urine samples specifically used for 8-iso-$PGF_{2\alpha}$ analysis also included 10μg/mL meclofenamic acid to prevent any *in vitro* isoprostane formation.

Plasma and/or urinary samples were subsequently analysed for protein, cholesterol, triglycerides, creatinine, and 8-iso-Prostaglandin $F_{2\alpha}$, (8-iso-$PGF_{2\alpha}$) concentration. Plasma samples were also analysed for its antioxidant capacity to scavenge superoxide.

2.5.2. Enzyme Immunoassay of Plasma and Urinary 8-iso-$PGF_{2\alpha}$

Plasma total (i.e. esterified plus free) and urine free 8-iso-$PGF_{2\alpha}$ concentrations were assayed using a competitive enzyme-immunoassay (Figure 2.3).

Essentially, plasma samples were thawed and incubated at 42°C for 10 min to melt any lipid or salt crystals. To release 8-iso-$PGF_{2\alpha}$ esterified to plasma lipids, 4 volumes of plasma were subjected to alkaline hydrolysis with 1 volume of 10M NaOH for 2 h at 45°C. The hydrolysis mixture was allowed to cool and neutralised by adding an equal volume of 2M HCl. The samples were then centrifuged at 500g for 5 min to remove any particulate matter. All analyses were carried out in duplicate as described in the kit manufacturers instructions; 25 μl of either sample and standards (range 0-50,000 pg 8-iso-$PGF_{2\alpha}$/mL) were incubated in 96 well plates coated with a goat antibody specific to rabbit IgG followed by addition of solutions of alkaline phosphatase conjugated 8-iso-$PGF_{2\alpha}$ and a polyclonal rabbit antibody to 8-iso-$PGF_{2\alpha}$. Wells were then washed thoroughly and incubated with a p-nitrophenyl phosphate substrate solution for 45 min at

room temperature. A stop solution was added to all wells and the absorbance was read immediately at 405nm using a microtiter plate reader. Results were expressed as nmol/L plasma ± standard error of the mean (SEM) (pg/mL x 0.003 = nmol/L). A typical standard curve is displayed in Appendix V.

Urine samples were thawed and incubated at 42°C for 10 min to melt any lipid or salt crystals, centrifuged at 500g for 5 min to remove any particulate matter and diluted 1:10 with Tris-buffered saline, pH 7.4. All samples and standards (range 0-100,000 pg 8-iso-$PGF_{2\alpha}$/mL) were assayed in duplicate as described previously. Because 24 hour urine collections were not possible, urinary creatinine concentrations (mg/mL) were used to standardise urinary 8-iso-$PGF_{2\alpha}$ output. Results are expressed as pmol/mmol of creatinine ± SEM (pg/mg creatinine x 0.32 = pmol/mmol creatinine). A typical standard curve is displayed in Appendix VI.

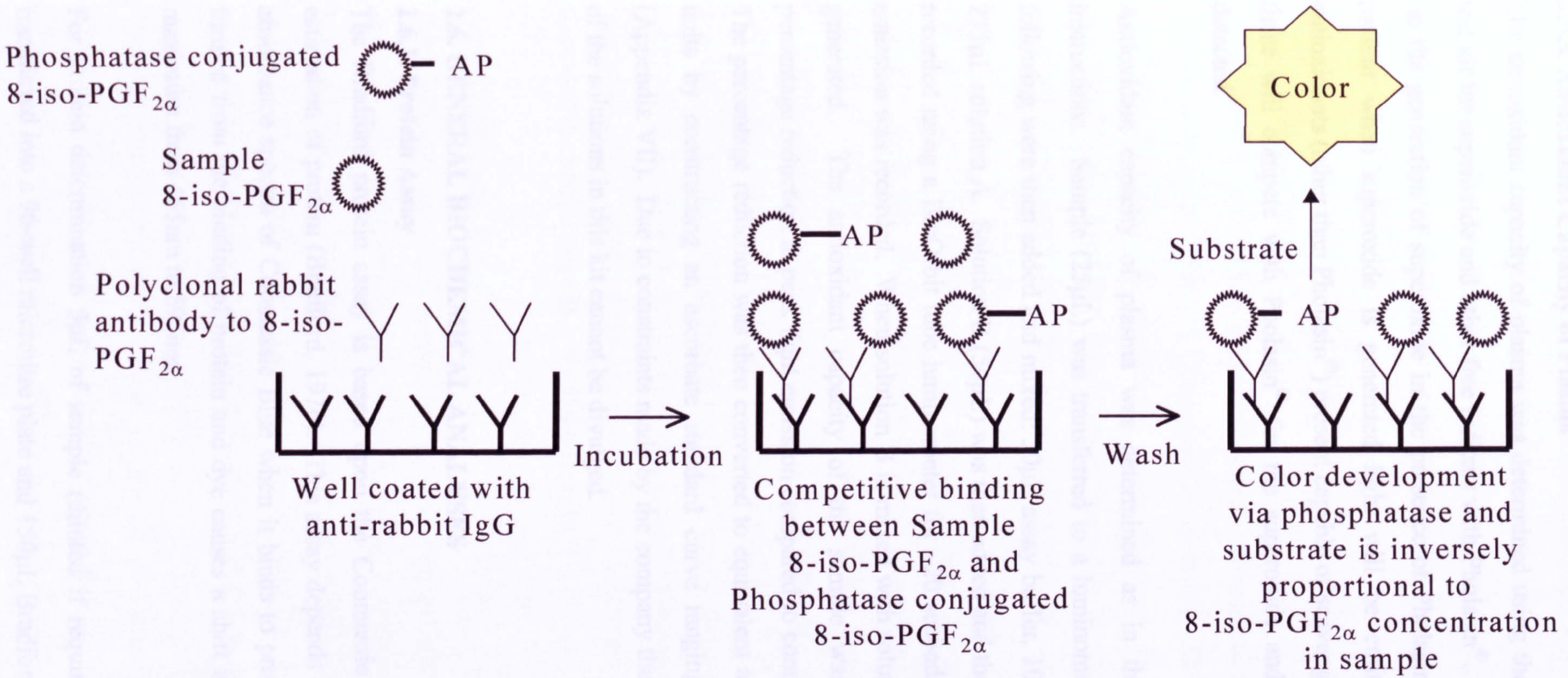


Figure 2.3. Principles of 8-iso-$PGF_{2\alpha}$ Competitive Enzyme Immunoassay. The polyclonal rabbit antibody to 8-iso-$PGF_{2\alpha}$ binds to the wells coated with anti-rabbit IgG. There is also competition between sample 8-iso-$PGF_{2\alpha}$ and phosphatase conjugated 8-iso-$PGF_{2\alpha}$ for binding to the polyclonal rabbit antibody to 8-iso-$PGF_{2\alpha}$. The phosphatase substrate is subsequently added and colour development occurs. The amount of colour developed is inversely proportional to the amount of 8-iso-$PGF_{2\alpha}$ in the sample.

2.5.3. Antioxidant Capacity of Plasma

The antioxidant capacity of plasma was determined using the Abel® antioxidant test kit for superoxide and other free radicals with Pholasin®. This assay is based on the generation of superoxide in the presence of Pholasin®. If Pholasin® is present when superoxide is generated light will be emitted. If there are antioxidants (other than Pholasin®) present capable of scavenging superoxide then these will compete with Pholasin® for the superoxide and less light will be detected.

Antioxidant capacity of plasma was determined as in the kit manufacturer instructions. Sample (25µL) was transferred to a luminometer cuvette and the following were then added and mixed: 50µL assay buffer, 100µL Pholasin®, and 275µL solution A. Solution B (50µL) was then added and the light emission was recorded using a BioOrbit tube luminometer for ~20 seconds and the peak light emission was recorded. When solution B is mixed with solution A superoxide is generated. The antioxidant capacity of the sample was expressed as the percentage reduction in peak light emission compared to controls without sample. The percentage reduction was then converted to equivalent ascorbate antioxidant units by constructing an ascorbate standard curve ranging from 0-80µmol/L (Appendix VII). Due to constraints made by the company the precise constituents of the solutions in this kit cannot be divulged.

2.6. GENERAL BIOCHEMICAL ANALYSES

2.6.1. Protein Assay

The Bradford protein assay is based upon the Coomassie Blue technique for estimation of protein (Bradford, 1976). This assay depends on the change in the absorbance spectra of Coomassie Blue when it binds to proteins. The complex formed from the binding of protein and dye causes a shift in the dye absorption maximum from 465nm to 595nm.

For protein determination 5µL of sample (diluted if required with saline) was transferred into a 96-well microtitre plate and 150µL Bradford reagent was added.

The plate was incubated for 10 minutes at room temperature and the absorbance read at 550nm. Protein concentration was determined by constructing a standard curve using human serum albumin (HSA) from 0 to 1mg/mL (Appendix VIII). Plasma protein concentration was expressed as g/L and all other samples were expressed as mg/mL.

2.6.2. Cholesterol Assay

Total plasma and lipoprotein cholesterol concentration was determined enzymatically using Infinity™ Cholesterol Reagent kit. This assay is based on enzymatic hydrolysis of cholesterol esters followed by oxidation of free cholesterol and subsequent chromophore formation (Katterman et al, 1984), which can be measured spectrophotometrically at 500-550nm (Figure 2.4).

For cholesterol determination 10µL of sample was transferred into a 96-well microtitre plate and 200µL cholesterol reagent (containing 100U/L cholesterol oxidase; 1250U/L cholesterol esterase; 800U/L horseradish peroxidase; 0.25mmol/L 4-aminoantipyrine; 10mmol/L hydroxybenzoic acid) was added. The plate was incubated for 15 minutes at room temperature and the absorbance read at 540nm. Plasma and lipoprotein cholesterol concentration was determined by constructing a standard curve using cholesterol calibrators from 0-300mg/dL (Appendix IX). Total plasma and lipoprotein cholesterol concentrations were converted to mmol/L (mg/dL x 0.0259 = mmol/L)

2.6.3. Triglyceride Assay

Total plasma and lipoprotein triglyceride concentration was determined enzymatically using Peridochrom® Triglycerides GPO-PAP kit. This assay is based on enzymatic hydrolysis of triglycerides followed by phosphorylation, and oxidation of glycerol and subsequent chromophore formation (Nagele et al., 1984), which can be measured spectrophotometrically at 492nm (Figure 2.5).

For triglyceride determination 10µL of sample was transferred into a 96-well microtitre plate and 200µL triglyceride reagent (containing 0.5mmol/L ATP; 0.35mmol/L 4-aminophenazone; 3000U/L lipase; 2500U/L glycerol phosphate

oxidase; 200U/L glycerol kinase; 150U/L peroxidase; 3.5mmol/L 4-chlorophenol) was added. The plate was incubated for 10 minutes at room temperature and the absorbance read at 492nm. Plasma and lipoprotein triglyceride concentration was determined by constructing a standard curve using triglyceride calibrators from 0-300mg/dL (Appendix X). Total plasma and lipoprotein triglyceride concentrations were converted to mmol/L (mg/dL x 0.0115 = mmol/L).

Figure 2.4. Enzymatic Determination of Cholesterol

		CE		
Step 1.	Cholesterol Esters	→	→	Cholesterol + Fatty acids
		CO		
Step 2.	Cholesterol + O_2	→	→	Cholest-4-en-3-one + H_2O_2
		POD		
Step 3.	$2H_2O_2$ + HBA + 4AAP	→	→	Quinoneimine dye + $4H_2O$

Step 1: Cholesterol esters are hydrolysed by cholesterol esterase (CE) to cholesterol and free fatty acids. Step 2: Free cholesterol, including that originally present, is then oxidised by cholesterol oxidase (CO) to cholest-4-en-3-one and hydrogen peroxide (H_2O_2). Step 3: The hydrogen peroxide combines with hydroxybenzoic acid (HBA) and 4-aminoantipyrine (4AAP) in the presence of peroxidase (POD) to form a chromophore (quinoneimine dye) which may be quantitated at 500-550nm.

Figure 2.5. Enzymatic Determination of Triglycerides

	lipase		
Step 1. Triglycerides + $3H_2O$	→	→	Glycerol + Fatty acids
	GK		
Step 2. Glycerol + ATP	→	→	Glycerol-3-phosphate + ADP
	GPO		
Step 3. Glycerol-3-phosphate + O_2		→	Dihydroxyacetone phosphate + H_2O_2
	POD		
Step 4. H_2O_2 + 4AP + 4CP	→	→	4-(p-benzoquinone-mono-imino)-phenazone + $2H_2O$ + HCl

Step 1: Triglycerides are hydrolysed by lipase to glycerol and free fatty acids. Step 2: Glycerol is then phophorylated by glycerol kinase (GK). Step 3: Glycerol-3-phosphate is then oxidised by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). Step 4: The hydrogen peroxide combines with 4-aminophenazone (4AP) and 4-chlorophenol (4CP) in the presence of peroxidase (POD) to form a chromophore (4-(p-benzoquinone-mono-imino)-phenazone) which may be quantitated at 492nm.

2.6.4. Creatinine Assay

Plasma and urinary creatinine were determined using a creatinine clinical diagnostic kit. This method is based upon creatinine reacting with alkaline picrate to form an orange/yellow coloured complex. The colour derived from creatinine is then destroyed at acidic pH (Heinegard & Tiderstrom, 1973). The difference in colour intensity measured at or near 500nm before and after acidification is proportional to the creatinine concentration.

For creatinine determination 150µL of sample (diluted if required with saline) was transferred into a cuvette and 1.5mL of alkaline picrate solution (prepared by mixing 5 volumes of creatinine colour reagent (0.6% picric acid) with 1 volume of 1M NaOH) was added then mixed and incubated at room temperature for 10 minutes. The absorbance at 500nm was then recorded (Initial A). 50µL of acid reagent (mixture of sulphuric acid and acetic acid) was then added, mixed thoroughly and allowed to stand at room temperature for 5 minutes. The absorbance at 500nm was then recorded (Final A). Final A was then subtracted from Initial A to give actual absorbance. Plasma and urinary creatinine concentration was determined by constructing a standard curve using creatinine calibrators from 0-10mg/dL (Appendix XI). Plasma and urinary creatinine concentration were converted to µmol/L (mg/dL x 88.4).

Urinary creatinine concentration was used to standardise urinary concentrations of 8-iso-Prostaglandin $F_{2\alpha}$. Plasma creatinine concentration (mg/dL) was used to estimate creatinine clearance (mL/min) which is a measure of glomerular filtration rate. This was determined using the following calculation were creatinine is related to lean body mass (Cockcroft & Gault, 1976):

$$\text{Creatinine clearance (mL/min)} = \frac{(140\text{-age in years}) \times (\text{weight in kg})}{72 \times \text{plasma creatinine in mg/dL}}$$

Note. For women multiply above calculation by 0.85.

2.7. STATISTICAL ANALYSIS

Results are expressed as means ± standard error of the mean (SEM). The significance of differences was assessed using the student's two-tailed, paired/unpaired t-test, $p<0.05$ was considered statistically significant. Analysis of correlation was performed using the Pearson correlation test, which generates an r-value. R-values close to 1 denote good correlation. A negative r-value denoted inverse correlation. Analyses were performed using Microsoft Excel-97 statistical package.

CHAPTER 3

THE *IN VITRO* ANTIOXIDANT NATURE OF AGED GARLIC EXTRACT

3. THE *IN VITRO* ANTIOXIDANT NATURE OF AGED GARLIC EXTRACT

3.1. INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced metabolically by the human body have been implicated in the pathogenesis of several human diseases, including atherosclerosis (Halliwell & Gutteridge, 1998). Endogenous human defences against ROS- and RNS-induced damage include antioxidant enzymes such as the superoxide dismutases, glutathione peroxidases, and catalase, and antioxidant compounds such as bilirubin, uric acid, and glutathione (Becker et al., 1991, Mates & Sanchez-Jimenez, 1999, Stocker et al, 1990). This defence system is supported by dietary antioxidants such as vitamin E, vitamin C, carotenoids, and flavonoids (Bendich & Langseth, 1995, Evstigneeva et al., 1998, Krinsky, 1998, Rice-Evans, 1995). Mechanisms and evaluation of antioxidant action can include scavenging of reactive species, inhibiting the formation of reactive species, recycling of other antioxidants, upregulation of endogenous antioxidant defences, and binding catalytic metal ions (Halliwell et al, 1995, Halliwell, 1997).

An important mechanism in the development of atherosclerosis is the oxidation of low-density lipoproteins (LDL), which due to its pro-atherogenic properties results in atherosclerotic plaque formation, progression, and subsequent coronary complications (Keaney, 2000). Because the oxidative modification of LDL appears to be a central mechanism for the development of atherosclerosis many studies have focused on the ability of nutritional antioxidants to directly inhibit this oxidation process *in vitro* or following dietary supplementation (Bowen et al., 1998, Fuhrman et al, 1997b, 1997d, Fuller et al, 2000).

Over the last decade many studies have focused on the antioxidant nature of garlic and it's numerous preparations and constituents. Fresh garlic and garlic powder extracts have been demonstrated to scavenge hydroxyl and peroxyl radicals in cell free assays and prevent hydroxyl radical-induced lipid peroxidation in rat liver homogenates (Aruoma et al., 1997, Prasad et al., 1995, 1996). Supplementation with garlic extracts has been shown to preserve or increase antioxidant enzyme

activity in animal models of oxidative stress (Helen et al., 1999, Pedraza-Chaverri et al., 2000). These latter studies noted preserved or increased activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase, and catalase. Dietary supplementation studies with garlic powder extracts have proven inconsistent with regards to reducing the susceptibility of subsequently isolated LDL to oxidative modification (Byrne et al., 1999, Orekhov et al., 1996, Phelps & Harris, 1993, Simons et al., 1995).

Similar antioxidant properties have been published for AGE. AGE and one of its constituents S-allylcysteine (SAC) have been shown to scavenge the ROS hydrogen peroxide and *t*-butyl-hydroperoxide and hence prevent oxidant injury to endothelial cells, liver microsomes, and erythrocytes (Ide & Lau, 1999a, Ide et al., 1996, Imai et al., 1994, Moriguchi et al., 2001, Yamasaki et al., 1994). Interestingly, one of these studies reported that water extracts of raw and heat-treated garlic enhanced peroxidation of liver microsomes induced by *t*-butyl-hydroperoxide (Imai et al., 1994). Studies have shown that AGE and SAC protect endothelial cells from oxidant-induced injury by increasing the activity of SOD and preventing depletion of the endogenous antioxidant compound glutathione (GSH) (Geng & Lau, 1997, Ide & Lau, 1999a, 1999b, 2001). This preservation of GSH levels by AGE is due to increased activity of glutathione disulphide (GSSG) reductase and hence modulation of the glutathione redox cycle (Geng & Lau, 1997). *In vitro* lipid peroxidation of LDL induced by Cu^{2+} was significantly reduced in the presence of AGE and some of its constituents and dietary supplementation with AGE was shown to significantly reduce the susceptibility of subsequently isolated LDL to Cu^{2+}-mediated lipid peroxidation (Ho et al., 2001, Ide et al., 1997, Munday et al., 1999). This latter study observed that dietary supplementation with raw garlic had no effect on the susceptibility of subsequently isolated LDL to Cu^{2+}-mediated lipid peroxidation.

Oxidation of apolipoprotein B-100 in LDL appears to be important for scavenger receptor recognition and subsequent uptake of oxidised LDL by macrophages, this uptake by macrophages can occur in the absence of lipid peroxidation (Hunt et al., 1994). With most studies focusing on lipid peroxidation of LDL the effect of AGE and other garlic preparations on inhibiting protein oxidation of LDL are

lacking. While AGE and other garlic preparations have been shown to increase the activity of certain antioxidant enzymes and reduce metal ion-induced oxidation of LDL (Geng & Lau, 1997, Helen et al., 1999, Ide et al., 1997, Pedraza-Chaverri et al., 2000), to date the ability of garlic preparations to prevent enzyme-mediated oxidation of LDL has not been examined.

The aim of this study was to investigate the *in vitro* antioxidant potential of aged garlic extract (AGE). Initial studies examined the ability of AGE to scavenge superoxide ions and inhibit lipid hydroperoxide formation in cell-free systems. Subsequent studies utilized isolated human LDL, which was challenged with Cu^{2+}, xanthine-xanthine oxidase, or 15-lipoxygenase, and AGE was examined for its ability to prevent the oxidative modification of LDL. These studies are schematically represented in Figure 3.1.

3.1.1. Overview of Study

Superoxide ions were generated during the xanthine oxidase-catalyzed breakdown of xanthine to uric acid. This enzymatic reaction can be monitored by two methods, namely superoxide production via cytochrome C reduction at 550nm and xanthine oxidase activity via uric production at 284nm (Edwards et al., 1987, Mondal & Mitra, 1994, see Chapter 2, pp 41-42 for detailed methods). The ability to monitor this reaction by using two methods is important in assessing the mechanism by which AGE interferes with this assay i.e. direct scavenging of superoxide or inhibition of xanthine oxidase itself.

In another set of experiments lipid hydroperoxides were generated during the 15-lipoxygenase-mediated oxygenation of the PUFA linoleic acid. This reaction begins with the formation of a lipid peroxyl radical, which in turn abstracts a hydrogen atom from a neighbouring PUFA to yield a lipid hydroperoxide and a carbon centred radical. This lipid peroxidation process is monitored via molecular rearrangement of the carbon-centred radical to a conjugated diene compound at 234nm (Esterbauer et al, 1989, see Chapter 2, pp 43-44 for detailed methods). AGE was assessed for its ability to prevent lipid hydroperoxide formation.

In the final series of experiments human lipoproteins were isolated by density ultracentrifugation and LDL was differentiated from other lipoprotein fractions by measuring the amount of cholesterol, triglycerides, and protein present and observing its electrophoretic mobility on agarose gels. LDL was oxidatively modified by both enzymic (xanthine oxidase/xanthine and 15-lipoxygenase) and non-enzymic methods (Cu^{2+}) in the absence and presence of AGE. Measuring TBARS production assessed lipid peroxidation (Mao et al., 1994) and protein oxidation was assessed by increased electrophoretic mobility on agarose gels. The effect of AGE on the characteristic lag phase and propagation phase of Cu^{2+}-mediated lipid peroxidation (Esterbauer & Ramos, 1995) was also assessed. For detailed methods see Chapter 2, pp 44-47.

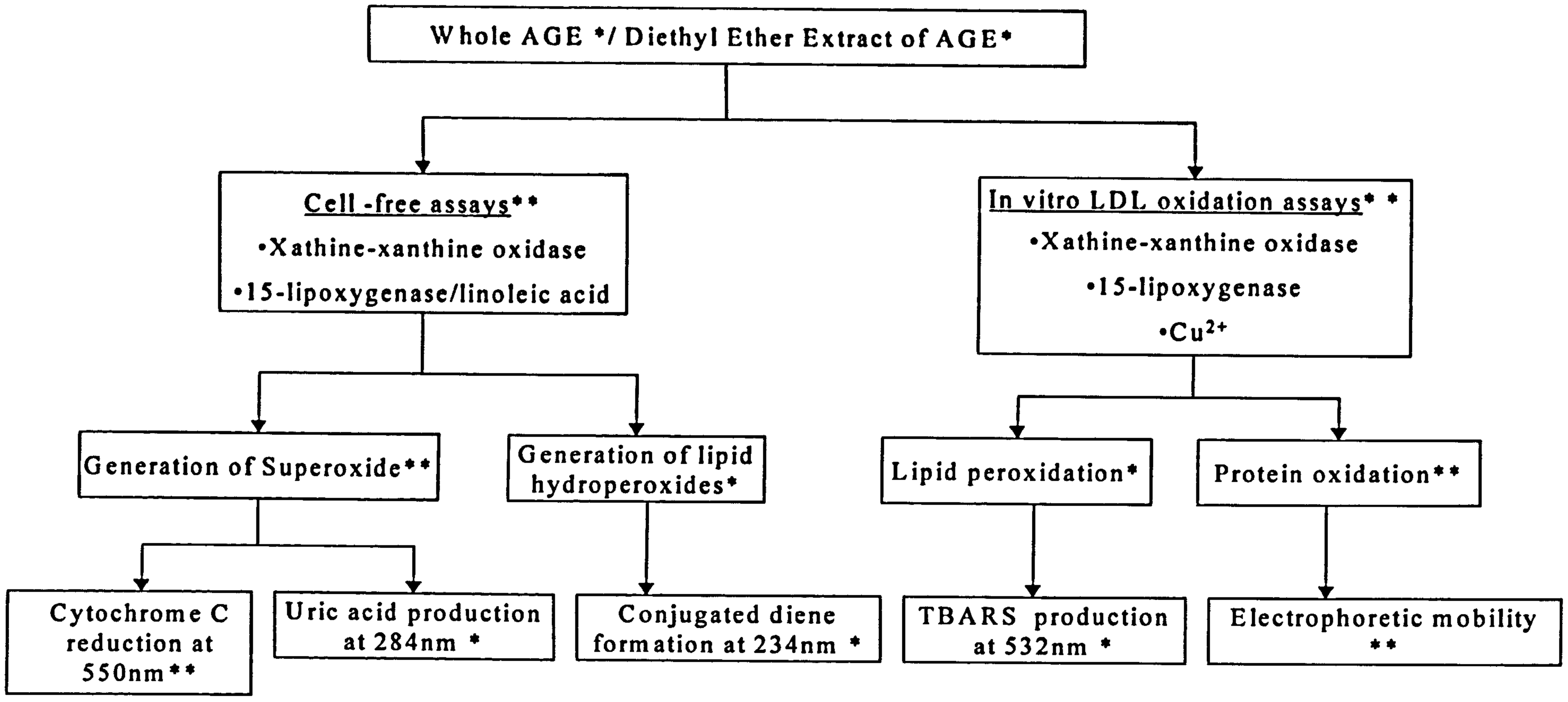


Figure 3.1. Overview of Study.
The cell-free and *in vitro* LDL oxidation assays employed for assessing the antioxidant nature of whole AGE (*) and a diethyl ether extract of AGE (*). It is important to note that whole AGE could not be used in most of these spectrophotometric assays due to excessive interference. Whole AGE could only be used to examine its effect on superoxide production from xanthine-xanthine oxidase and protein oxidation of LDL by enzymic and non-enzymic methods.

3.2. RESULTS

3.2.1. Superoxide Scavenging Ability of AGE

Superoxide production by xanthine-xanthine oxidase measured by the reduction of cytochrome gave a reaction rate of 0.040 ± 0.001 ΔA550nm/min. The inclusion of 3mM ascorbic acid, a known superoxide scavenger (Nishikimi, 1975), inhibited the reduction of cytochrome C by approximately 98% (Table 3.1). Similarly, the presence of 50U of superoxide dismutase inhibited the reduction of cytochrome C by 98%; this confirms the production of superoxide in the enzyme reaction. Xanthine oxidase activity measured by the production of uric acid gave a reaction rate of 0.060 ± 0.002 ΔA284nm/min and was not significantly affected by the presence of superoxide dismutase (Table 3.1).

At 10% (v/v) of the reaction volume, both whole AGE and the diethyl ether extract of AGE significantly inhibited superoxide production i.e. the reduction of cytochrome C by 100% and 34% respectively. Superoxide production was inversely related to the concentration of AGE or the diethyl ether extract of AGE ($r = -0.96$ and -0.97 respectively, Figure 3.2). Uric acid production was not significantly affected in the presence of the diethyl extract of AGE. These results suggest that components of AGE present in the diethyl ether extract of AGE do not inhibit xanthine oxidase, rather they scavenge superoxide generated in this reaction as evidenced by their inhibition of cytochrome C reduction (Figure 3.2).

Table 3.1. The effect of SOD, ascorbic acid, whole AGE and a diethyl ether extract of AGE on superoxide and uric acid production by xanthine-xanthine oxidase. Values are means of three experiments ± SEM. Significant differences ($p < 0.05$) from control[1] (xanthine-xanthine oxidase only) are indicated by *.

Extract/Agent	Superoxide Production (ΔA550nm/min)	Uric Acid Production (ΔA284nm/min)
None[1]	0.040 ± 0.001	0.060 ± 0.002
50U SOD	0.001 ± 0.001*	0.057 ± 0.003
3mM ascorbic acid	0.001 ± 0.001*	N.D.
10% whole AGE	0*	N.D.
10% diethyl ether extract of AGE	0.027 ± 0.001*	0.053 ± 0.005

N.D.: not determined.

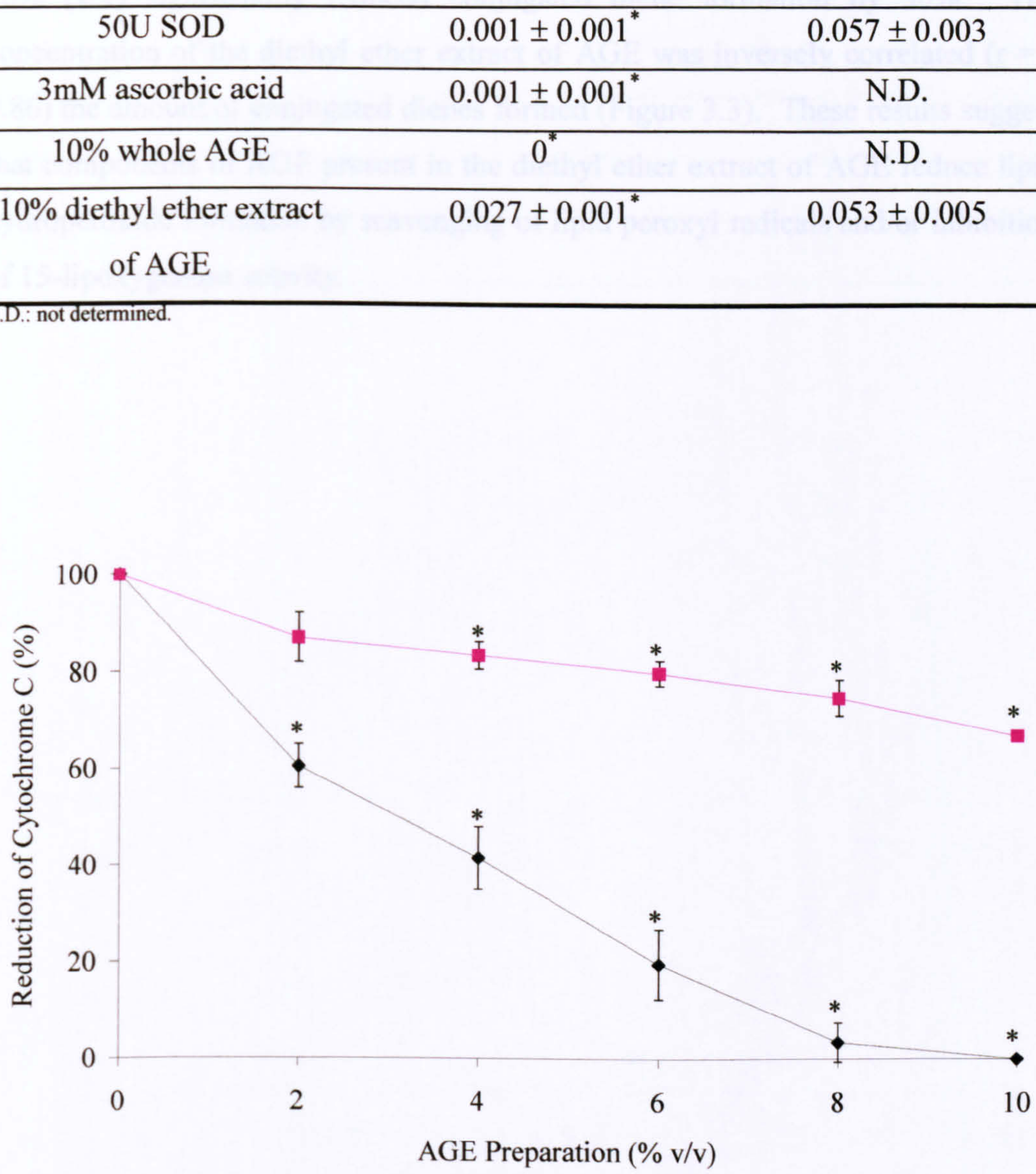


Figure 3.2. Concentration dependant inhibition by whole AGE (◆) and a diethyl ether extract of AGE (■) used at 0-10% (v/v) on reduction of cytochrome C by superoxide ions generated by xanthine-xanthine oxidase. Values are means of three separate experiments ± SEM. Pearson correlation coefficient (r) for AGE and diethyl ether extract of AGE was –0.96 and –0.97 respectively. Significant differences ($p < 0.05$) from control (0% AGE preparation) are indicated by *.

3.2.2. Inhibition of Lipid Hydroperoxide Formation by AGE

Lipid hydroperoxide formation by 15-lipoxygenase/linoleic acid measured as the production of conjugated diene compounds gave a reaction rate of 0.30 ± 0.01 ΔA234nm/min. The inclusion of 1.5mM Trolox®, a synthetic analogue of the lipophilic chain-breaking antioxidant vitamin E, completely inhibited conjugated diene formation (Table 3.2). The presence of the diethyl ether extract of AGE at 10% (v/v) significantly reduced conjugated diene formation by 50%. The concentration of the diethyl ether extract of AGE was inversely correlated (r = -0.86) the amount of conjugated dienes formed (Figure 3.3). These results suggest that components of AGE present in the diethyl ether extract of AGE reduce lipid hydroperoxide formation by scavenging of lipid peroxyl radicals and/or inhibition of 15-lipoxygenase activity.

Table 3.2. The effect of Trolox® and a diethyl ether extract of AGE on lipid hydroperoxide formation measured as conjugated diene formation by 15-lipoxygenase/linoleic acid. Values are means of three experiments ± SEM. Significant differences ($p < 0.05$) from control[1] (15-lipoxygenase/linoleic acid only) are indicated by *.

Extract/Agent	Conjugated Diene Formation (ΔA234nm/min)
None[1]	0.30 ± 0.01
1.5mM Trolox®	0*
10% diethyl ether extract of AGE	0.15 ± 0.01*

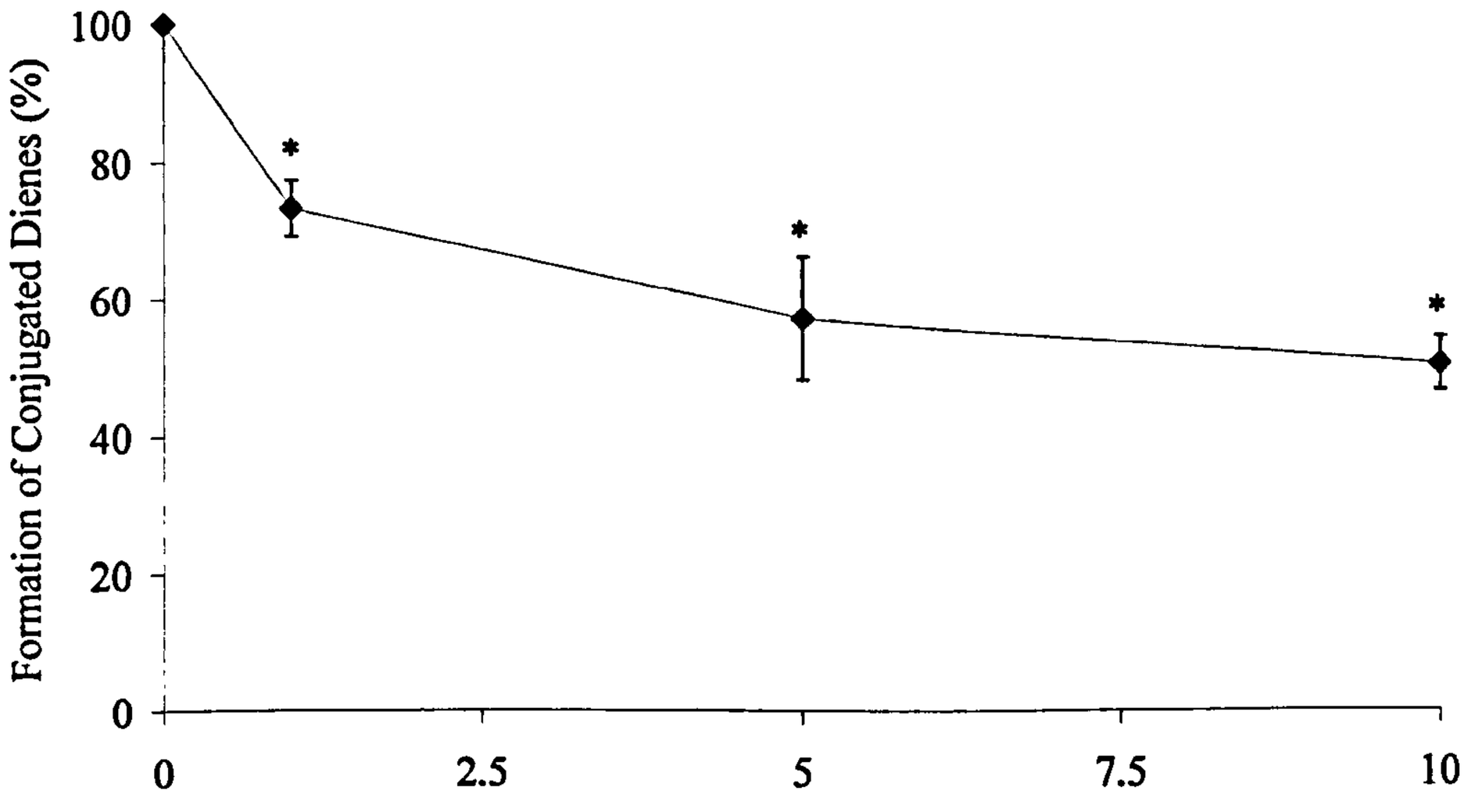


Figure 3.3. Concentration dependant effect of a diethyl ether extract of AGE used at 0-10% (v/v) on lipid hydroperoxide formation measured as conjugated diene formation by 15-lipoxygenase/linoleic acid substrate solution at 234nm. Values are means of three experiments ± SEM. Pearson correlation coefficient (r) was –0.086. Significant differences ($p < 0.05$) from control (0% diethyl ether extract of AGE) are indicated by *.

3.2.3. The Ability of AGE to Inhibit Oxidative Modification of Human LDL

3.2.3.1. *Characterisation of Human LDL*

Human lipoprotein fractions isolated by density ultracentrifugation were differentiated from each other by measuring the amount of protein, cholesterol, and triglycerides present and observing their electrophoretic mobility on agarose gels as described by Lowe et al, 1999 and Papadopoulos, 1985. The amount of protein present was determined using the Bradford assay and constructing a standard curve using human serum albumin (HSA) as the standard (appendix VIII). Cholesterol and triglyceride content was determined enzymatically and by constructing a standard curve using cholesterol and triglyceride calibrators (appendices IX & X, respectively).

LDL was distinguished from VLDL by its significantly higher cholesterol (3.54 ± 0.87 mmol/L vs. 1.20 ± 0.31 mmol/L) and protein concentration (0.65 ± 0.08 mg/mL vs. 0.29 ± 0.05 mg/mL) and its significantly lower triglyceride concentration (0.21 ± 0.11 mmol/L vs. 1.48 ± 0.75 mmol/L) (Figure 3.4). VLDL also had a slightly greater electrophoretic mobility than LDL on agarose gels (Figure 3.5). LDL was distinguished from HDL by its significantly lower protein concentration (0.65 ± 0.08 mg/mL vs. 2.34 ± 0.34 mg/mL) and HDL also had a much greater electrophoretic mobility than LDL on agarose gels (Figures 3.4 & 3.5).

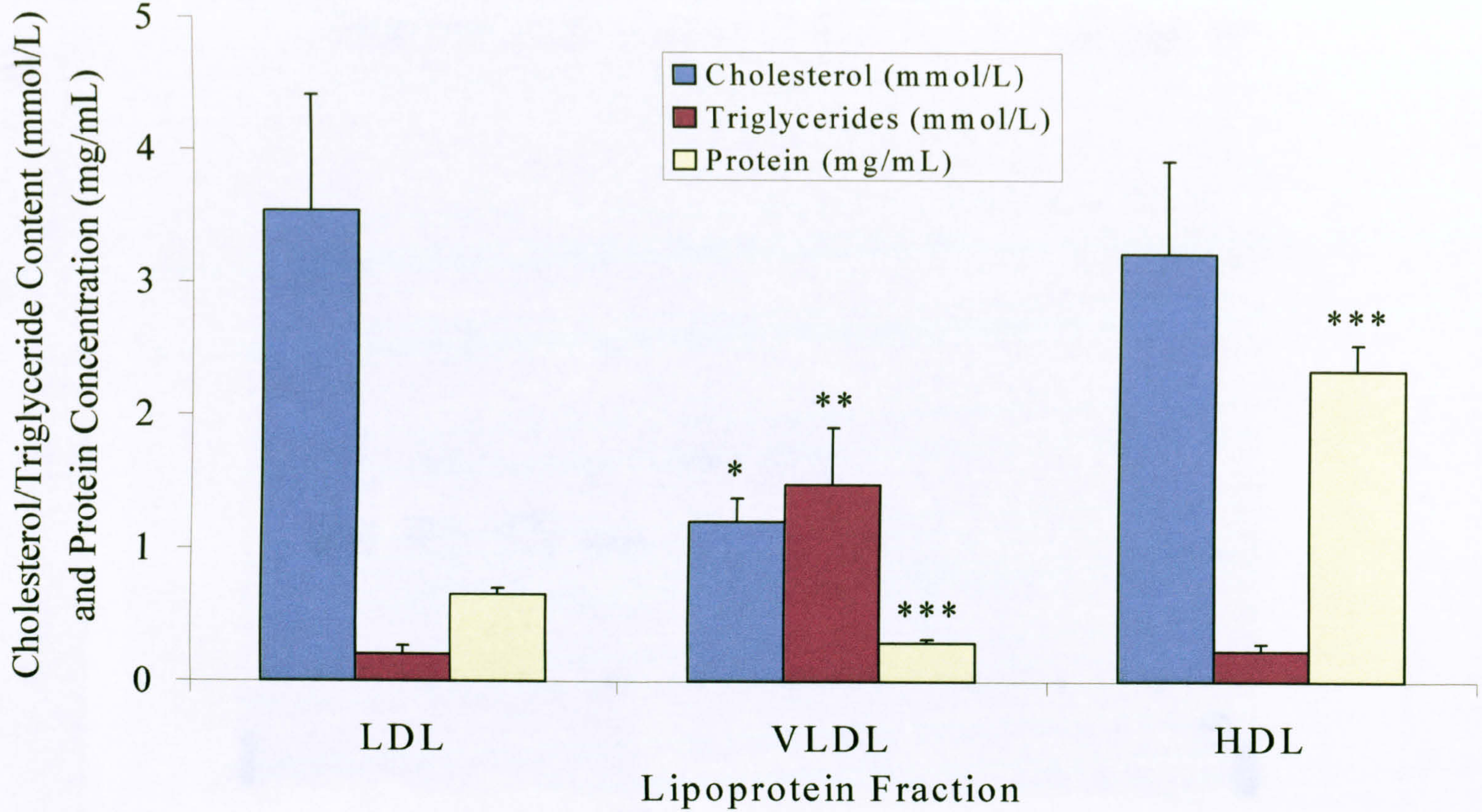


Figure 3.4. The Cholesterol, Triglyceride, and Protein Concentration of Human Lipoprotein Fractions. Values are the means of three experiments ± SEM. Significant difference ($p < 0.05$) from control (LDL-cholesterol) is indicated by *, from control (LDL-triglycerides) is indicated by **, and from control (LDL-protein) is indicated by ***.

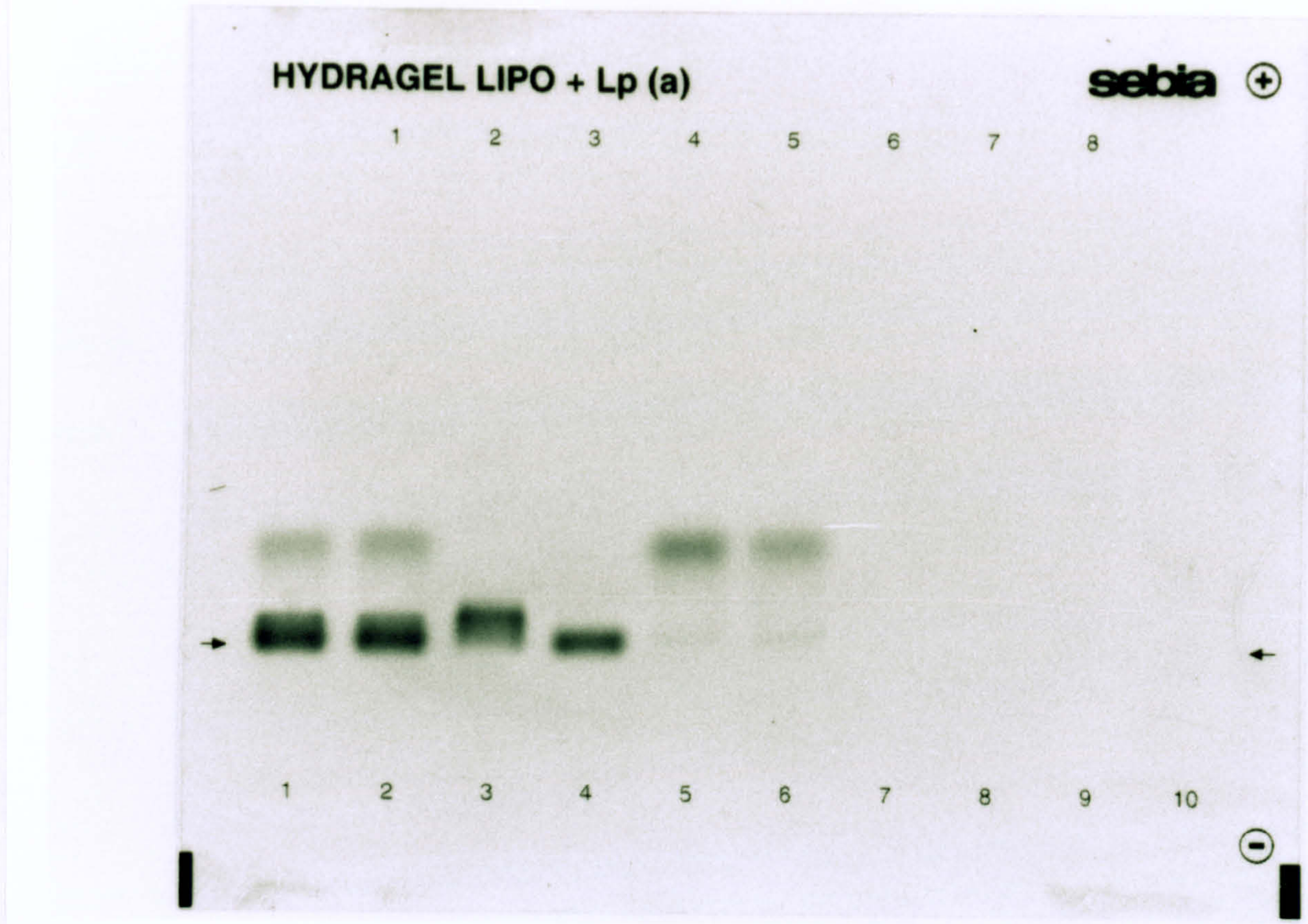


Figure 3.5. Electrophoretic Mobility of Human Lipoproteins on Agarose Gel. Samples were electrophoresed for 80mins at 27mA/gel and then stained with Sudan Black. (1+2) Whole plasma. (3) VLDL. (4) LDL. (5) HDL fraction 1. (6) HDL fraction 2. A typical lipoprotein profile is shown.

3.2.3.2. *Enzyme Mediated Oxidation of LDL*

LDL was used at 0.2 mg protein/mL as determined by the Bradford protein assay (appendix VIII). Lipid peroxidation of LDL was monitored by the production of TBARS at 532nm. This was converted to nmoles of MDA by constructing a standard curve using hydrolyzed tetraethoxypropane as the standard (appendix I). LDL incubated in PBS only produced 3.29 ± 0.27 nmol MDA/mg LDL protein (Figure 3.6). No significant lipid peroxidation as assessed by the formation of TBARS or protein oxidation as assessed by increased electrophoretic mobility of LDL was observed in the presence of xanthine-xanthine oxidase (Figures 3.6 & 3.7).

LDL incubated in borate buffer only produced 0.19 ± 0.10 nmol MDA/mg LDL protein (Figure 3.6). The extent of lipid peroxidation induced in LDL by 15-lipoxygenase was significantly increased by 34-fold to 6.46 ± 0.40 nmol MDA/mg LDL protein when compared to the LDL in borate buffer control ($p=0.002$, Figure 3.6). This was reduced by approximately 17% (5.39 ± 0.77 nmol MDA/mg LDL protein) in the presence of 5% (v/v) diethyl ether extract of AGE, and significantly reduced by approximately 37% (4.10 ± 1.35 nmol MDA/mg LDL protein) in the presence of 10% (v/v) diethyl ether extract of AGE ($p=0.03$, Figure 3.6). There was no alteration in the electrophoretic mobility of LDL incubated in the presence of 15-lipoxygenase (Figure 3.7).

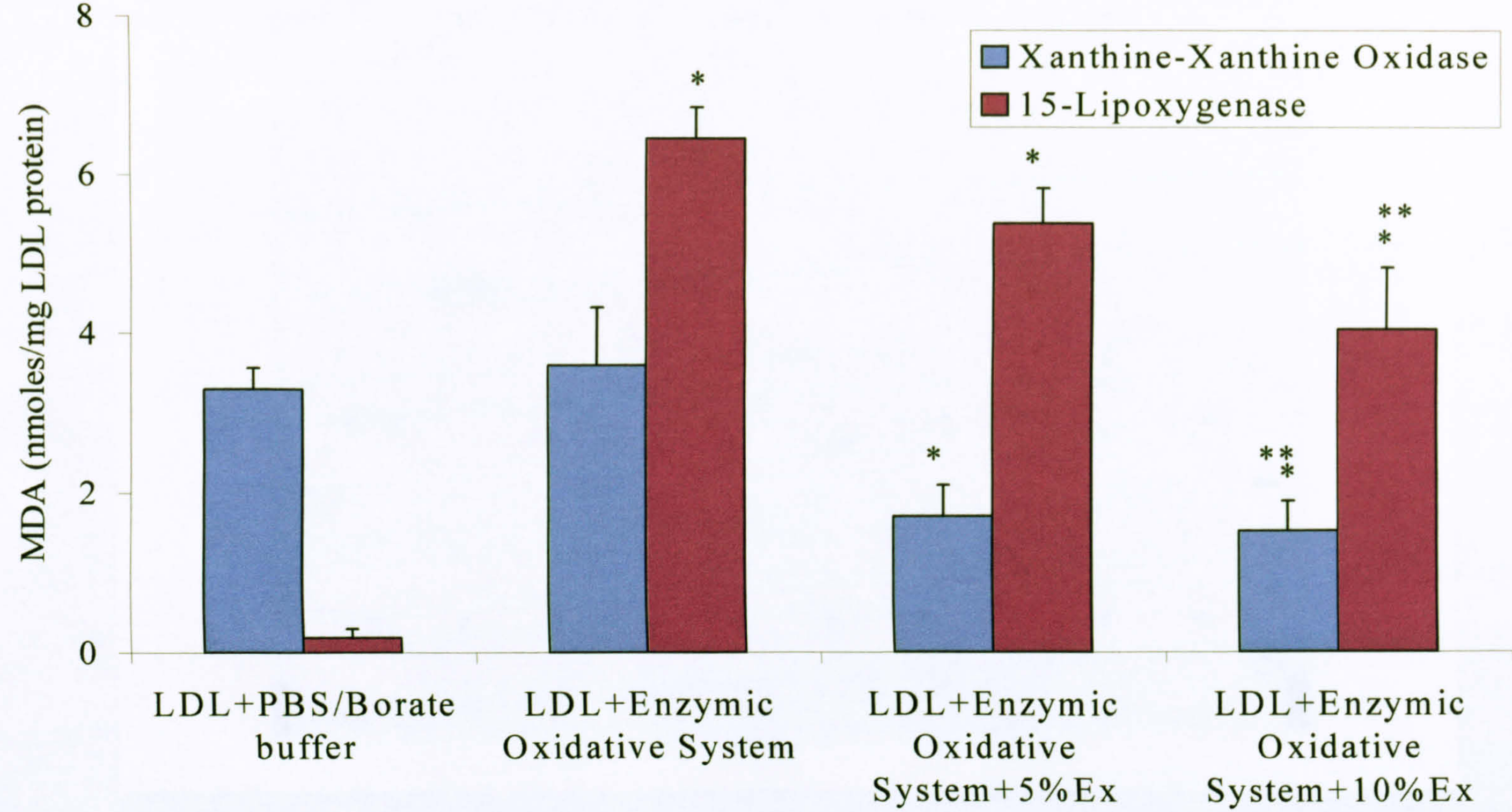


Figure 3.6. The Effect of a Diethyl Ether Extract of AGE (Ex) on the Oxidative Modification of LDL Induced by Xanthine-Xanthine Oxidase and 15-Lipoxygenase. Values are means of three experiments ± SEM. Significant differences ($p < 0.05$) from control (LDL + PBS/Borate buffer) are indicated by *, and control (LDL + enzymic oxidative system) are indicated by **.

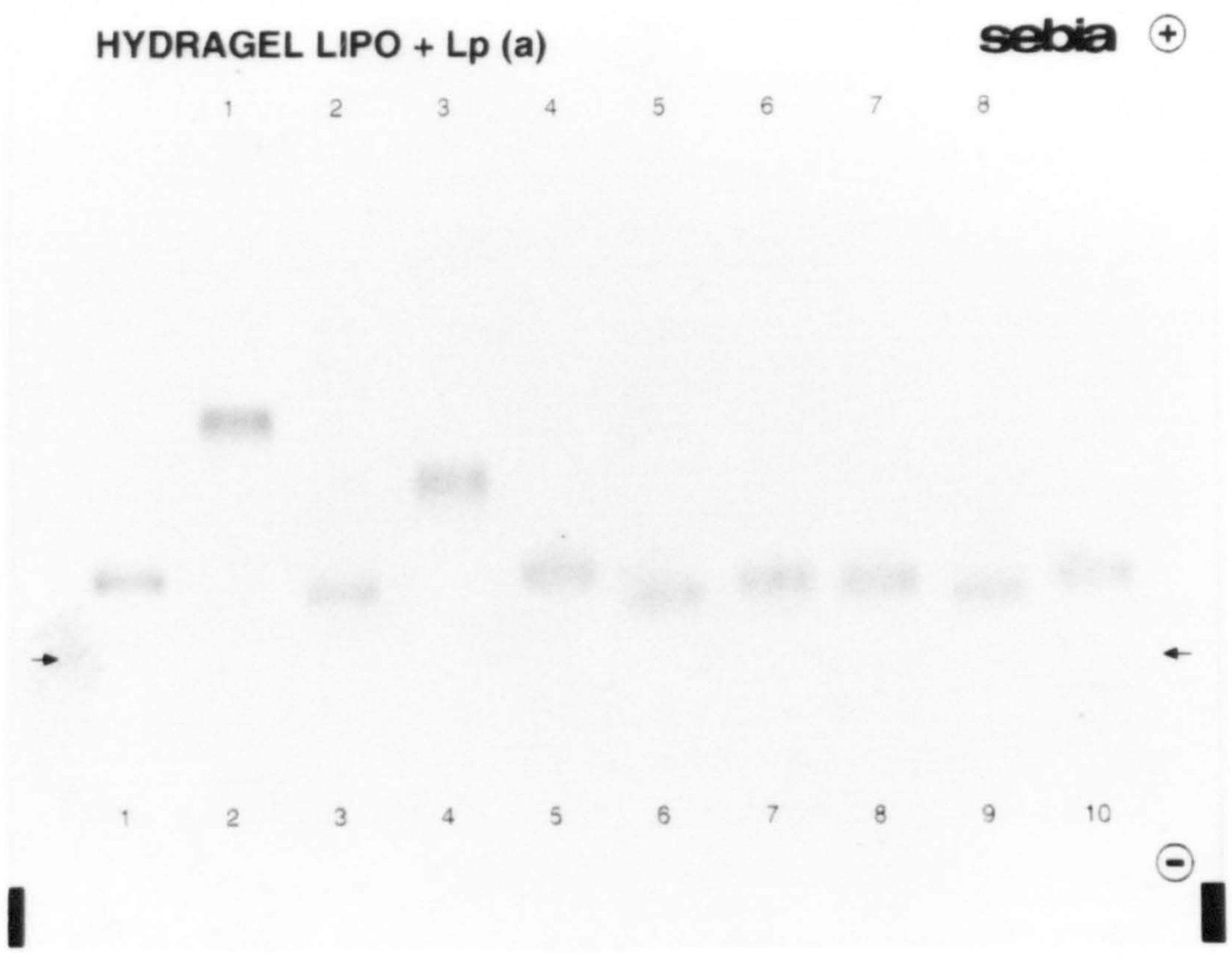


Figure 3.7. Electrophoretic Mobility of Oxidised LDL on Agarose Gel. Samples were electrophoresed for 80mins at 27mA/gel and then stained with Sudan Black. (1) LDL only. (2) LDL incubated with $CuSO_4$ for 5h at 37°C in the presence of 10% whole AGE (3) and 10% diethyl ether extract of AGE (4). (5) LDL incubated with 15-lipoxygenase for 16h at 37°C in the presence of 10% whole AGE (6) and 10% diethyl ether extract of AGE (lane 7). (8) LDL incubated with xanthine oxidase-xanthine for 5h at 37°C in the presence of 10% whole AGE (9) and 10% diethyl ether extract of AGE (10). Experiments were repeated three times and the gel shown is typical of the results obtained.

3.2.3.3. *Cu^{2+}-Mediated Oxidation of LDL*

Lipid peroxidation of LDL incubated in the presence of Cu^{2+} was significantly increased by approximately 20-fold in comparison with LDL incubated in PBS only (3.29 ± 0.27 nmol MDA/mg LDL protein vs. 60.39 ± 7.55 nmol MDA/mg LDL protein, p=0.017). The presence of 5% or 10% (v/v) diethyl ether extract of AGE significantly inhibited lipid peroxidation by approximately 70% and 81%, respectively (18.38 ± 2.28 nmol MDA/mg LDL protein and 11.22 ± 3.64 nmol MDA/mg LDL protein, respectively, p=0.019 and 0.006, respectively) (Figure 3.8).

Oxidation of the LDL lipoprotein by Cu^{2+} greatly increased its electrophoretic mobility (Figure 3.7). This increase in electrophoretic mobility was completely abolished in the presence of 10% (v/v) whole AGE (Figure 3.7). However, this effect was not observed with 10% (v/v) diethyl ether extract of AGE (Figure 3.7). While the diethyl ether extract of AGE displayed a concentration dependent decrease in electrophoretic mobility of LDL even at concentrations of 10% the extract had not abolished the Cu^{2+}-induced increase in electrophoretic mobility.

The kinetics of Cu^{2+} mediated oxidation of LDL is characterized by two phases, a lag phase and a propagation phase (Esterbauer & Ramos, 1995). The presence of the diethyl ether extract of AGE extended the lag phase to such an extent that there was no observable propagation phase (Figure 3.9).

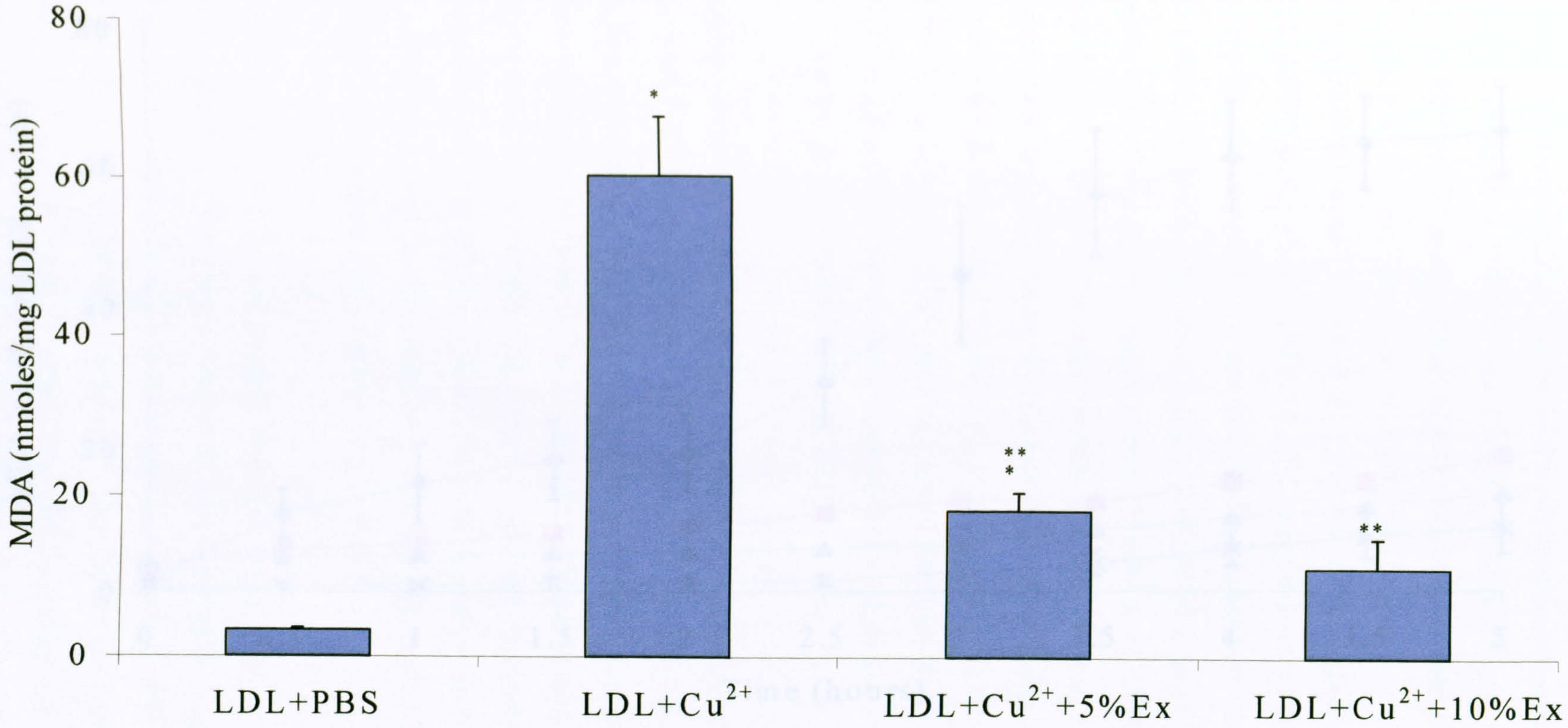


Figure 3.8. The Effect of a Diethyl Ether Extract of AGE (Ex) on the Oxidative Modification of LDL Induced by Cu^{2+}. Values are means of three experiments ± SEM. Significant differences ($p < 0.05$) from control (LDL + PBS) are indicated by *, and control (LDL + Cu^{2+}) are indicated by **.

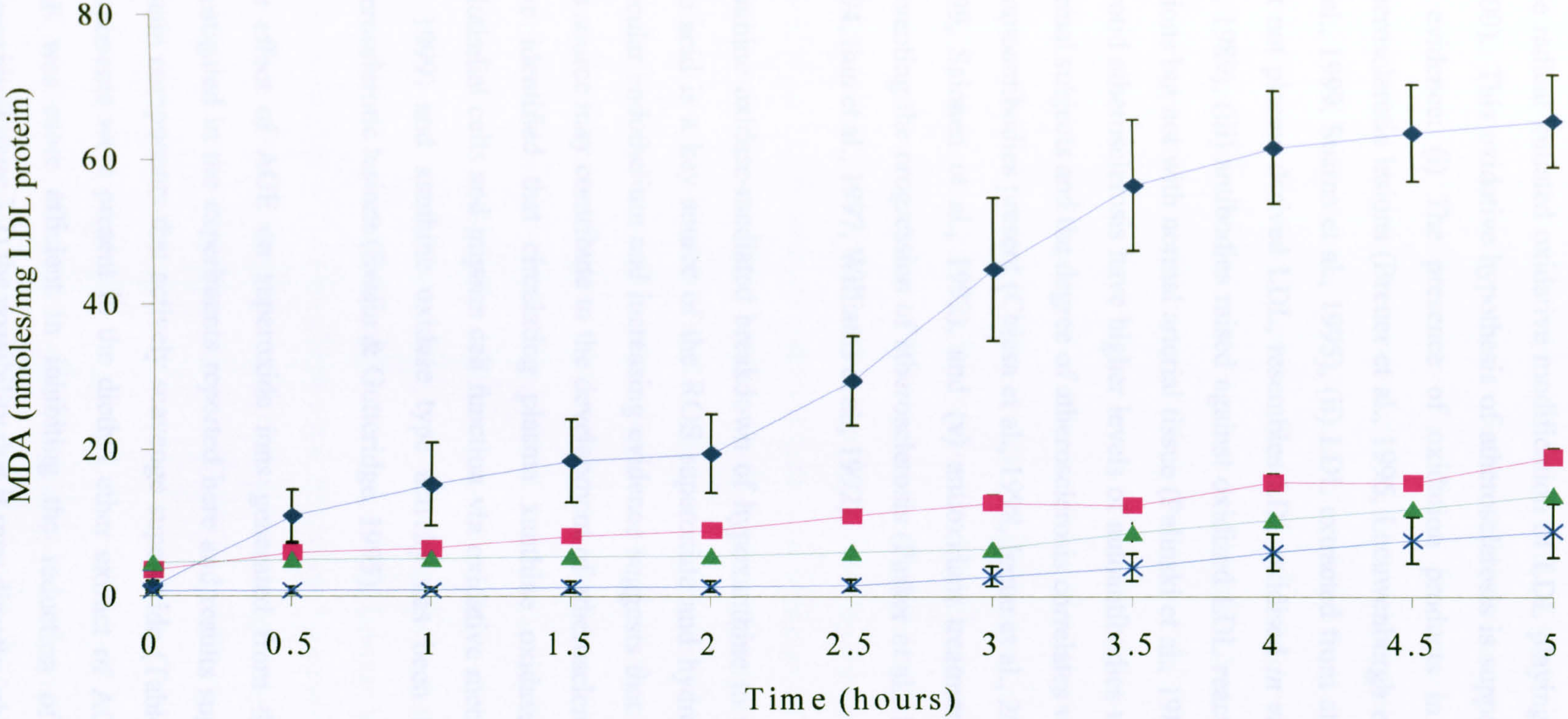


Figure 3.9. Time course for the oxidative modification of LDL induced by Cu^{2+} (♦) in the presence of 5% (■) and 10% (▲) diethyl ether extract of AGE (Ex). Control was LDL in PBS only (×). Values are means of three experiments ± SEM. Significant difference ($p<0.05$) from control (LDL + PBS) was observed from 1.5 h onwards in the presence of Cu^{2+}, and from 4 h onwards in the presence of Cu^{2+} and 5% or 10% diethyl ether extract of AGE. Significant difference ($p<0.05$) from control (LDL + Cu^{2+}) was observed from 3 h onwards in the presence of Cu^{2+} and 5% diethyl ether extract of AGE, and from 2.5 h onwards in the presence of Cu^{2+} and 10% diethyl ether extract of AGE.

3.3. DISCUSSION

Free radicals have been implicated in the pathogenesis of atherosclerosis with the free radical initiated oxidative modification of LDL playing a key role (Keaney, 2000). This oxidative hypothesis of atherosclerosis is supported by several lines of evidence: (i) The presence of oxidation products in human and animal atherosclerotic lesions (Breuer et al., 1996, Leeuwenburgh et al., 1997, Oguogho et al., 1999, Suarna et al., 1995), (ii) LDL extracted from atherosclerotic lesions, but not plasma derived LDL, resembles LDL oxidised *in vitro* (Yla-Herttuala et al., 1989), (iii) antibodies raised against oxidized LDL react with atherosclerotic lesions but not with normal arterial tissue (Palinski et al., 1989), (iv) patients with carotid atherosclerosis have higher levels of autoantibodies to oxidized LDL than normal subjects and the degree of atherosclerosis correlates with the concentration of autoantibodies present (Chiesa et al., 1998, Inoue et al., 2001, Lehtimaki et al., 1999, Salonen et al., 1992), and (v) antioxidant treatment is consistent with preventing the progression of atherosclerosis (Parker et al., 1995, Sasahara et al., 1994, Sun et al., 1997, Williams et al., 1992).

Xanthine oxidase-mediated breakdown of hypoxanthine to xanthine and then to uric acid is a key source of the ROS superoxide and hydrogen peroxide in the vascular endothelium and increasing evidence suggests that ROS generated from this source may contribute to the development of atherosclerosis. *In vitro* studies have identified that circulating plasma xanthine oxidase binds to vascular endothelial cells and impairs cell function via oxidative mechanisms (Houston et al., 1999) and xanthine oxidase type activity has been identified in human atherosclerotic lesions (Swain & Gutteridge, 1995).

The effect of AGE on superoxide ions generated from this source has been investigated in the experiments reported here and results suggest that AGE may contain components that actively scavenge superoxide (Table 3.1). These active components were present in the diethyl ether extract of AGE and while whole AGE was more efficient in inhibiting the reduction of cytochrome C by superoxide (Figure 3.2) the possibility that it may directly inhibit xanthine oxidase itself and not scavenge superoxide could not be excluded. These results

contradict other studies, which have demonstrated that AGE does not possess any significant superoxide scavenging ability (Ide et al., 1996). More comparative observations cannot be made between the methods and results presented here and those reported by Ide et al. (1996) due to absence of methods and data. However, this group has repeatedly demonstrated that AGE can scavenge the ROS hydrogen peroxide (Ide & Lau, 1999a, Ide et al., 1996). The results from the experiments presented here suggest that superoxide generated by xanthine-xanthine oxidase alone is not sufficient to induce oxidative modification of LDL (Figures 3.6 & 3.7) and it may be that superoxide interacts with other reactive species such as nitric oxide to produce extremely potent free radicals (i.e. peroxynitrite) capable of inducing LDL oxidation (Hogg & Kalyanaraman, 1998, Violi et al., 1999).

15-Lipoxygenase oxygenates PUFAs such as linoleic acid resulting in the formation of lipid peroxyl radicals. These lipid peroxyl radicals are capable of initiating the chain propagating process of lipid peroxidation by abstracting hydrogen from an adjacent PUFA generating a lipid hydroperoxide and a new PUFA radical. This PUFA radical propagates the lipid peroxidation process and the lipid hydroperoxide decomposes into a wide range of products capable of modifying proteins (Yamamoto, 1992). Substantial evidence now exists implicating 15-lipoxygenase in the pathogenesis of atherosclerosis. Using animal models of atherosclerosis increased expression of 15-lipoxygenase mRNA and protein and colocalization with oxidised LDL has been demonstrated in atherosclerotic lesions (Harats et al., 2000, Hiltunen et al., 1995, Yla-Herttuala et al., 1990). 15-lipoxygenase inhibitors and disruption of 15-lipoxygenase gene expression attenuates atherosclerosis in animal models of atherosclerosis (Bocan et al., 1998, Cyrus et al., 1999). Activation of 15-lipoxygenase by oxidised LDL has been demonstrated to be one of the mechanisms by which vascular endothelial cells may contribute to oxidation of LDL and lipoxygenase-type activity has been demonstrated in macrophages capable of oxidising LDL (Derian & Lewis, 1992, Rankin et al., 1991).

This present study demonstrates that AGE is an efficient inhibitor of 15-lipoxygenase-mediated peroxidation of linoleic acid using a cell free system and lipid peroxidation of isolated human LDL (Table 3.2 & Figures 3.3 & 3.6).

Oxidation of the protein component of LDL was not observed with 15-lipoxygenase (Figure 3.7). These inhibitory effects were observed using the diethyl ether extract of AGE and could be due to either to inhibition of 15-lipoxygenase itself or scavenging of lipid peroxyl radicals. In both cases the chain propagation process of lipid peroxidation mediated by 15-lipoxygenase would be terminated. The results presented here are one of the first studies that have examined the effect of AGE on 15-lipoxygenase mediated lipid peroxidation of linoleic acid and LDL.

Although there is little evidence for the actual involvement of Cu^{2+} in modifying LDL *in vivo,* atherosclerotic lesions have been shown to contain detectable amounts of redox reactive copper (Evans et al., 1995, Smith et al., 1992, Swain & Gutteridge, 1995). This catalytic copper may contribute to the pro-oxidant environment of the atherosclerotic lesion where extensive LDL oxidation takes place. *In vitro* studies have demonstrated that Cu^{2+} induces LDL oxidation by binding to discrete sites on apolipoprotein B-100 of LDL (Gieseg & Esterbauer, 1994, Kuzuya et al., 1992) where it becomes reduced by abstraction of a hydrogen atom from apolipoprotein B-100 itself (Lynch & Frei, 1995), or from preformed lipid hydroperoxides (Patel et al., 1997), or from α-tocopherol (Iwatsuki et al., 1995, Yoshida et al., 1994). The most important of these being lipid hydroperoxides, which decompose into products capable of initiating rapid lipid peroxidation and protein oxidation. Thus, the initial amount of lipid hydroperoxides present in LDL is important when examining oxidative modification induced by Cu^{2+}. Lengthy centrifugation processes requiring additional salt removal by dialysis is utilised in many LDL isolation procedures and this may result in artificially high levels of lipid hydroperoxides. In an effort to overcome this problem a novel method was employed where LDL was isolated within 3.5 h using iodixanol as the centrifugation medium. The presence of iodixanol which is inert and non-toxic in the LDL sub-fraction did not interfere with any of the subsequent assays therefore no dialysis step was required and the LDL was not subjected to harsh salt solutions (Graham et al., 1996, Lowe et al., 1999). This method has proven to be a less harsh and more rapid procedure for

the isolation of lipoprotein subfractions because the centrifugation step is only 3.5 h and a dialysis step was not required.

Oxidation of LDL by Cu^{2+} has become a routine method for assessing the antioxidant potential of dietary phytochemicals and the studies reported here confirm the efficacy of AGE in inhibiting this oxidation process. Whole AGE completely abolished the Cu^{2+}-induced electrophoretic shift in LDL, indicating there was no oxidative modification of the LDL lipoprotein (Figure 3.7). However, the diethyl ether extract of AGE only partially reversed the electrophoretic shift in LDL (Figure 3.7). The diethyl ether extract of AGE significantly reduced Cu^{2+}-induced lipid peroxidation of LDL as assessed by TBARS production to the extent that the propagation phase characteristic of Cu^{2+}-mediated lipid peroxidation of LDL (Esterbauer & Ramos, 1995) was completely abolished (Figure 3.9). These observations may reflect a different mechanism for whole AGE and the diethyl ether extract of AGE. The diethyl ether extract may act to preserve the lipid soluble antioxidants of LDL and hence prolong the lag phase of Cu^{2+}-mediated lipid peroxidation of LDL. These results are in agreement with other studies that have shown AGE to be an efficient inhibitor of Cu^{2+}-induced lipid peroxidation of LDL *in vitro* and following dietary supplementation (Ide et al., 1997, Munday et al., 1999). In contrast to these previous studies, the results presented here also confirm that AGE is an efficient inhibitor of Cu^{2+}-mediated protein oxidation of apolipoprotein B-100 in isolated LDL.

Collectively these results have demonstrated that AGE may inhibit the atherogenic oxidation of LDL by (i) scavenging of superoxide, (ii) inhibiting the formation of lipid hydroperoxides, and (iii) inhibiting Cu^{2+}-mediated oxidative modification of LDL by possibly two different mechanisms.

CHAPTER 4

THE ANTIOXIDANT MECHANISMS OF AGED GARLIC EXTRACT

4. THE ANTIOXIDANT MECHANISMS OF AGED GARLIC EXTRACT

4.1. INTRODUCTION

Epidemiological evidence, although inconclusive suggests that a diet rich in antioxidants such as vitamin E, vitamin C, β-carotene and flavonoids may play a role in preventing the development and progression of atherosclerosis (Ascherio et al., 1999, Geleijnse et al., 1999, Hennekens et al., 1996, Hertog et al., 1993, Klipstein-Grobusch et al., 1999, Knekt et al., 1994, 1996, Kushi et al., 1996, Rimm et al., 1993, Stampfer et al., 1993). The anti-atherogenic properties exhibited by these compounds may be attributed to their ability to prevent LDL oxidation. Many biochemical studies have focused on this using single dietary antioxidants and there is evidence to show that their presence increases the *in vitro* resistance of LDL to oxidation (Aviram & Fuhrman, 1998, Bowen et al., 1998, Jialal & Fuller, 1995).

Studies suggest that plant polyphenols such as the flavonoids are potent anti-atherosclerotic and antioxidant compounds both *in vitro* and *in vivo*. They have been shown to scavenge reactive species, chelate metal ions, and inhibit enzymes implicated in the pathogenesis of atherosclerosis (Brown et al., 1998, Chang, 1993, Hanasaki et al., 1994, Miller et al., 1996, Robak, 1996). Their consumption by humans and atherosclerosis-prone mice significantly reduces the susceptibility of isolated LDL to lipid peroxidation (Aviram et al., 2000, Belinky et al., 1998, Fuhrman et al., 1997d, Hayek et al., 1997, Hodgson et al., 2000), and the suggested binding or absorption of plant extracts and plant flavonoids to LDL may explain their effectiveness in inhibiting the oxidation of LDL (Belinky et al., 1998, Grassmann et al., 2001, Hayek et al., 1997, Ivanov et al., 2001, Vinson et al., 1995, 1998).

Garlic is a rich source of phenolic compounds (Vinson et al., 1998). Numerous phenolic acids have been identified which include p-hydroxybenzoic acid, caffeic acid, ferulic acid, vanillic acid, sinapinic acid, p-coumaric acid, and salicylic acid (Lawson, 1996, Swain et al., 1995). The concentration of these phenolic acids ranges from 1-13μg/g fresh garlic. More recently, flavonoids have been identified

and quantified in whole garlic bulbs following acid-hydrolysis pre-treatment (Miean & Mohamed, 2001). The flavonoids identified include quercetin (47μg/g), apigenin (217μg/g), and myricetin (693μg/g). There is considerable interest in plant polyphenolic flavonoids and phenolic acids due to their antioxidant activity and protective effect against the development of cardiovascular disease (Rice-Evans, 1995, Visioli et al, 2000a). In spite of this, and the relatively high and varied phenolic content of garlic, little work has been reported which attributes any of the antioxidant effects of garlic and its preparations to its phenolic components.

Many studies have focused on the efficacy of plant extracts in inhibiting oxidative modification of LDL by Cu^{2+}. However, with regards to whole plant extracts identifying how these extracts interfere with Cu^{2+}-mediated oxidation of LDL is rarely assessed. AGE and one of its antioxidant constituents *S*-allylcysteine (SAC) inhibit the *in vitro* lipid peroxidation of isolated LDL by Cu^{2+} (Ho et al., 2001, Ide et al., 1997). Dietary intervention with AGE or garlic powder extracts has been shown to reduce the susceptibility of isolated LDL to undergo lipid peroxidation by extending the lag time of Cu^{2+}-induced lipid peroxidation (Munday et al., 1999, Orekhov et al., 1996, Phelps & Harris, 1993). This observed elongation of the lag time of Cu^{2+}-induced oxidation suggests that garlic extracts may act to prevent depletion of endogenous LDL antioxidants. Other studies have assessed the ability of pure plant phenolic compounds to bind Cu^{2+} and hence prevent any initiation of LDL oxidation using spectroscopic assays which are unsuitable for whole plant extracts (Brown et al., 1998, Miller et al., 1996). However, the role of garlic extracts in chelating unbound Cu^{2+} or preventing the depletion of endogenous LDL antioxidants has yet to be addressed.

The aim of the study reported in this chapter was to investigate the antioxidant properties of AGE, and the mechanisms by which AGE exerts its antioxidant effects. Initial studies addressed the role of AGE in chelating unbound Cu^{2+}. Subsequent studies analysed the phenolic content of AGE using acid-hydrolysed extracts, spectrophotometric assays, thin-layer chromatography (TLC), and high-pressure liquid-chromatography (HPLC). Finally, the role of AGE in inhibiting

Cu^{2+}-mediated oxidation of LDL by preventing the depletion of endogenous LDL antioxidants and binding directly to LDL was addressed. The studies are shown schematically in Figure 4.1.

4.1.1. Overview of Study

The copper chelating properties of AGE were assessed using a novel approach based on restoring the activity of xanthine oxidase. Xanthine oxidase activity monitored via the production of superoxide or uric acid is completely inhibited in the presence of 50μM $CuSO_4$. If whole AGE or the diethyl ether extract of AGE should chelate Cu^{2+} then xanthine oxidase activity would be restored (see Chapter 2, pp 43 for detailed methods).

AGE was assessed for its ability to prevent the depletion of endogenous LDL antioxidants during Cu^{2+}-mediated oxidation. Prior to extraction of antioxidants, LDL was oxidised in the presence and absence of the diethyl ether extract of AGE and samples were removed between 0-4 h and subsequently analysed for TBARS. α-Tocopherol and carotenoids were extracted from LDL and analysed by HPLC at 290nm and 460nm, respectively (see Chapter 2, pp 48-49 for detailed methods).

The nature of the phenolic content of AGE was assessed using quantitative and qualitative methods. Acid-hydrolysed extracts were compared with untreated extracts. The amount of phenolics in AGE was assessed using the Folin-Ciocalteu phenol assay and more qualitative information on the phenolics in AGE was assessed by TLC analysis using ferric chloride/UV detection systems. Phenolic compounds have a characteristic absorbance at 280nm and AGE was examined at this wavelength by HPLC (see Chapter 2, pp 49-51 for detailed methods).

AGE was assessed for its ability to directly bind to LDL and hence reduce the oxidative susceptibility of LDL to Cu^{2+} mediated oxidation. Whole plasma was incubated in the presence and absence of whole AGE and the diethyl ether extract of AGE prior to LDL isolation. Isolated LDL was subsequently oxidized by Cu^{2+} and samples were removed every 30 min and subsequently analysed for TBARS. (see Chapter 2, pp 45, 48 for detailed methods).

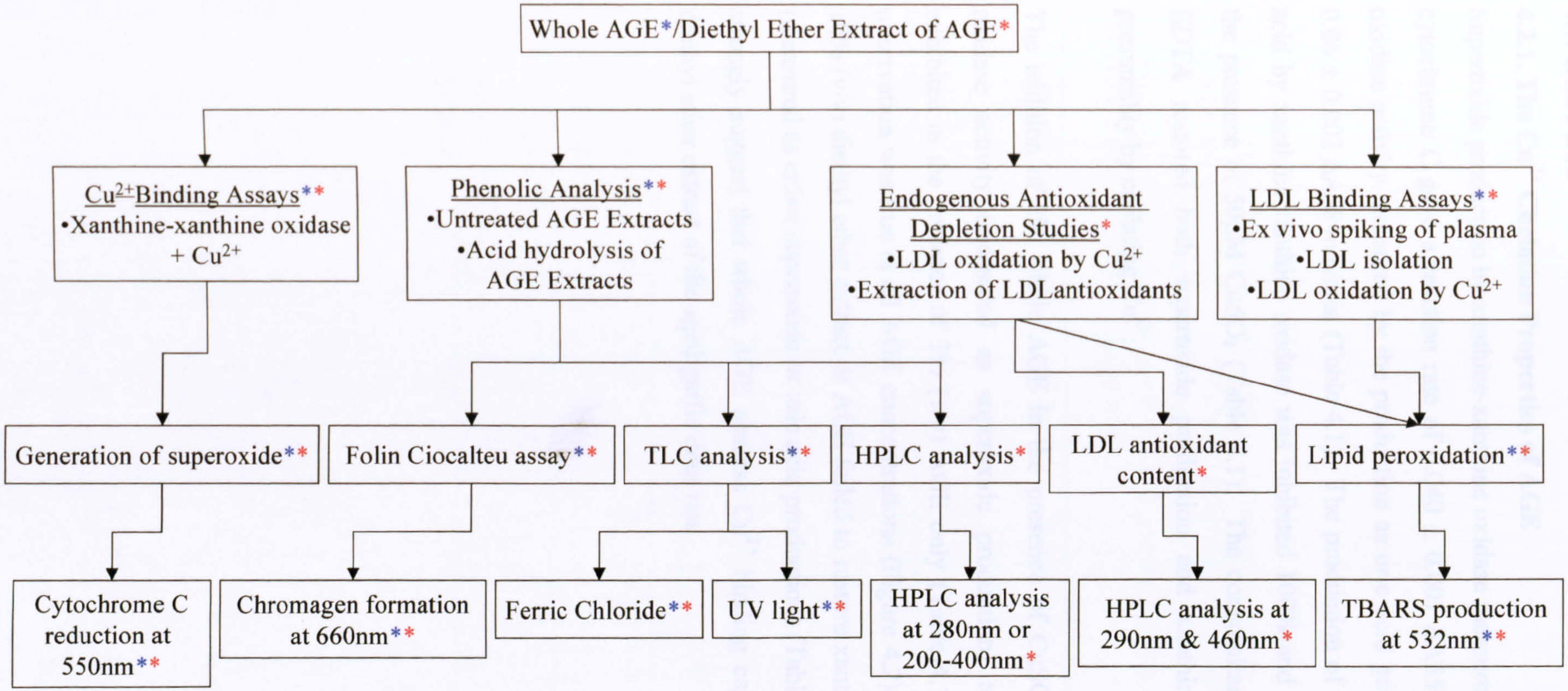


Figure 4.1. Overview of study.
The assays employed for assessing the antioxidant properties and mechanisms of antioxidant effect of whole AGE (*) and the diethyl ether extract of AGE (*). Due to the complex nature of whole AGE analyses involving HPLC analysis could not be employed.

4.1. RESULTS

4.2.1. The Cu^{2+} Chelation Properties of AGE

Superoxide production by xanthine-xanthine oxidase measured as the reduction of cytochrome C gave a reaction rate of 0.040 ± 0.001 ΔA550nm/min. Xanthine oxidase activity measured by the production as uric acid gave a reaction rate of 0.06 ± 0.002 ΔA284nm/min (Table 4.1). The production of superoxide, and uric acid by xanthine-xanthine oxidase was inhibited 100% and 63% respectively in the presence of 50μM $CuSO_4$ (Table 4.1). The concomitant addition of 60μM EDTA restored both superoxide production and xanthine oxidase activity, presumably by chelating Cu^{2+}.

The addition of 2% whole AGE in the presence of $CuSO_4$ restored xanthine oxidase activity (measured as superoxide production) to comparable rates exhibited in the presence of 2% (v/v) AGE only (Table 4.1, Figure 4.2). This observation was true at all AGE concentrations (Figure 4.2). In contrast, 2% or 10% (v/v) diethyl ether extract of AGE failed to restore xanthine oxidase activity (measured as either superoxide or uric acid production) (Table 4.1). These results strongly suggest that whole AGE possess Cu^{2+} binding capabilities, whilst the diethyl ether extract of the aged garlic does not.

Table 4.1. The effect of whole AGE, a diethyl ether extract of AGE, and EDTA on superoxide and uric acid production by xanthine-xanthine oxidase (X-XO) in the presence of Cu^{2+}. Values are means of three experiments ± SEM. Significant differences ($p < 0.05$) from control[1] (X-XO) are indicated by * and from control[2] (X-XO + 50μM $CuSO_4$) are indicated by **.

Extract/Agent	$CuSO_4$ (50μM)	Superoxide Production (ΔA550nm/min)	Uric Acid Production (ΔA284nm/min)
NONE[1]	-	0.040 ± 0.001	0.060 ± 0.002
2% Whole AGE	-	0.024 ± 0.001*	ND
NONE[2]	+	0*	0.022 ± 0.002*
60μM EDTA	+	0.039 ± 0.004**	0.062 ± 0.003**
2% Whole AGE	+	0.026 ± 0.002*,**	ND
2% Diethyl Ether Extract of AGE	+	0*	0.025 ± 0.001*
10% Diethyl Ether Extract of AGE	+	0*	0.025 ± 0.003*

ND: Not Determined

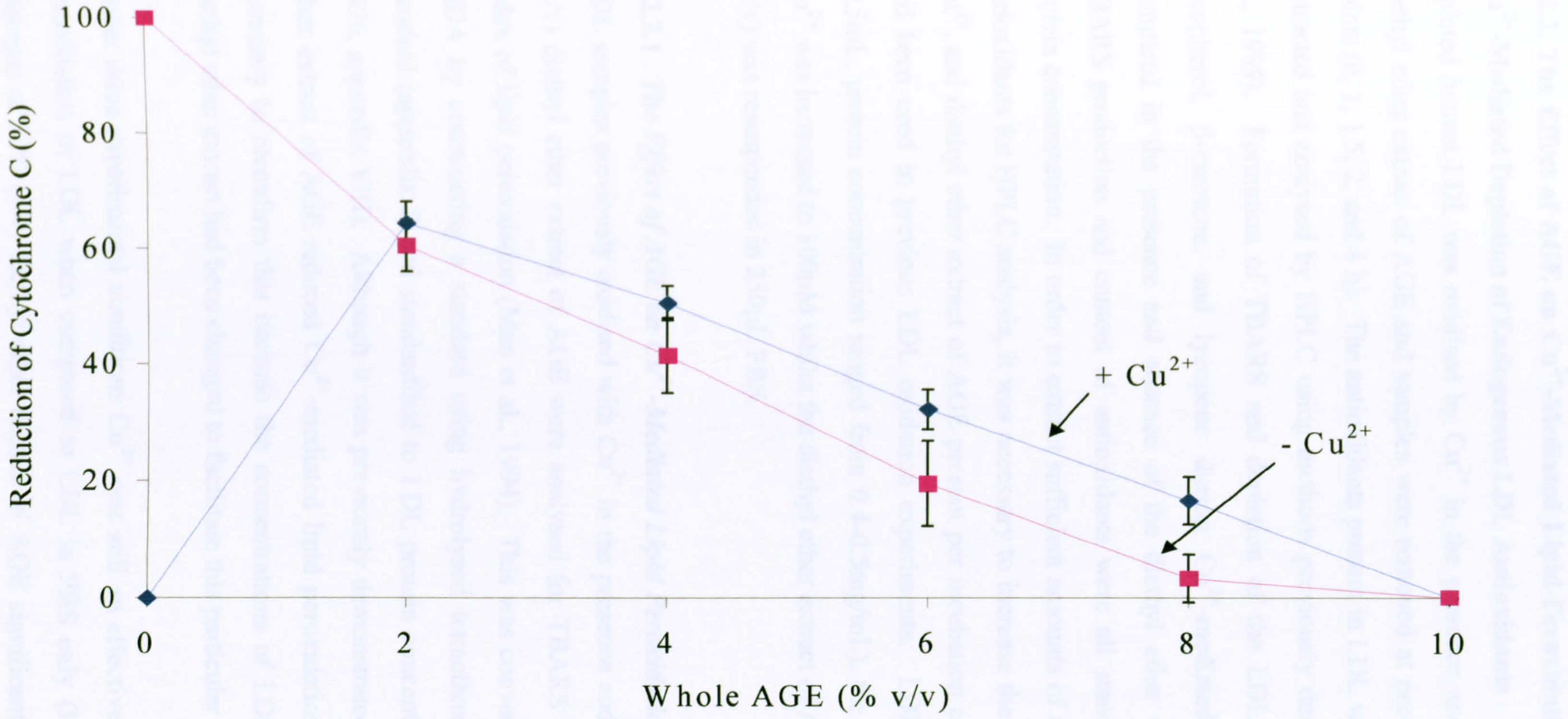


Figure 4.2. The effect of whole AGE on Cu^{2+}-mediated inhibition of reduction of cytochrome C by superoxide ions generated from xanthine-xanthine oxidase. The restoration of superoxide-mediated reduction of cytochrome C by whole AGE at 2% - 10% (v/v) by chelation of Cu^{2+} (◆) was not significantly different and comparable to that observed in the presence of 2% -10% (v/v) whole AGE only (■). Values are the means of three experiments ± SEM.

4.2.2. The Effect of AGE on Cu^{2+}-Mediated Lipid Peroxidation of LDL and Cu^{2+}-Mediated Depletion of Endogenous LDL Antioxidants

Isolated human LDL was oxidised by Cu^{2+} in the presence and absence of the diethyl ether extract of AGE and samples were removed at pre-determined time points (0, 1, 1.5, 2, and 4 h). The antioxidants present in LDL were subsequently extracted and analysed by HPLC using methods previously described (Lowe et al., 1999). Formation of TBARS and depletion of the LDL antioxidants α-tocopherol, β-carotene, and lycopene during Cu^{2+}-mediated oxidation was compared in the presence and absence of the diethyl ether extract of AGE. TBARS production and content of antioxidants were all standardised to LDL protein concentration. In order to extract sufficient amounts of endogenous LDL antioxidants for HPLC analysis, it was necessary to increase the amount of LDL, Cu^{2+}, and diethyl ether extract of AGE present per incubation compared to what had been used in previous LDL oxidation experiments. LDL was used neat (0.5mL, protein concentration ranged from 0.4-0.5mg/mL), the concentration of Cu^{2+} was increased to 100μM whilst the diethyl ether extract of AGE used at 10% (v/v) was resuspended in 250μL PBS.

4.2.2.1 . *The Effect of AGE on Cu^{2+}-Mediated Lipid Peroxidation of LDL*

LDL samples previously oxidised with Cu^{2+} in the presence and absence of 10% (v/v) diethyl ether extract of AGE were analysed for TBARS production as an index of lipid peroxidation (Mao et al., 1994). This was converted to nmoles of MDA by constructing a standard using hydrolysed tetraethoxypropane as the standard (appendix I) and standardised to LDL protein concentration (Bradford, 1976, appendix VIII). Although it was previously demonstrated that the diethyl ether extract of AGE reduced Cu^{2+}-mediated lipid peroxidation of LDL it was necessary to reconfirm this because the concentrations of LDL, Cu^{2+}, and the diethyl ether extract had been changed to facilitate this particular experiment.

Under these experimental conditions Cu^{2+} was still an effective initiator of lipid peroxidation of LDL when compared to LDL in PBS only (Figure 4.3). The presence of 10% (v/v) diethyl ether extract of AGE significantly reduced Cu^{2+}-mediated lipid peroxidation by 23% at 2 hours (15.17 ± 2.21 nmol MDA/mg LDL

protein vs. 12.09 ± 0.89 nmol MDA/mg LDL protein, $p<0.05$), and 44% at 4 hours (30.37 ± 4.04 nmol MDA/mg LDL protein vs. 17.06 ± 2.48 nmol MDA/mg LDL protein, $p<0.05$) (Figure 4.3). This inhibition of lipid peroxidation observed in the presence of the diethyl ether extract of AGE and Cu^{2+} remained significantly different from LDL in PBS only ($p<0.05$, Figure 4.3).

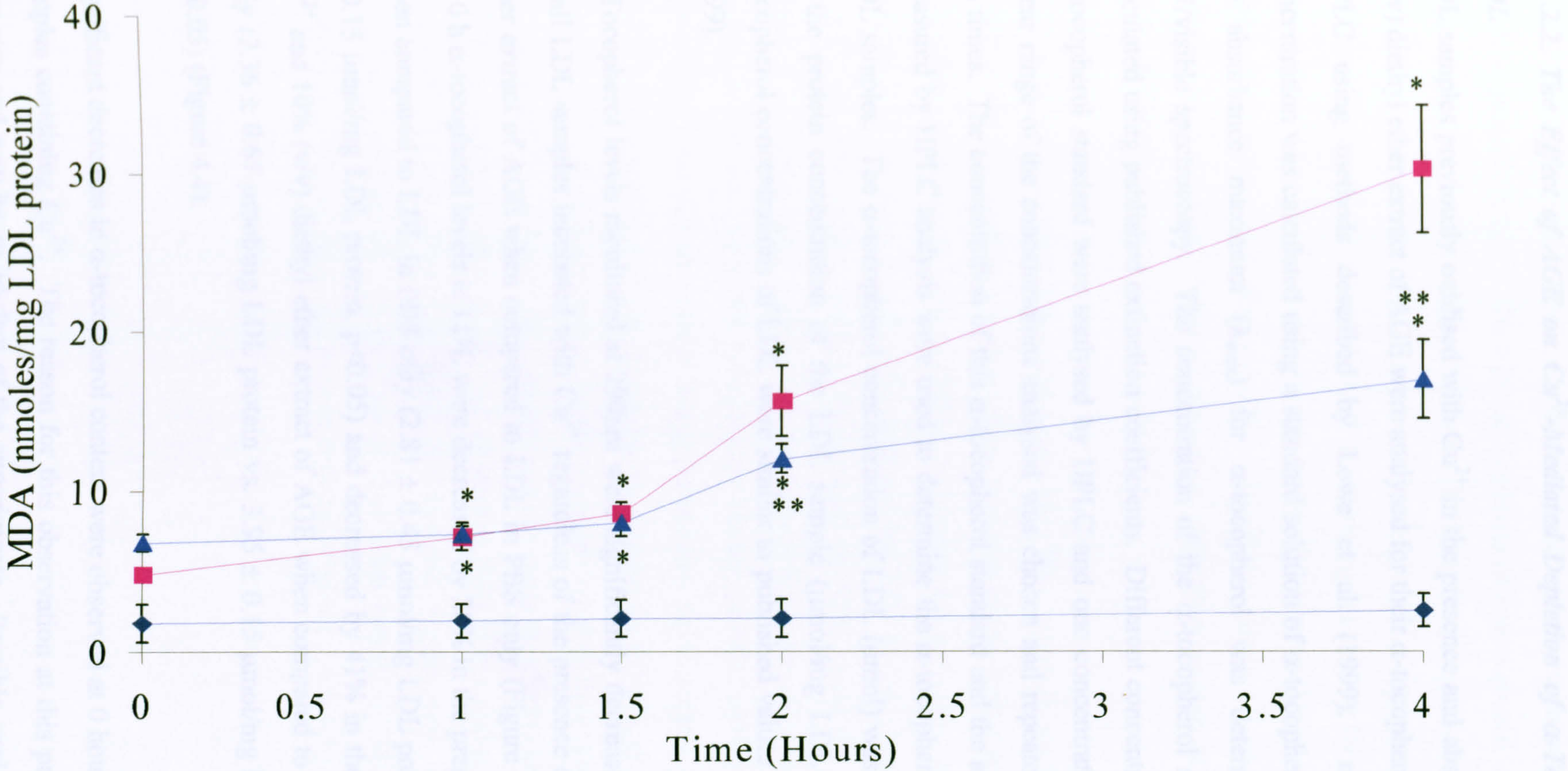


Figure 4.3. Time course for the oxidative modification of LDL induced by Cu^{2+} (■) and in the presence of 10% (v/v) diethyl ether extract of AGE (▲). Control was LDL in PBS only (◆). Values are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *, and control (LDL + Cu^{2+}) are indicated by **.

4.2.2.2. *The Effect of AGE on Cu^{2+}-Mediated Depletion of α-Tocopherol in LDL*

LDL samples previously oxidised with Cu^{2+} in the presence and absence of 10% (v/v) diethyl ether extract of AGE were analysed for their α-tocopherol content by HPLC using methods described by Lowe et al. (1999). α-Tocopherol concentration was calculated using a standard solution of α-tocopherol. In brief, the absorbance maximum (λ_{max}) for α-tocopherol was determined using UV/visible spectroscopy. The concentration of the α-tocopherol standard was calculated using published extinction coefficients. Different concentrations of the α-tocopherol standard were analysed by HPLC and one concentration from the linear range of the concentrations analysed was chosen and repeatedly analysed ten times. The concentration of this α-tocopherol standard and the arbitrary units measured by HPLC analysis were used to determine the α-tocopherol content of LDL samples. The α-tocopherol concentration of LDL (μmol) was standardised to the protein concentration of the LDL sample (μmol/mg LDL protein). α-Tocopherol concentrations of LDL were similar to published values (Lowe et al., 1999).

α-Tocopherol levels monitored at 290nm were significantly decreased at 0 hours in all LDL samples incubated with Cu^{2+} regardless of the presence of the diethyl ether extract of AGE when compared to LDL in PBS only (Figure 4.4, $p<0.05$). At 0 h α-tocopherol levels in LDL were decreased by 28% in the presence of Cu^{2+} when compared to LDL in PBS only (2.81 ± 0.45 μmol/mg LDL protein vs. 3.95 ± 0.15 μmol/mg LDL protein, $p<0.05$) and decreased by 41% in the presence of Cu^{2+} and 10% (v/v) diethyl ether extract of AGE when compared to LDL in PBS only (2.36 ± 0.67 μmol/mg LDL protein vs. 3.95 ± 0.15 μmol/mg LDL protein, $p<0.05$) (Figure 4.4).

Significant decreases in α-tocopherol content were observed at 0 hours in all LDL samples containing Cu^{2+}. The reason for this observation at this present time is unknown and may be an artefact of the experiments. Possible explanations may be that rapid freezing did not result in rapid termination of Cu^{2+}-induced oxidation

of LDL and/or Cu^{2+} may have reacted extremely rapidly with preformed lipid peroxides present in LDL. This initial depletion of α-tocopherol observed at 0 hours in all samples containing Cu^{2+} was also true for β-carotene and lycopene (Figures 4.6 & 4.8). Further analysis will be required to explain the reason for this observation. The addition of a Cu^{2+} chelating agent such as EDTA may have been more suitable for these experiments were high concentrations of Cu^{2+} were employed to induce oxidation of LDL. For these reason all results are also presented as percentage change compared to respective controls at 0 h (Figures 4.5, 4.7 & 4.9).

α-Tocopherol levels were decreased at all other time points in all LDL samples incubated with Cu^{2+} again regardless of the presence of the diethyl ether extract of AGE when compared to LDL in PBS only (Figure 4.4, $p<0.05$). At 2 hours α-tocopherol levels were significantly depleted by 70% in the presence of Cu^{2+} ($p=0.03$) and even more significantly depleted by 92% when the diethyl ether extract of AGE was present ($p=0.01$). At 4 hours depletion of α-tocopherol was maximal in the presence of the diethyl ether extract of AGE and/or Cu^{2+}. When values were presented as percent change compared to respective controls at 0 hours α-tocopherol levels were depleted in the presence of Cu^{2+} and this depletion appeared to be slightly enhanced by the presence of a diethyl ether, extract of AGE. (Figure 4.5). These results suggest that although the diethyl ether extract of AGE is an efficient inhibitor of Cu^{2+}-mediated lipid peroxidation of LDL the mechanism of this protection does not involve preservation of the LDL antioxidant α-tocopherol. In fact, the diethyl ether extract of AGE appears to enhance Cu^{2+}-induced depletion of α-tocopherol (Figures 4.4 & 4.5).

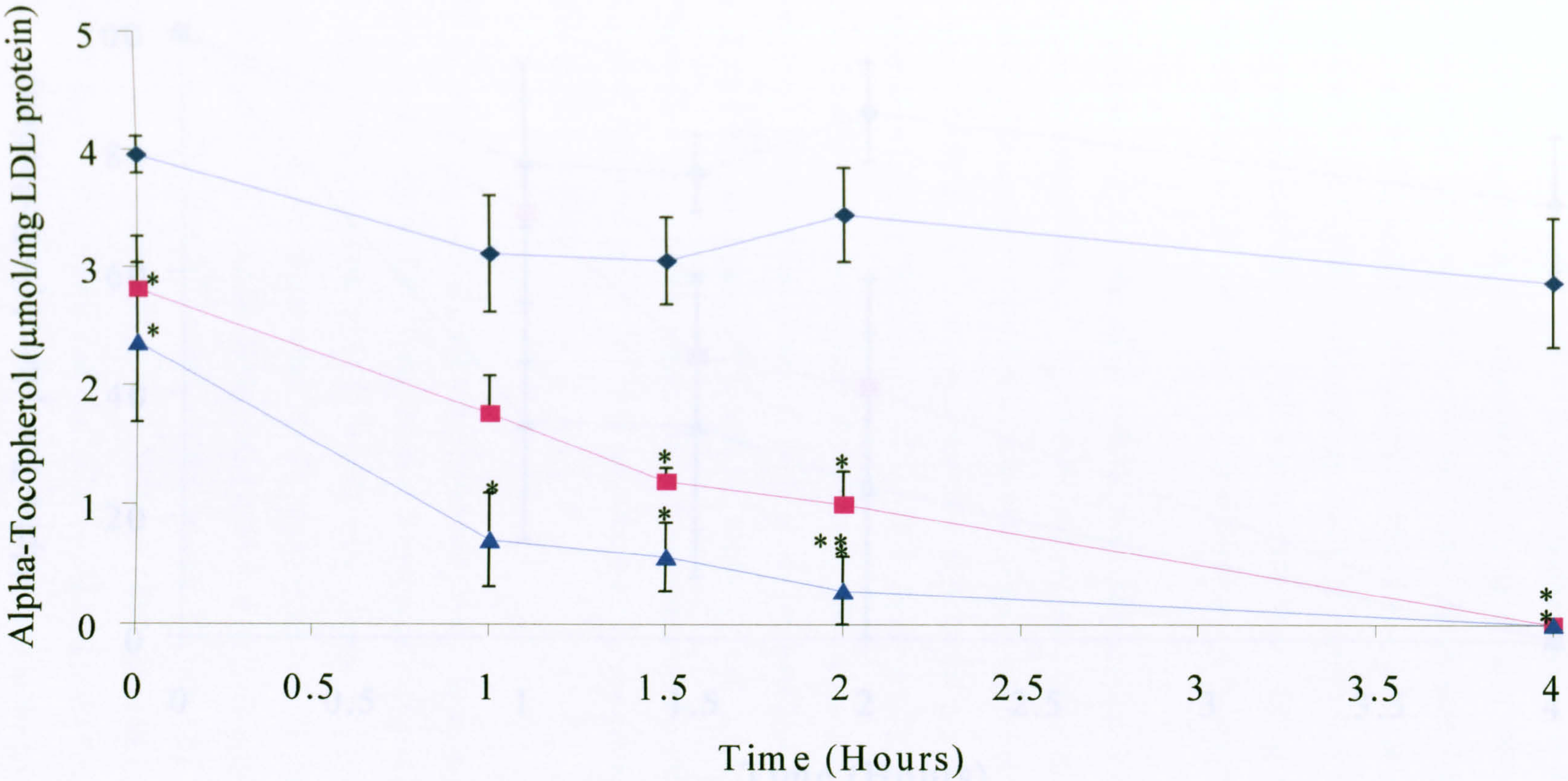


Figure 4.4. Time course for the depletion of α-tocopherol during the oxidative modification of LDL induced by Cu^{2+} (■) and in the presence of 10% (v/v) diethyl ether extract of AGE (▲). Control was LDL in PBS only (◆). Values are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *, and control (LDL + Cu^{2+}) are indicated by **.

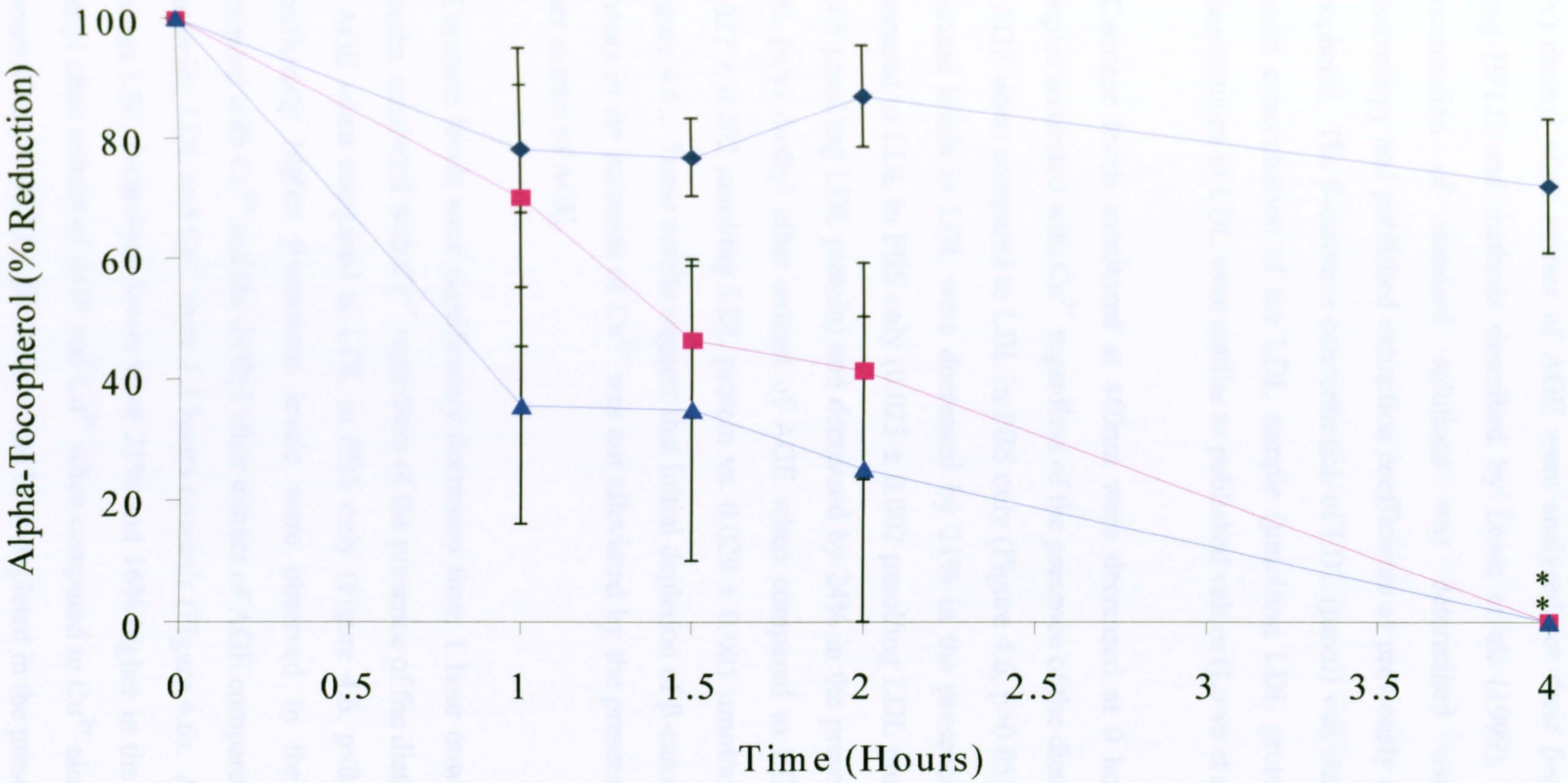


Figure 4.5. Change in α-Tocopherol Levels at all Time Points Expressed as Percent Reduction Compared with Respective Controls at 0 hours. Control was LDL in PBS only (◆), LDL + Cu^{2+} (■), and LDL + Cu^{2+} + 10% (v/v) diethyl ether extract of AGE (▲). Values are presented as % change compared to respective controls at 0 hours and are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *.

4.2.2.3. *The Effect of AGE on Cu^{2+}-Mediated Depletion of β-Carotene in LDL*

LDL samples previously oxidised with Cu^{2+} in the presence and absence of 10% (v/v) diethyl ether extract of AGE were analysed for their β-carotene content using HPLC and methods described by Lowe et al. (1999). The β-carotene concentration of standard solutions was determined using UV/visible spectroscopy and published extinction coefficients as previously described for α-tocopherol. The β-carotene concentration of LDL (μmol) was standardised to the protein concentration of the LDL sample (μmol/mg LDL protein). β-Carotene concentrations of LDL were similar to published values (Lowe et al., 1999).

β-Carotene levels monitored at 460nm were decreased at 0 hours in all LDL samples incubated with Cu^{2+} regardless of the presence of the diethyl ether extract of AGE when compared to LDL in PBS only (Figure 4.6, $p>0.05$). At 0 hours β-carotene levels in LDL were decreased by 21% in the presence of Cu^{2+} when compared to LDL in PBS only (0.023 ± 0.002 μmol/mg LDL protein vs. 0.029 ± 0.005 μmol/mg LDL protein) and decreased by 24% in the presence of Cu^{2+} and 10% (v/v) diethyl ether extract of AGE when compared to LDL in PBS only (0.022 ± 0.002 μmol/mg LDL protein vs. 0.029 ± 0.005 μmol/mg LDL protein) (Figure 4.6). These results suggest that initial depletion of β-carotene observed at 0 hours in the presence of Cu^{2+} was not alleviated by the presence of the diethyl ether extract of AGE.

β-Carotene levels were significantly decreased from 1 hour onwards in all LDL samples incubated with Cu^{2+} regardless of the presence of the diethyl ether extract of AGE when compared to LDL in PBS only (Figure 4.6, $p<0.05$). However, significantly higher β-carotene levels were observed in the LDL samples incubated with Cu^{2+} and the diethyl ether extract of AGE compared to the samples containing LDL and Cu^{2+} from 1.5 hours onwards (Figure 4.6). At 1.5 hours and 2 hours LDL β-carotene levels were 21% and 16% higher in the presence of the diethyl ether extract of AGE and Cu^{2+} when compared to Cu^{2+} alone ($p<0.05$). At 4 hours virtually all LDL β-carotene had been depleted in the presence of Cu^{2+} but was 82% higher when the diethyl ether extract of AGE was present (0.004 ± 0.001

μmol/mg LDL protein vs. 0.001 ± 0.001 μmol/mg LDL protein, $p<0.05$) (Figure 4.6). When values were presented as percent change compared to respective controls at 0 hour's β-carotene was gradually depleted from 1 hour onwards but this depletion was significantly prevented by the diethyl ether extract of AGE from 1.5 hours onwards (Figure 4.7). These results suggest that the diethyl ether extract of AGE was an efficient inhibitor of Cu^{2+}-mediated lipid peroxidation of LDL and the mechanism of this protection may involve preservation of the LDL antioxidant β-carotene.

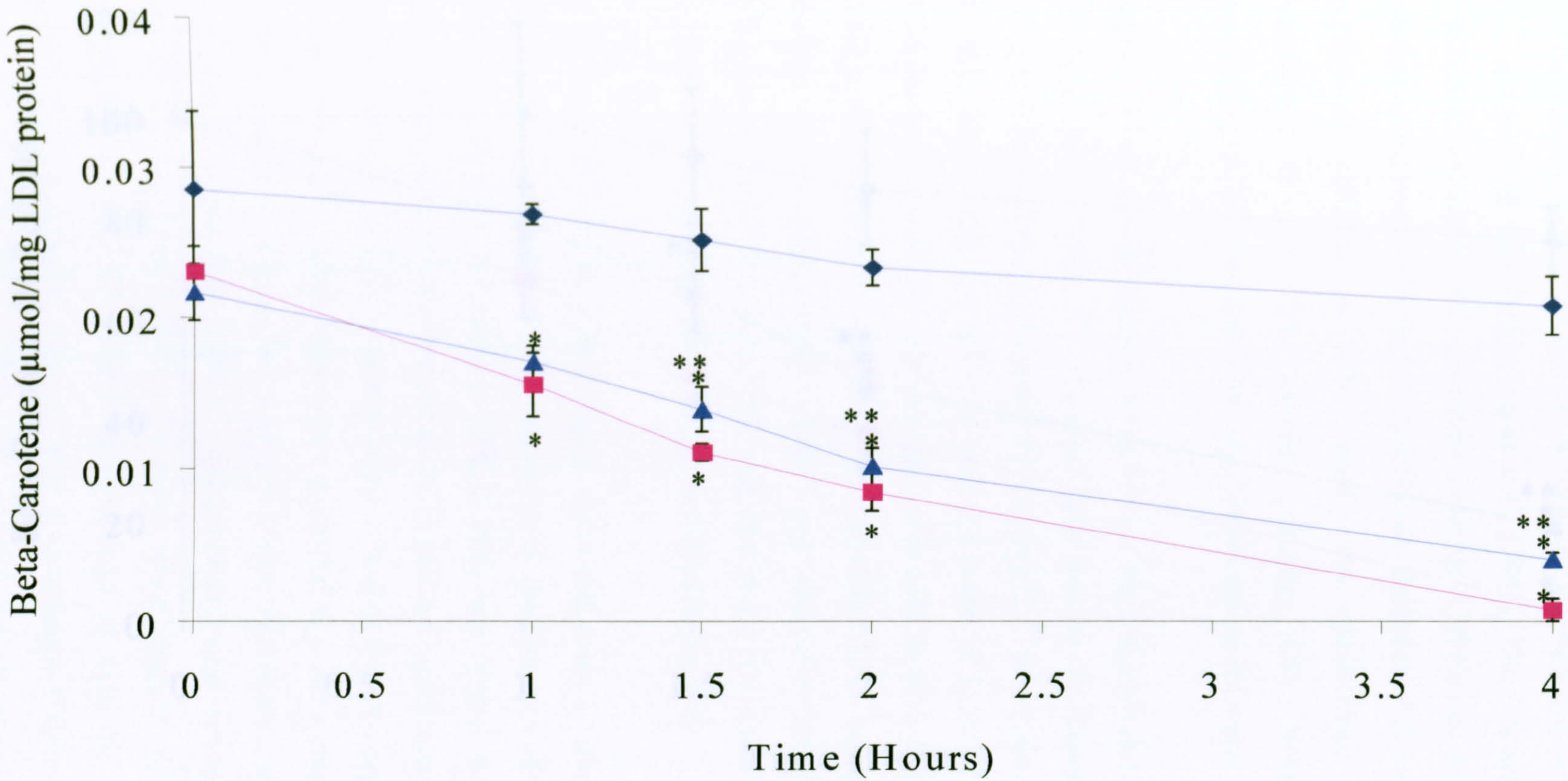


Figure 4.6. Time course for the depletion of β-carotene during oxidative modification of LDL induced by Cu^{2+} (■) and in the presence of 10% (v/v) diethyl ether extract of AGE (▲). Control was LDL in PBS only (◆). Values are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *, and control (LDL + Cu^{2+}) are indicated by **.

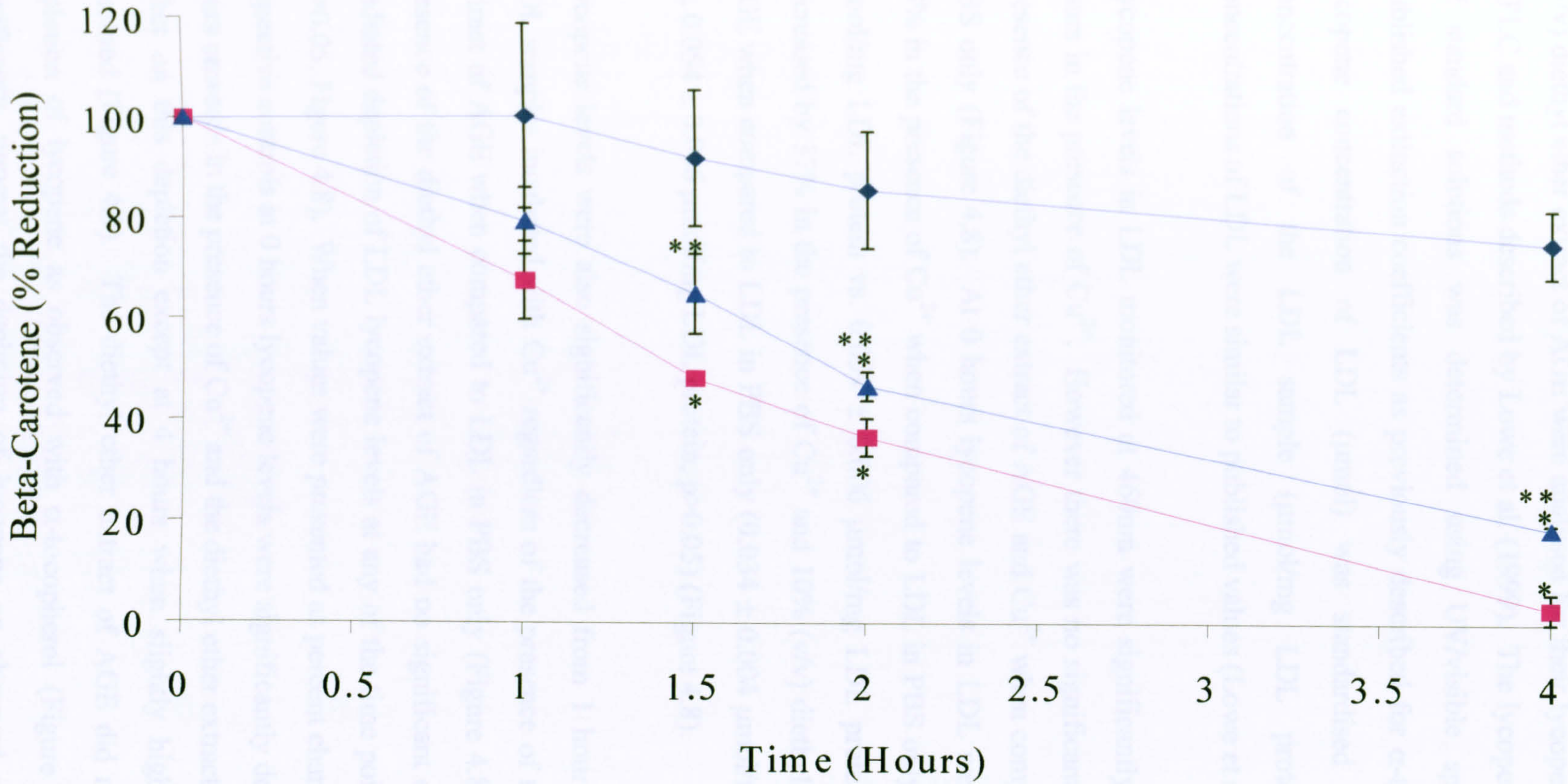


Figure 4.7. Change in β-carotene Levels at all Time Points Expressed as Percent Reduction Compared with Respective Controls at 0 hours. Control was LDL in PBS only (◆), LDL + Cu^{2+} (■), and LDL + Cu^{2+} + 10% (v/v) diethyl ether extract of AGE (▲). Values are presented as % change compared to respective controls at 0 hours and are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *, and control (LDL + Cu^{2+}) are indicated by **.

4.2.2.4. *The Effect of AGE on Cu^{2+}-Mediated Depletion of Lycopene in LDL*

LDL samples previously oxidised with Cu^{2+} in the presence and absence of 10% (v/v) diethyl ether extract of AGE were analysed for their lycopene content using HPLC and methods described by Lowe et al. (1999). The lycopene concentration of standard solutions was determined using UV/visible spectroscopy and published extinction coefficients as previously described for α-tocopherol. The lycopene concentration of LDL (μmol) was standardised to the protein concentration of the LDL sample (μmol/mg LDL protein). Lycopene concentrations of LDL were similar to published values (Lowe et al., 1999).

Lycopene levels in LDL monitored at 460nm were significantly decreased at 0 hours in the presence of Cu^{2+}. However there was no significant decrease in the presence of the diethyl ether extract of AGE and Cu^{2+} when compared to LDL in PBS only (Figure 4.8). At 0 hours lycopene levels in LDL were decreased by 43% in the presence of Cu^{2+} when compared to LDL in PBS only (0.031 ± 0.002 μmol/mg LDL protein vs. 0.054 ± 0.006 μmol/mg LDL protein, $p<0.05$) and decreased by 37% in the presence of Cu^{2+} and 10% (v/v) diethyl ether extract of AGE when compared to LDL in PBS only (0.034 ± 0.004 μmol/mg LDL protein vs. 0.054 ± 0.006 μmol/mg LDL protein, $p>0.05$) (Figure 4.8).

Lycopene levels were also significantly decreased from 1 hour onwards in all LDL samples incubated with Cu^{2+} regardless of the presence of the diethyl ether extract of AGE when compared to LDL in PBS only (Figure 4.8, $p<0.05$). The presence of the diethyl ether extract of AGE had no significant effects on Cu^{2+}-mediated depletion of LDL lycopene levels at any of the time points investigated ($p>0.05$, Figure 4.8). When values were presented as percent change compared to respective controls at 0 hours lycopene levels were significantly depleted from 1.5 hours onwards in the presence of Cu^{2+} and the diethyl ether extract of AGE had no effect on this depletion except at 4 hours when slightly higher levels were observed (Figure 4.9). The diethyl ether extract of AGE did not enhance the depletion of lycopene as observed with α-tocopherol (Figure 4.4 & 4.5) or significantly prevent the depletion of lycopene as observed with β-carotene (Figure 4.6 & 4.7).

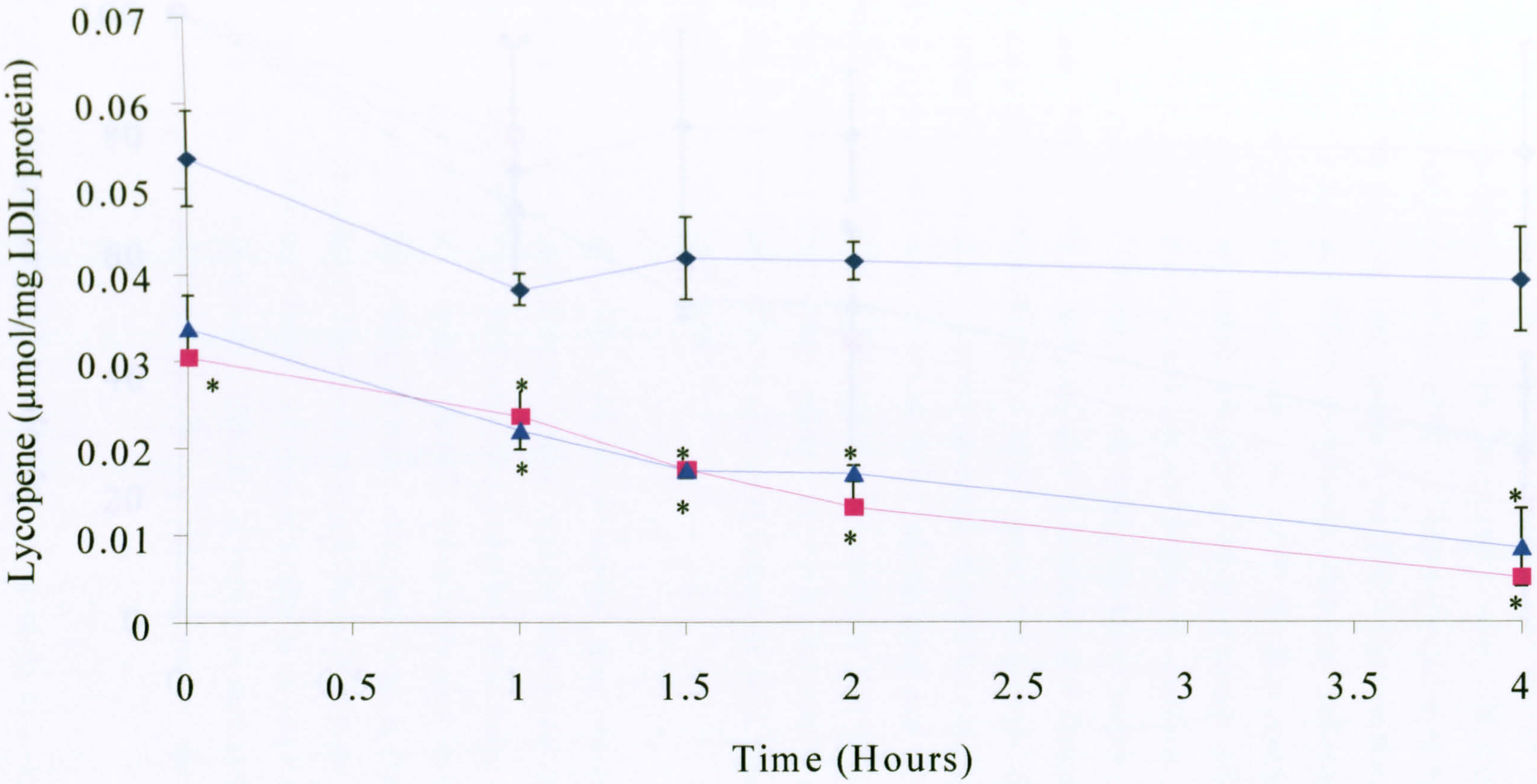


Figure 4.8. Time course for the depletion of lycopene during oxidative modification of LDL induced by Cu^{2+} (■) and in the presence of 10% (v/v) diethyl ether extract of AGE (▲). Control was LDL in PBS only (◆). Values are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *.

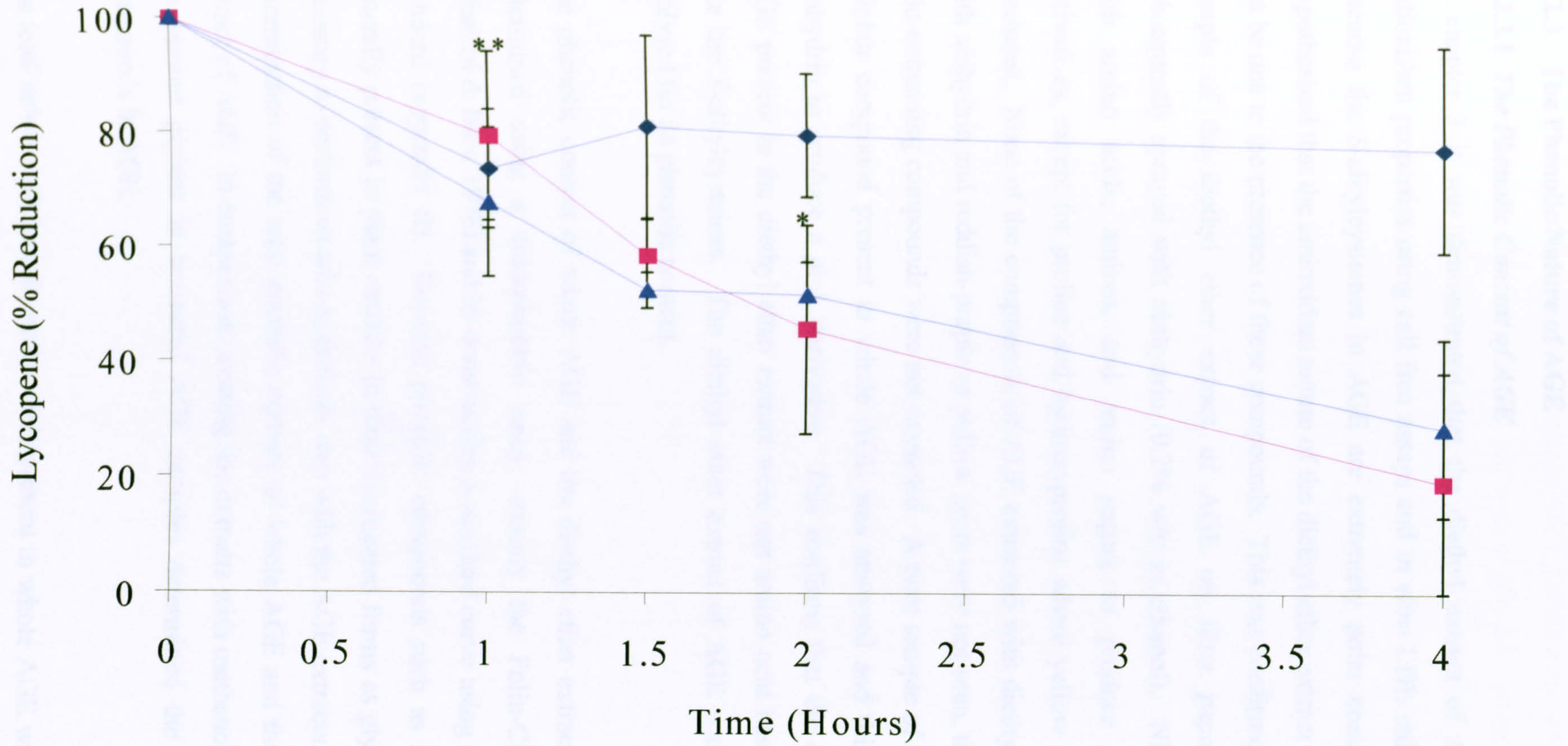


Figure 4.9. Change in Lycopene Levels at all Time Points Expressed as Percent Reduction Compared with Respective Controls at 0 hours. Control was LDL in PBS only (◆), LDL + Cu^{2+} (■), and LDL + Cu^{2+} + 10% (v/v) diethyl ether extract of AGE (▲). Values are presented as % change compared to respective controls at 0 hours and are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *, and control (LDL + Cu^{2+}) are indicated by **.

4.2.3. The Phenolic Nature of AGE

4.2.3.1 *The Phenolic Content of AGE*

In chapter 3 it was demonstrated that the diethyl extract of AGE possesses antioxidant properties using cell free assays and *in vitro* LDL oxidation studies. Because the *S*-alkylcysteines in AGE are extremely polar compounds it was hypothesised that the antioxidant nature of the diethyl ether extract of AGE would not be due to the presence of these compounds. This was confirmed by blotting a sample of the diethyl ether extract of AGE on filter paper, which was subsequently sprayed with ninhydrin (0.2% w/v in ethanol). Ninhydrin reacts with amino acids, amines, and amino sugars to produce reddish-purple derivatives, except for proline and hydroxyproline where yellow derivatives are produced. None of the components of AGE extracted with diethyl ether reacted with ninhydrin and reddish-purple or yellow spots were not seen, therefore amino acid-containing compounds were not extracted. A pure sample of SAC, the main sulphur compound present in whole AGE was analysed and this reacted with ninhydrin to produce a pink derivative. This confirms that the components of AGE present in the diethyl ether extract were not amino acid based compounds like the *S*-alkylcysteines. The diethyl ether extract of AGE was subsequently analysed for its phenolic content.

The phenolic content of whole AGE and the diethyl ether extract of AGE was determined using a colourimetric assay, namely the Folin-Ciocalteu assay (Ohnishi & Barr, 1978) and by constructing a standard curve using catechin as the standard (appendix II). Because phenolic compounds such as flavonoids are generally present in plant extracts in their conjugated forms as glycosides it was necessary to perform an acid-hydrolysis step with the AGE extracts. This allowed determination of the total phenolic content of whole AGE and the diethyl ether extract of AGE. In comparison, treating the extracts with methanol or measuring the amount present in untreated AGE samples determined the free phenolic compounds in AGE.

The total amount of phenolic compounds present in whole AGE was found to be 22.70 ± 1.57 mg/mL (Table 4.2). Conjugated phenolic compounds accounted for

77% of the total phenolic content of whole AGE, the remaining 23% being free phenolic compounds. As expected, the amount of phenolic compounds detected in untreated whole AGE was not significantly different from the values obtained with the methanol-treated whole AGE (6.10± 0.22 mg/mL vs. 5.26 ± 0.98 mg/mL, $p>0.05$) (Table 4.2). The total amount of phenolic compounds present in the diethyl ether extract of AGE was determined to be 1.10 ± 0.05 mg/mL and was much less than that observed for whole AGE (Table 4.2). Conjugated phenolic compounds accounted for 72% and free phenolic compounds accounted for the remainder of the total phenolic content (28%). As observed with whole AGE the amount of phenolic compounds detected in untreated diethyl ether extract of AGE was not significantly different from the values obtained with the methanol-treated diethyl ether extract of AGE (0.24 ± 0.04 mg/mL vs. 0.30 ± 0.05 mg/mL, $p>0.05$) (Table 4.2).

Table 4.2. The phenolic content of whole AGE and a diethyl ether extract of AGE. In order to measure total phenolics in the extracts, acid hydrolysis at 90°C for 2 h was required. Free phenolics were measured in extracts treated with methanol at 90°C for 2 h and in untreated extracts for comparison. Values are the means of three experiments ± SEM.

Phenolic Compounds	Whole AGE (mg/mL)	Diethyl Ether Extract of AGE (mg/mL)
Free phenolics in untreated samples	6.10 ± 0.22	0.24 ± 0.04
Free phenolics in methanol treated samples (A)	5.26 ± 0.98	0.30 ± 0.08
Total phenolics in acid-hydrolysed samples (B)	22.70 ± 1.57	1.10 ± 0.05
Conjugated phenolics (B-A)	17.46 ± 1.27	0.80 ± 0.04

4.2.3.2. *Analysis of Phenolic Compounds in AGE By TLC*

Thin-layer chromatography (TLC) analysis was performed to compare the properties of the phenolic compounds present in AGE with a battery of known standard phenolic compounds. The Folin-Ciocalteu assay confirmed that the majority of the phenolic compounds in whole AGE and the diethyl ether extract of AGE were present in their conjugated form. For a comparative TLC analysis acid-hydrolysed whole AGE and a diethyl ether extract of this were employed as well as untreated whole AGE and its diethyl ether extract. Silica gel 60 plates and an organic solvent system (ethylacetate:methanol:water (10:2:1, v/v/v)) were employed for TLC analysis and phenolic compounds were visualised with UV light and ferric chloride reagent.

Whereas untreated whole AGE did not display any separation of phenolic compounds under this system, the diethyl ether extract of AGE contained three well-resolved bands detected with ferric chloride and four well-resolved blue fluorescent bands detected with UV light (Table 4.3 & Figure 4.10). In contrast, the acid-hydrolysed whole AGE and its diethyl ether extract displayed a much greater mobility of phenolic compounds to the extent that that a smear of compounds were detected with ferric chloride. This smear contained a pink band and a brown band separated by a discreet brown band which were more concentrated in the diethyl ether extract suggesting a more efficient extraction under these acidic conditions (Table 4.3 & Figure 4.10). However, a small group of bands identified in the acid-hydrolysed whole AGE with R_f values of 1.8-2.2 were not present in the diethyl ether extract (Figure 4.10). Numerous phenolic compounds present in the acid-hydrolysed AGE sample and its diethyl ether extract were also detected under UV light. Five bands were detected in the acid-hydrolysed whole AGE and six bands were detected in its diethyl ether extract (Table 4.3). These compounds exhibited blue, white, and purple fluorescence indicating they were phenolic in nature.

Table 4.3. The R_f values for standard phenolic compounds and AGE samples. H^+ designates acid-hydrolysed AGE samples. The R_f values for samples/standards were calculated by measuring the distance of the compound moved (measured to the centre of spot/smear) and dividing this value by the distance moved by the solvent front in millimetres. All experiments were repeated three times and values shown are typical of the results obtained.

Sample/Standard	Ferric Chloride R_f values	Sample/Standard	UV Light R_f values
<u>Standards</u>		<u>Standards</u>	
Caffeic acid (grey)	0.67	Caffeic acid (white)	0.65
Catechin (grey-brown)	0.70	Catechin (dull)	0.71
p-Coumaric acid (brown)	0.72	*p*-Coumaric acid (blue)	0.71
Ferulic acid (pink-brown)	0.70	Ferulic acid (blue)	0.67
Kaempferol (brown)	0.81	Kaempferol (dull)	0.84
Quercetin (grey)	0.77	Quercetin (dull)	0.80
Salicylic acid (pink)	0.39	Salicylic acid (white)	0.38
<u>Diethyl extract of AGE</u>		<u>Diethyl extract of AGE</u>	
Band 1 (brown)	0.55	Band 1 (blue)	0.52
Band 2 (brown)	0.64	Band 2 (blue)	0.62
Band 3 (brown)	0.71	Band 3 (blue)	0.69
Band 4	-	Band 4 (blue)	0.77
<u>Whole Age (H^+)</u>		<u>Whole Age (H^+)</u>	
Distinct group of 3 brown bands	0.18 0.20 0.22	Band 1 (white) Band 2 (white) Band 3 (blue) Band 4 (blue-purple)	0.17 0.40 0.65 0.73
Pink band	0.58	Band 5 (blue-purple)	0.81
Discreet brown band	0.61		
Brown Smear	0.62-0.77		
<u>Diethyl Ether Extract of Age (H^+)</u>		<u>Diethyl Ether Extract of Age (H^+)</u>	
Pink band	0.56	Band 1 (white)	0.18
Discreet brown band	0.61	Band 2 (white)	0.40
Brown Smear	0.62-0.75	Band 3 (white)	0.54
		Band 4 (blue)	0.65
		Band 5 (blue-purple)	0.73
		Band 6 (blue-purple)	0.81

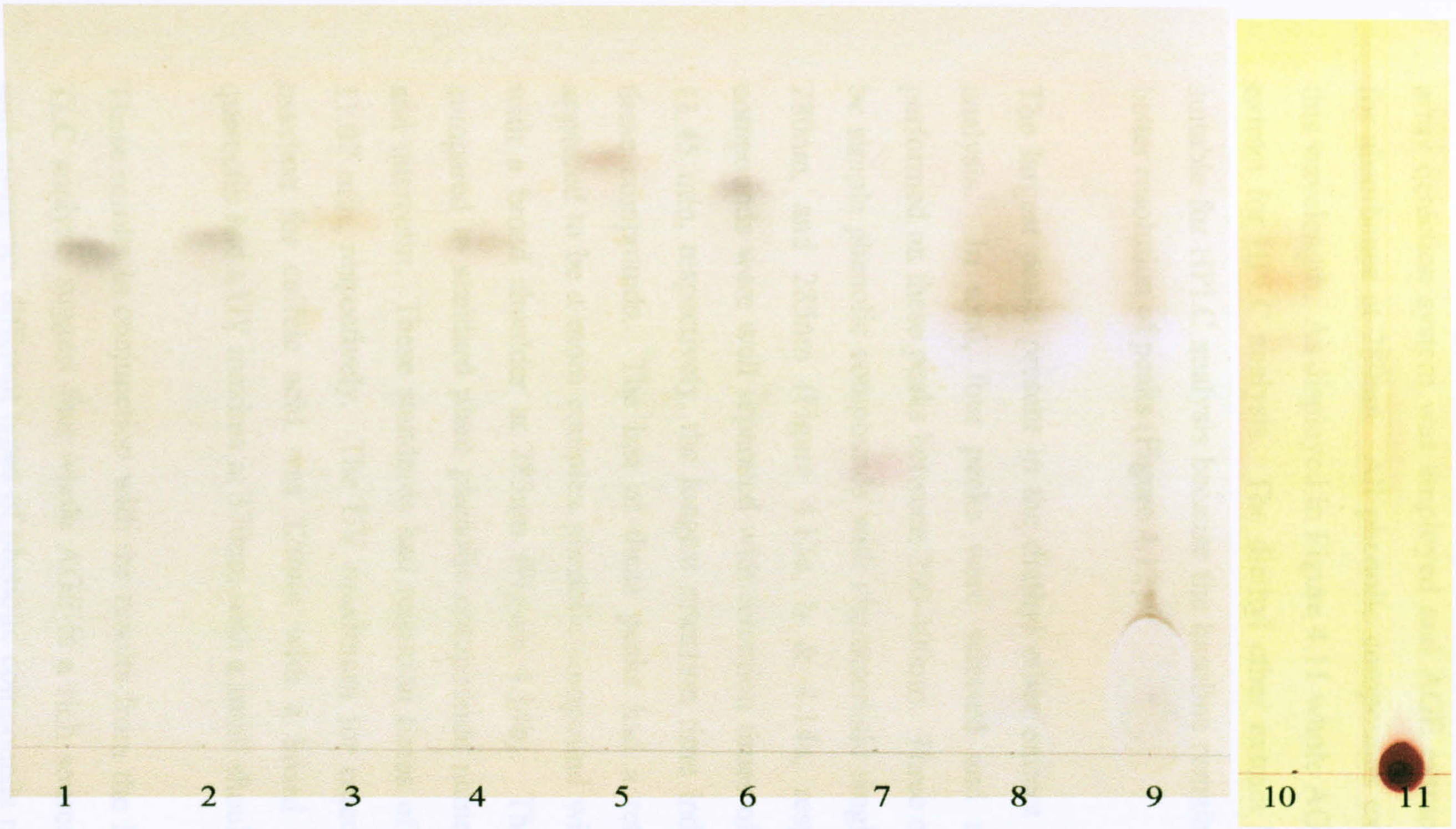


Figure 4.10. TLC analysis of standard phenolic compounds and AGE samples using a ferric chloride detection system. (1) Caffeic acid, (2) Catechin, (3) *p*-Coumaric acid, (4) Ferulic acid, (5) Kaempferol, (6) Quercetin, (7) Salicylic acid, (8) Diethyl ether extract of acid-hydrolysed AGE, (9) Acid-hydrolysed AGE, (10) Diethyl ether extract of AGE, (11) Whole AGE. The distance moved by phenolic compounds and solvent system was measured in millimetres and used to calculate R_f values. All experiments were repeated three times and the TLC plates shown were typical of the results obtained.

4.2.3.3. *Analysis of Phenolic Compounds in AGE by HPLC*

High-pressure liquid-chromatography (HPLC) analysis is a useful technique for investigating complex mixtures of phenolic compounds. The acetonitrile/water gradient system used allowed separation of phenolic compounds present in AGE by polarity with the most polar compounds eluted first. A Waters photo diode array detection system was employed and AGE extracts were initially analysed for absorbance at 280nm. All phenolic compounds exhibit some absorbance at this wavelength. As displayed in Figure 4.11 whole AGE was far too complex an extract for HPLC analysis. The diethyl ether extract however was much more suitable for HPLC analysis because the baseline remained relatively level with a better resolution of peaks (Figure 4.12).

The largest peaks present in the diethyl ether extract were selected for further analysis. In total, four peaks were selected and an absorbance scan was performed on these peaks between 200-400nm. Three of these peaks appeared to be simple phenolic compounds with characteristic single UV maxima at 275nm, 280nm, and 285nm (Figure 4.13a, b & 4.14a, respectively). These three compounds were well separated with retention times of 2.23 min, 4.17 min, and 11.45 min, respectively, the longest retention time indicating the least polar of these compounds. The last of these peaks had a retention time of 8.12 and appeared to be a more complex phenolic compound with UV maxima at 320nm with a broad shoulder at 285nm (Figure 4.14b). These retention times were compared to standard plant phenolic compounds namely catechin, caffeic acid, and quercetin. These standards had retention times of 5.87 min, 6.64 min, and 11.07 min, respectively. The UV maximum for catechin was 279nm, the UV maxima for caffeic acid was 320nm with a broad shoulder at 295nm, and quercetin had a UV maxima at 370nm with a small shoulder at 300nm.

These results in conjunction with the results from the Folin-Ciocalteu assay and TLC analysis suggest that whole AGE is a rich source of phenolic compounds with numerous different types of phenolic compounds present and these phenolic compounds were extracted with diethyl ether.

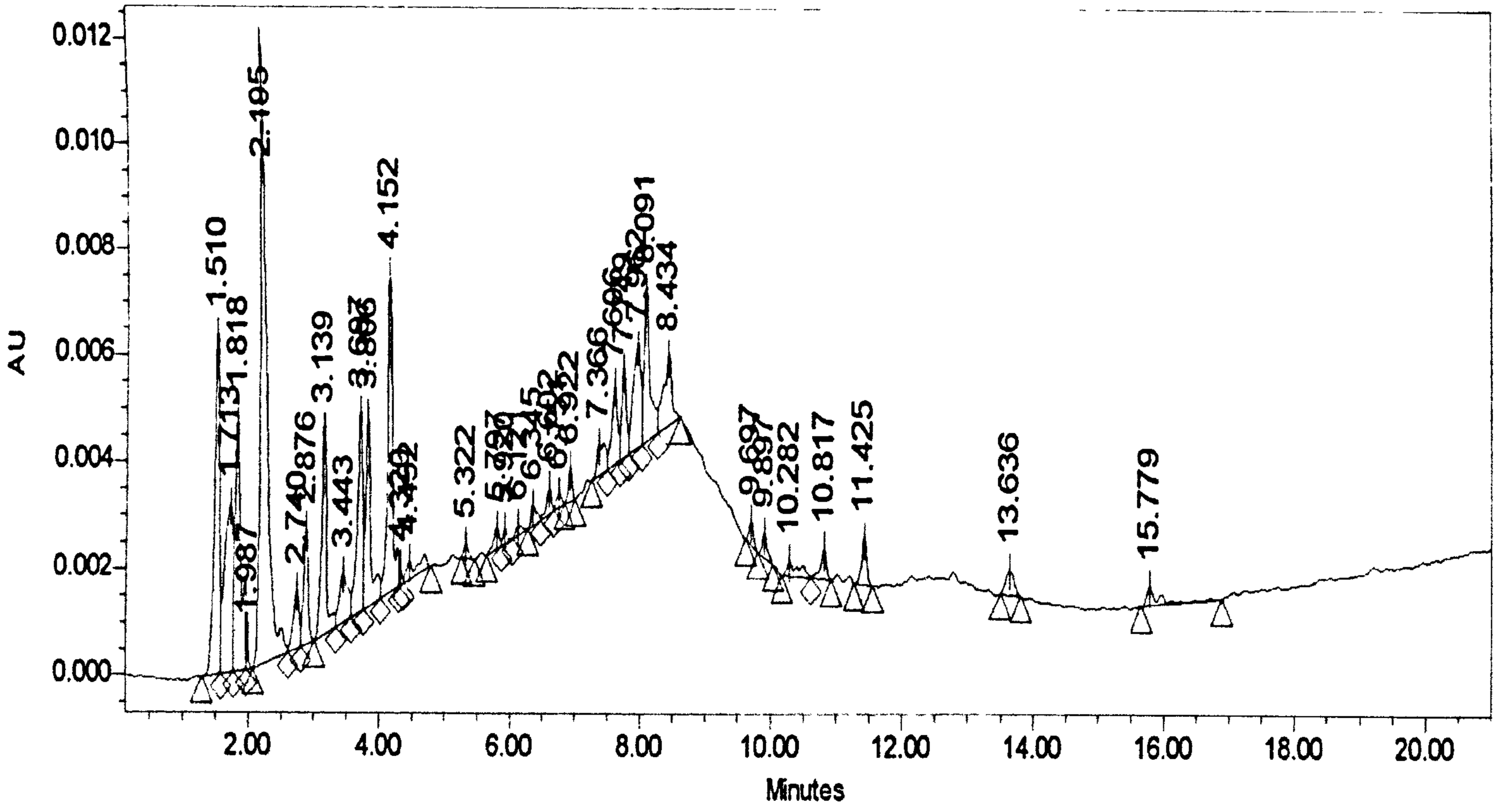


Figure 4.11. HPLC Analysis of Whole AGE at 280nm. Whole AGE was diluted 1/50 with methanol. The unstable baseline clearly suggested that whole AGE was far too complex for HPLC analysis.

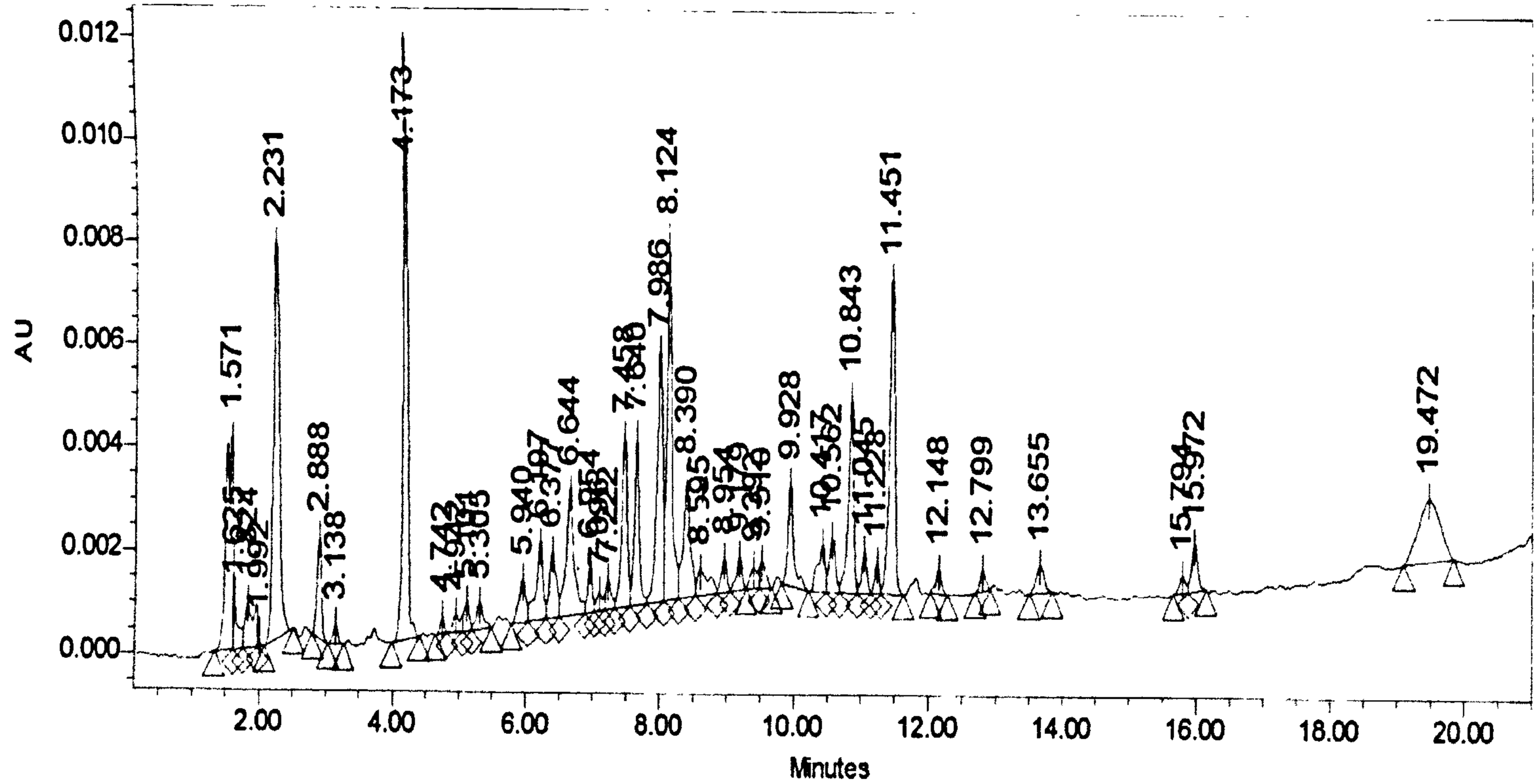


Figure 4.12. HPLC Analysis of a Diethyl Ether Extract of AGE. The diethyl ether extract of AGE was re-suspended in 1mL methanol. The baseline remained relatively low and at a level which provided adequate resolution of peaks. Of the numerous peaks identified the four main peaks with retention times of 2.231, 4.173, 8.124, and 11.451 min were selected for further analysis.

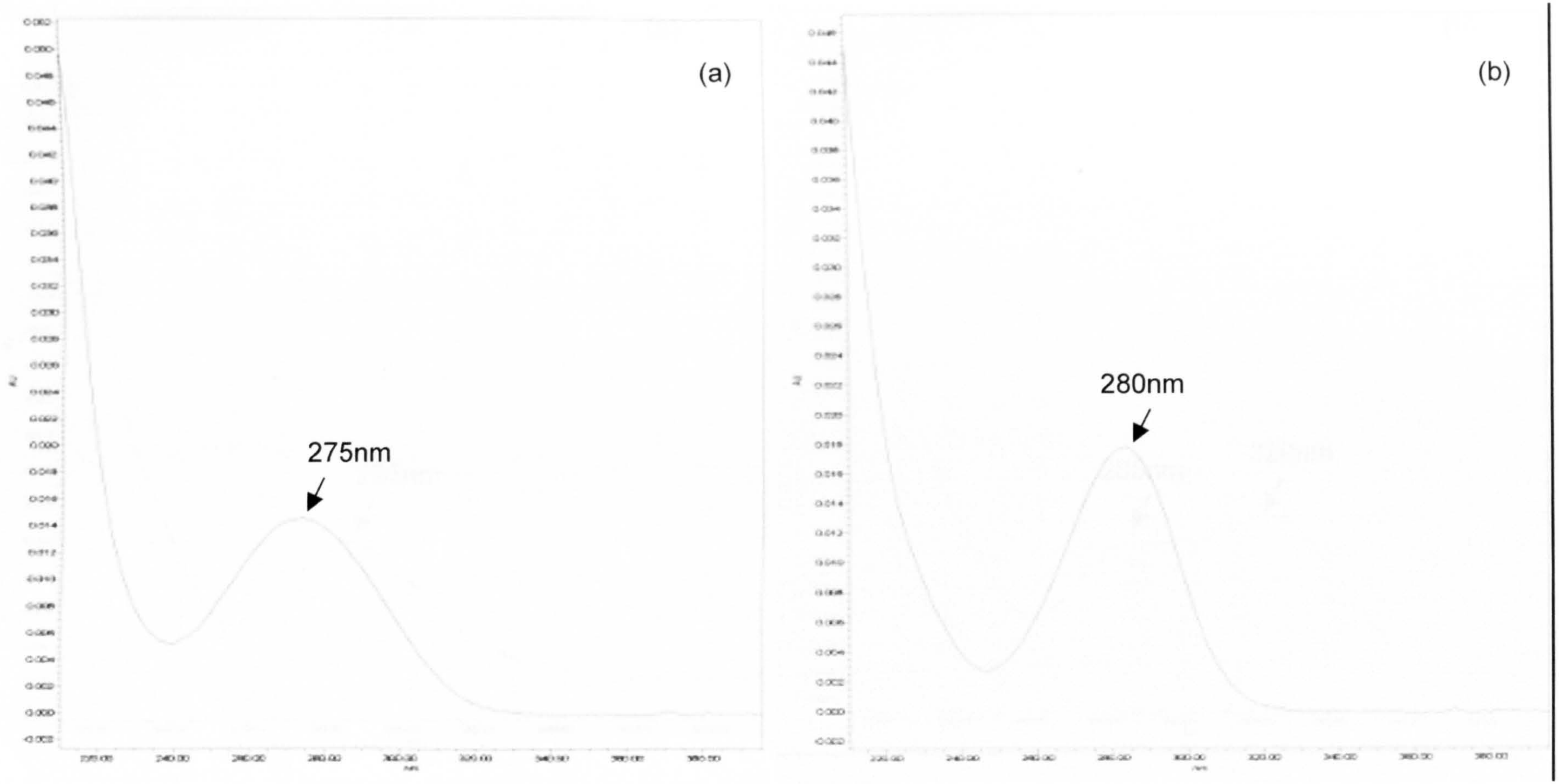
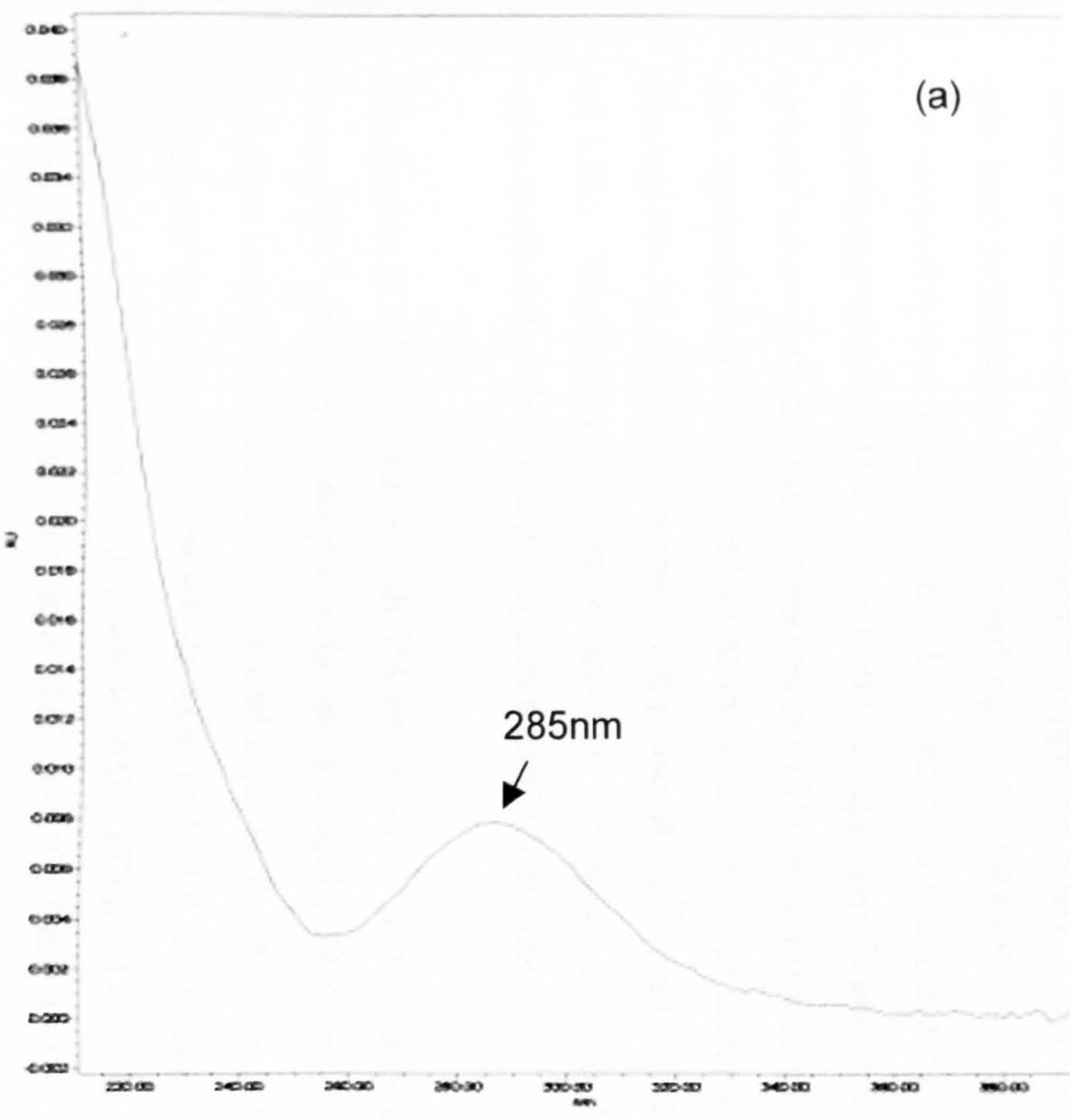


Figure 4.13. Absorbance Spectra (200nm-400nm) of Two of the Four Main Peaks Identified in a Diethyl Ether Extract of AGE. (a) Phenolic compound with a retention time of 2.231 min and a UV maxima of 275nm. (b) Phenolic compound with a retention time of 4.173 min and UV maxima of 280nm.



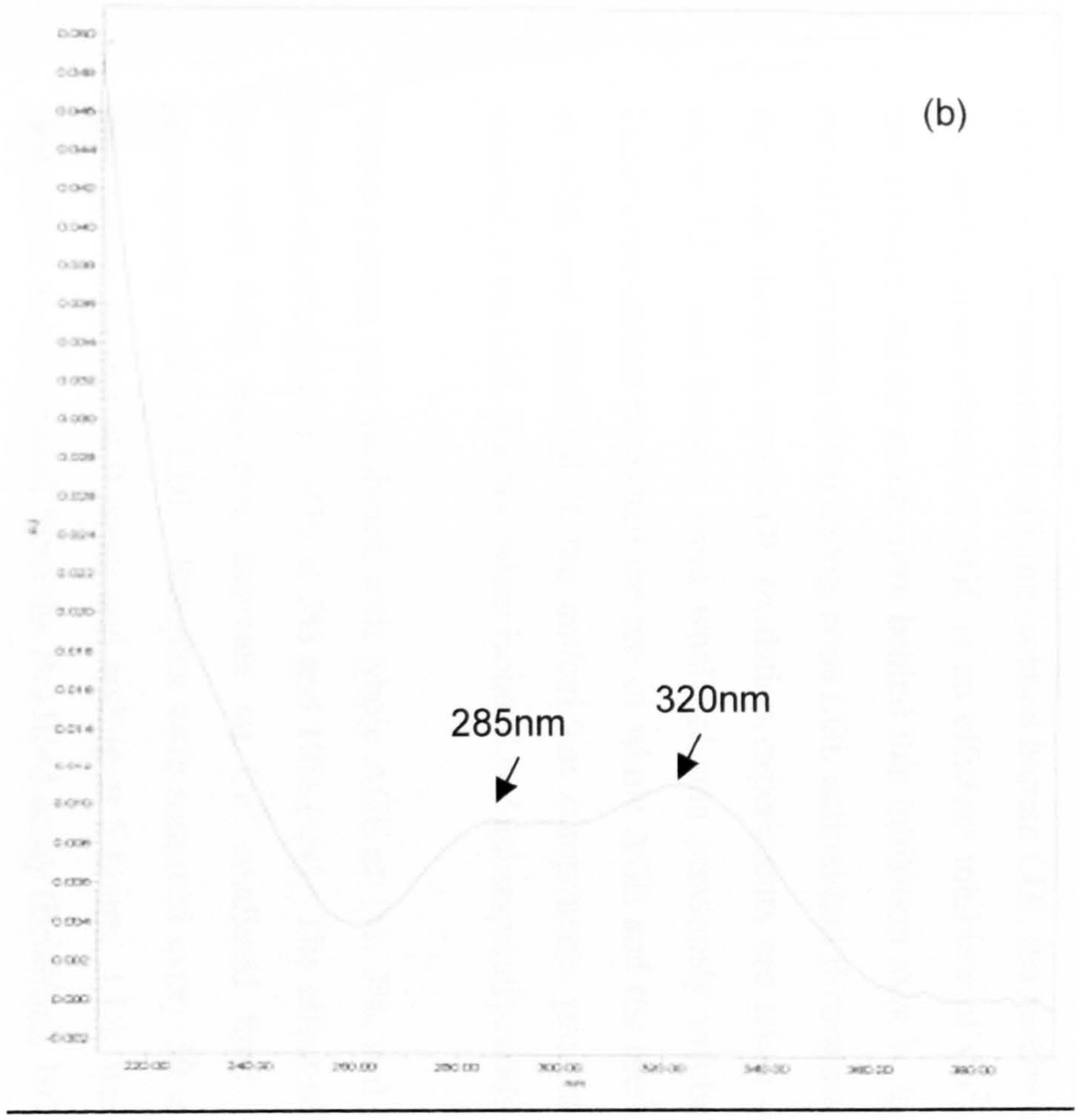


Figure 4.14. Absorbance Spectra (200nm-400nm) of the Remaining Two main peaks Identified in a Diethyl Ether Extract of AGE. (a) Phenolic compound with a retention time of 11.451 min and UV maxima of 285nm. (b) Phenolic compound with a retention time of 8.124 min and UV maxima of 320nm and a broad shoulder at 285nm.

4.2.4. The LDL-Binding Properties of AGE

In previous experiments utilising isolated human LDL the results have shown that the diethyl ether extract of AGE is an efficient inhibitor of Cu^{2+}-mediated lipid peroxidation and the mechanism behind this inhibition may be in part due to the partial preservation of the endogenous LDL antioxidant β-carotene. In this part of the study these *in vitro* LDL oxidation experiments are taken one step further where LDL was isolated from whole plasma previously incubated with AGE. These experiments permitted the use of whole AGE and the diethyl ether extract of AGE and identified if the antioxidant components present in AGE were retained in the LDL fraction when isolated and subsequently oxidised with Cu^{2+}.

Whole plasma was incubated with whole AGE at 1%, 3%, and 5% (v/v) or the diethyl ether extract of AGE at 5% and 10% (v/v). The effect of this incubation step with AGE was then assessed on Cu^{2+}-mediated lipid peroxidation of subsequently isolated LDL. Samples were removed every 30 minutes with the time course starting at 0 hours and ending at 5 hours. LDL was used at 200μg protein/mL as determined from the Bradford assay (Bradford, 1976) using human serum albumin (HSA) as the standard (appendix VIII). $CuSO_4$ was used at 10μM and lipid peroxidation was assessed using the TBARS assay (Mao et al., 1994) and converted to nmoles of MDA by constructing a standard using hydrolysed tetraethoxypropane (MDA) as the standard (appendix I) and standardised to LDL protein concentration. MDA (nmoles/mg LDL protein) was plotted versus time and lag times were determined from the interception of linear trend lines drawn through the lag phase and propagation phase of Cu^{2+}-mediated lipid peroxidation of LDL. Where there was no observable propagation phase the lag time was taken at the end of the experiment, this being 300 minutes.

Incubation of whole plasma with 1% or 3% (v/v) whole AGE extended the lag time of Cu^{2+}-mediated lipid peroxidation of LDL by 24% and 26%, respectively. Lag time was non-significantly increased from 96.0 ± 4.9 minutes to 123.0 ± 17.4 minutes in the presence of 1% whole AGE and 87.0 ± 4.8 minutes to 118.0 ± 3.8 minutes in the presence of 3% whole AGE (Table 4.4). There was a trend towards significance with 3% whole AGE ($p=0.07$, Table 4.4).

Incubation of whole plasma with 5% (v/v) whole AGE significantly extended the lag time of Cu^{2+}-mediated lipid peroxidation of LDL by 71%. Lag time was increased from 86.0 ± 9.5 minutes to 300 minutes in the presence of 5% whole AGE (p=0.017, Table 4.4). In all experiments incubation of plasma with 5% whole AGE consistently extended the lag time of Cu^{2+}-mediated lipid peroxidation of LDL to the final time point (300 minutes, Figure 4.15).

Incubation of whole plasma with 5% or 10% (v/v) diethyl ether extract of AGE extended the lag time of Cu^{2+}-mediated lipid peroxidation of LDL by 20% and 32%, respectively. Lag time was non-significantly increased from 71.0 ± 8.1 minutes to 89.0 ± 7.2 minutes in the presence of 5% diethyl ether extract of AGE (Table 4.4) and significantly increased from 62.0 ± 3.5 minutes to 90.0 ± 4.2 minutes in the presence of 10% diethyl ether extract of AGE (Table 4.4, Figure 4.16).

In summary, these results demonstrate that the antioxidant properties of AGE with regards to inhibiting lipid peroxidation induced by Cu^{2+} remain even when AGE is not directly incubated with LDL i.e. LDL is isolated from plasma incubated with AGE. These results suggest that components of AGE that are also present in the diethyl ether extract bind to or become incorporated within the LDL particle.

Table 4.4. The effect of incubation of whole plasma with whole AGE or a diethyl ether extract of AGE on the lag time of Cu^{2+}-mediated lipid peroxidation of subsequently isolated LDL. Values are means of three experiments ± SEM. Significant differences ($p < 0.05$) from control (LDL + Cu^{2+}) are indicated by *.

LDL Sample	Lag Time (minutes)	P value
LDL + Cu^{2+}	96.0 ± 4.9	
LDL + Cu^{2+} + 1% whole AGE	123.0 ± 17.4	0.21
LDL + Cu^{2+}	87.0 ± 4.8	
LDL + Cu^{2+} + 3% whole AGE	118.0 ± 3.8	0.07
LDL + Cu^{2+}	86.0 ± 9.5	
LDL + Cu^{2+} + 5% whole AGE	300	0.017*
LDL + Cu^{2+}	71.0 ± 8.1	
LDL + Cu^{2+} + 5% diethyl ether extract of AGE	89.0 ± 7.2	0.18
LDL + Cu^{2+}	62.0 ± 3.5	
LDL + Cu^{2+} + 10% diethyl ether extract of AGE	90.0 ± 4.2	0.016*

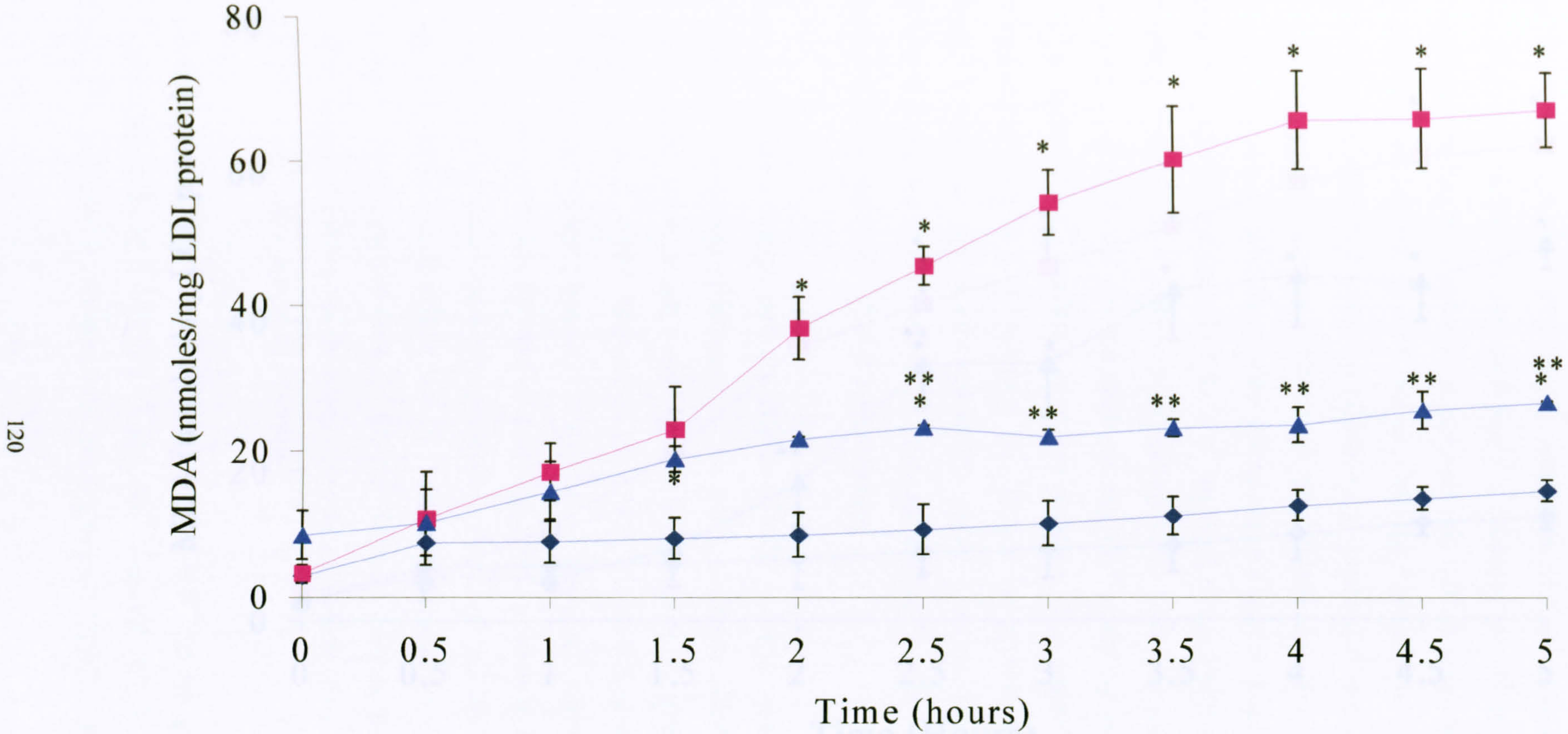


Figure 4.15. Time course of Cu^{2+}-induced oxidation of LDL isolated from whole plasma previously incubated with (▲) or without (■) 5% (v/v) whole AGE. Control was LDL in PBS only (◆). Values are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *, and control (LDL + Cu^{2+}) are indicated by **.

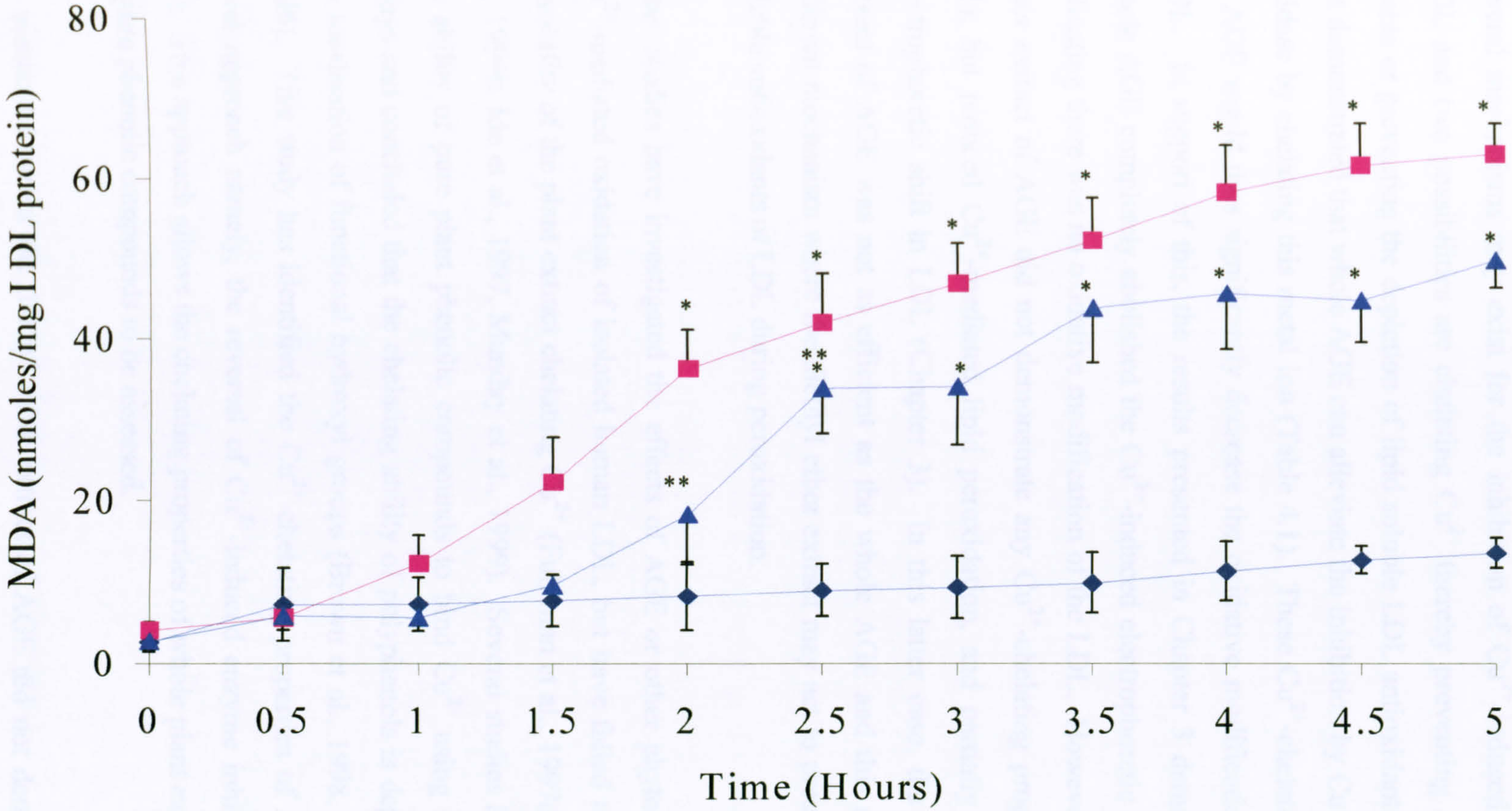


Figure 4.16. Time course of Cu^{2+}-induced oxidation of LDL isolated from whole plasma previously incubated with (▲) or without (■) 10% (v/v) diethyl ether extract of AGE. Control was LDL in PBS only (◆). Values are the means of three experiments ± SEM. Significant differences ($p<0.05$) from control (LDL + PBS) are indicated by *, and control (LDL + Cu^{2+}) are indicated by **.

4.3. Discussion

AGE is a complex mixture of phytochemicals. Therefore, it is not surprising that several mechanisms may exist for the inhibition of Cu^{2+}-induced oxidation of LDL and two possibilities are chelating Cu^{2+} thereby preventing any initiating events or preventing the depletion of lipid soluble LDL antioxidants. This study has demonstrated that whole AGE can alleviate the inhibition by Cu^{2+} of xanthine oxidase by chelating this metal ion (Table 4.1). These Cu^{2+}-chelating properties of AGE would thus significantly decrease the oxidative modification of isolated LDL. In support of this, the results presented in Chapter 3 demonstrated that whole AGE completely abolished the Cu^{2+}-induced electrophoretic shift in LDL, indicating there was no oxidative modification of the LDL. However, the diethyl ether extract of AGE did not demonstrate any Cu^{2+}-chelating properties (Table 4.1), but reduced Cu^{2+}-mediated lipid peroxidation, and partially reversed the electrophoretic shift in LDL (Chapter 3). In this latter case, the diethyl ether extract of AGE was not as efficient as the whole AGE and this may reflect a different mechanism where the diethyl ether extract may act to preserve the lipid soluble antioxidants of LDL during peroxidation.

Other studies have investigated the effects of AGE or other phytochemicals on Cu^{2+}-mediated oxidation of isolated human LDL, but have failed to address the possibility of the plant extract chelating Cu^{2+} (Fuhrman et al., 1997d, Hodgson et al., 1999b, Ide et al., 1997, Munday et al., 1999). Several studies have assessed the ability of pure plant phenolic compounds to bind Cu^{2+} using spectroscopic assays and concluded that the chelating ability of polyphenols is dependent upon the localisation of functional hydroxyl groups (Brown et al., 1998, Miller et al., 1996). This study has identified the Cu^{2+}-chelating properties of AGE using a novel approach namely, the reversal of Cu^{2+}-induced enzyme inhibition (Table 4.1). This approach allows the chelating properties of whole plant extracts as well as pure phenolic compounds to be assessed.

As mentioned earlier the diethyl ether extract of AGE did not demonstrate any Cu^{2+}-chelating properties (Table 4.1), but reduced Cu^{2+}-mediated lipid peroxidation, and partially reversed the electrophoretic shift in LDL (Chapter 3).

If this diethyl ether extract reduces Cu^{2+}-mediated oxidation of LDL and this effect was not due to chelation of Cu^{2+} it can be hypothesised that this antioxidant effect may be due to preservation of endogenous LDL antioxidants such as α-tocopherol, β-carotene, and lycopene. Before rapid lipid peroxidation occurs, LDL becomes depleted of its antioxidants, with α-tocopherol being consumed first and β-carotene last (Esterbauer & Ramos, 1995); this is the lag phase of the process. It is not until the LDL has lost most of its antioxidant compounds that the propagation phase commences and the PUFAs in LDL are rapidly oxidised to lipid hydroperoxides. This is followed by the decomposition phase, when the lipid hydroperoxides break down and/or rearrange to form a wide range of products, including aldehydes, ketones, alcohols, and epoxides (Bhadra et al., 1991, Brown et al., 1997, Esterbauer et al, 1991a).

Interestingly, the diethyl ether extract of AGE did not prevent the depletion of α-tocopherol or lycopene during Cu^{2+}-mediated lipid peroxidation of LDL (Figures 4.4 & 4.8) even though there was substantial inhibition of lipid peroxidation during the same experiments (Figure 4.3). More importantly, the diethyl ether extract of AGE actually appeared to enhance depletion of α-tocopherol during Cu^{2+}-mediated lipid peroxidation of LDL (Figures 4.4 & 4.5). The reason for this is currently unknown, although other studies have also observed a similar effect (Belinky et al., 1998, Hayek et al, 1997). The *in vitro* oxidation of LDL isolated from atherosclerotic mice in the presence of the licorice flavonoid glabridin failed to protect α-tocopherol although oxidation was reduced (Belinky et al., 1998). Hayek et al (1997) observed that LDL isolated from atherosclerotic mice who had consumed the red wine flavonoids catechin and quercetin was more resistant to oxidation although this LDL had ~50% less α-tocopherol than the placebo group. A more recent study has shown that LDL isolated from human plasma previously incubated with lemon oil was more resistant to oxidation and this was independent of preservation of α-tocopherol (Grassmann et al., 2001).

In contrast, preservation of β-carotene was observed during Cu^{2+}-mediated lipid peroxidation of LDL in the presence of the diethyl ether extract of AGE (Figure 4.6 & 4.7). Because β-carotene is located within the core of LDL particles it is

improbable that there is direct interaction between the antioxidant components present in AGE and β-carotene. This suggests that the antioxidant components present in AGE extracted with diethyl ether may act to inhibit the chain propagation process of lipid peroxidation by scavenging of lipid peroxyl radicals and subsequently inhibiting the formation of lipid hydroperoxides; a role assigned to α-tocopherol. Under these circumstances preservation of β-carotene, the last antioxidant to be depleted during Cu^{2+}-mediated lipid peroxidation of LDL, may be observed. This preservation of β-carotene has also been observed in other studies where the *in vitro* oxidation of LDL isolated from atherosclerotic mice in the presence of the licorice flavonoid glabridin or LDL isolated from human plasma previously incubated with lemon oil inhibited the consumption of β-carotene and lycopene (Belinky et al., 1998, Grassmann et al., 2001).

Garlic is a rich source of plant polyphenols such as flavonoids and phenolic acids (Lawson, 1996, Miean & Mohamed, 2001, Swain et al., 1995, Vinson et al., 1998). The results presented in this study suggest the same is true for AGE. Using numerous methods of analysis this study has shown that AGE has a high phenolic content (Table 4.2) with many different types of polyphenols present in both whole AGE and the diethyl ether extract of AGE (Table 4.3, Figures 4.10, 4.12, 4.13, & 4.14). The antioxidant effect of the diethyl ether extract of AGE with regards to scavenging of superoxide, inhibiting lipid peroxide formation, and inhibiting oxidative modification of LDL may be attributed to these polyphenolic components. The high and varied polyphenolic content of AGE would not be true for all commercial garlic preparations due to processing and extraction methods. Polyphenolic compounds are relatively polar molecules but some do exhibit lipophilic tendencies, so their concentration and variability in different garlic extracts (aged extracts, oil-based preparations, and powdered extracts) would be expected to vary.

The potent effects of plant flavonoids in reducing the susceptibility of LDL to undergo oxidative modification may be related to the absorption or binding of these antioxidants to LDL (Belinky et al., 1998, Hayek et al., 1997). These studies have shown that LDL from plasma previously incubated with red wine,

catechin, quercetin, and glabridin is enriched with these polyphenols. Studies with whole plant extracts have also shown that antioxidant components present in these extracts exhibit lipoprotein-bound antioxidant activity (Grassmann et al., 2001, Ivanov et al., 2001, Vinson et al., 1995, 1998). Similar experiments were also performed with AGE. LDL isolated from whole plasma previously incubated with whole AGE or the diethyl ether extract of AGE significantly extended the lag time of Cu^{2+}-mediated lipid peroxidation (Tables 4.4 & 4.5). These results suggest that the potent effects of AGE with regards to inhibiting Cu^{2+}-mediated lipid peroxidation may in part be due to the ability of components of AGE to bind to or become absorbed by LDL. Identifying how these antioxidant components present in AGE become bound to or incorporated within LDL may help to explain the partial preservation of β-carotene observed in earlier experiments.

In summary, the results presented here have demonstrated that AGE may inhibit the atherogenic oxidation of LDL by Cu^{2+} by two different mechanisms: (i) by chelation of Cu^{2+} and hence preventing any initiation events and (ii) by preventing depletion of the LDL antioxidant β-carotene. This latter effect suggests that AGE may directly scavenge lipid peroxyl radical and subsequently prevent lipid hydroperoxide formation. The efficacy of AGE in inhibiting LDL oxidation may also in part be due to its ability to bind to or become absorbed by LDL and its high concentration of polyphenolic antioxidants.

CHAPTER 5

THE *IN VIVO* ANTIOXIDANT NATURE OF AGED GARLIC EXTRACT

5. THE *IN VIVO* ANTIOXIDANT NATURE OF AGED GARLIC EXTRACT

5.1. INTRODUCTION

Cigarette smoking is one of the highest risk factors for the development of atherosclerotic vascular disease and may be the most important risk factor as it contributes to a third of all deaths from this condition (Feeman, 1999, Tierney et al., 2000). As cigarette smoke is known to contain a large number of oxidants, it has been hypothesised that the adverse effects of smoking may result from oxidative damage to lipids, proteins, and DNA (Halliwell & Gutteridge, 1999). This damage could result from oxidants present in cigarette smoke or from activation of phagocytic cells that generate reactive species. Oxidation of LDL is an important mechanism in the development of atherosclerosis and exposure of LDL to cigarette smoke results in oxidative modification *in vitro* and *in vivo*, however evidence for this is controversial primarily due to different and unreliable methods of assessing lipid peroxidation (Chen & Loo, 1995, Valkonen & Kuusi, 1998, Yamaguchi et al., 2000).

The hypothesis that smokers are subject to increased oxidative stress is supported by more direct evidence: lower plasma levels of the antioxidants α-tocopherol (vitamin E), ascorbic acid (vitamin C) and glutathione have been observed in smokers when compared to non-smokers, increased plasma levels of lipid peroxides have been identified in smokers when compared to non-smokers although some results for this are conflicting, and smokers risk of cardiovascular disease correlates inversely with their intake of the antioxidants vitamin E and β-carotene (Ayaori et al., 2000, Banerjee et al., 1998, Liu et al., 1998, Nowak et al., 1999, Rimm et al., 1993).

Free radical-mediated peroxidation of membrane and lipoprotein lipids plays a pivotal role in the pathogenesis of many diseases including atherosclerosis (Esterbauer et al., 1993). Traditional methods of assessing lipid peroxidation *in vivo* have included the measurement of thiobarbituric acid-reactive substances (TBARS) or lipid peroxides both of which suffer from ex vivo artifactual

generation, instability and non-specificity of analytes (Halliwell, 2000, Jackson, 1999). Another common method of monitoring lipid peroxidation and assessing the efficacy of antioxidants *in vivo* is to estimate the susceptibility of isolated low-density lipoprotein (LDL) to Cu^{2+}-induced oxidation *in vitro*, the obvious problem with this method is that it does not directly relate to oxidant stress *in vivo* and the effect of non-lipoprotein associated antioxidants is removed (Fruebis et al., 1997). For these reasons monitoring lipid peroxidation *in vivo* and assessing the effects of dietary intervention with antioxidants on lipid peroxidation has been problematic.

F_2-isoprostanes are increased in human conditions thought to be associated with increased oxidative stress such as smoking, hypercholesterolemia, hypertension, diabetes, and alcoholic liver disease (Cracowski et al., 2001, Davi et al., 1997, 1999, Devaraj et al, 2001, Gopaul et al., 1995, Meagher et al., 1999, Morrow et al., 1995, Reilly et al., 1996, 1998). They are formed *in situ* from arachidonic acid through a non-enzymatic process of lipid peroxidation catalyzed by free radicals on cell membranes and LDL particles (Morrow et al., 1992, Pratico et al., 1998). They are cleaved presumably by phospholipases, and circulate in the plasma as the free form or esterified to phospholipids whilst the free form is excreted in urine (Morrow et al., 1990a, 1990b). F_2-isoprostanes are thought to be reliable and sensitive markers of lipid peroxidation *in vitro* and *in vivo, which* can be monitored non-invasively in the urine and are not confounded by lipid-rich diets (Gopaul et al., 2000a, 2000b, Pratico, 1999).

Due to its vasoconstrictor, platelet activation, and mitogenic properties the F_2-isoprostane 8-iso-Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) also known as 8-epi-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III has received much attention (Banerjee et al., 1992, Fukunaga et al., 1993, Janssen, 2000, Kang et al., 1993, Kromer & Tippins, 1996, Minuz et al., 1998, Takahashi et al., 1992). Elevated plasma and urinary levels of total F_2-isoprostanes and specifically 8-iso-$PGF_{2\alpha}$ have been demonstrated in smokers (Bachi et al., 1996, Morrow et al., 1995, Oguogho et al., 2000, Reilly et al., 1996). These studies also noted that upon cessation of smoking or supplementation with vitamin C alone or in conjunction with vitamin E the levels of total F_2-

isoprostanes or 8-iso-$PGF_{2\alpha}$ dramatically decreased (Morrow et al., 1995, Reilly et al., 1996). Also, in support of smoking as a mediator of oxidative stress and 8-iso-$PGF_{2\alpha}$ as marker for increased oxidative stress plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ normalise within two-four weeks of quitting cigarette smoking (Morrow et al., 1995, Oguogho et al., 2000, Pilz et al., 2000, Reilly et al., 1996).

Garlic has attracted considerable interest as a cardioprotective agent (Agarwal, 1996, Rahman, 2001). Its effects on risk factors for cardiovascular disease include reducing plasma lipids, preventing platelet aggregation and subsequent thrombus formation, enhancing fibrinolytic activity, and reducing blood pressure (Abuirmeileh et al., 1991, Bordia et al., 1998, Kannar et al., 2001, Rahman & Billington, 2000, Steiner & Li, 2001, Steiner et al., 1996, Thompson et al., 2000). The majority of the studies are human supplementation trials. The antioxidant property of garlic has also been well demonstrated but this has mainly been concentrated on *in vitro* studies or *in vivo* animal studies. These studies have demonstrated the AGE possesses substantial antioxidant activity in comparison with other garlic preparations, which includes scavenging of reactive species, preventing oxidative damage of lipoproteins and endothelial cells, and preservation of endogenous antioxidant defences (Geng & Lau, 1997, Ide & Lau, 1999a, 2001, Ide et al., 1997, Imai et al., 1994). Direct evidence for the antioxidant properties of AGE *in vivo* in humans is insubstantial with AGE supplementation reducing the *in vitro* susceptibility of LDL to oxidation (Steiner et al., 1996, Munday et al., 1999).

The aim of the study reported here was to investigate the *in vivo* antioxidant potential of AGE in human subjects. Initially it was identified whether increased oxidative stress due to cigarette smoking results in elevated levels of plasma and urinary 8-iso-$PGF_{2\alpha}$ and a decreased antioxidative capacity of plasma to scavenge superoxide when compared to non-smoking individuals. The antioxidant properties of AGE were then examined by monitoring the effects of AGE supplementation on plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ and the antioxidative capacity of plasma to scavenge superoxide in both smoking and

non-smoking individuals. These studies are schematically represented in Figure 5.1.

5.1.1. Overview of Study

Apparently healthy subjects recruited for this study (n = 20) were divided into two age- and sex-matched groups, 10 non-smokers and 10 smokers. All volunteers answered a simple questionnaire about their age, height, weight, average weekly alcohol consumption, and number of cigarettes smoked per day (Appendix IV). The duration of the trial was 28 days in total, starting with 14 days of AGE consumption followed by a 14 day washout period. The volume of AGE was 5 mL/day and blood and urine samples were collected at the start of the trial (day 0), on the last day of AGE consumption (day 14), and at the end of the washout period (day 28). Plasma and urine samples were stored at –70°C until analysis.

Routine biochemical measurements included total plasma cholesterol, triglycerides, and protein concentrations. Plasma and urinary creatinine concentrations were also monitored to assess kidney function and to standardise urinary concentrations of 8-iso-$PGF_{2\alpha}$, respectively. Total plasma and free urinary concentrations of 8-iso-$PGF_{2\alpha}$ were analysed using a competitive enzyme-linked immunoassay (ELISA) and the antioxidant capacity of plasma to scavenge superoxide was assessed using a chemiluminescence assay (see Chapter 2, pp 51-59 for detailed methods).

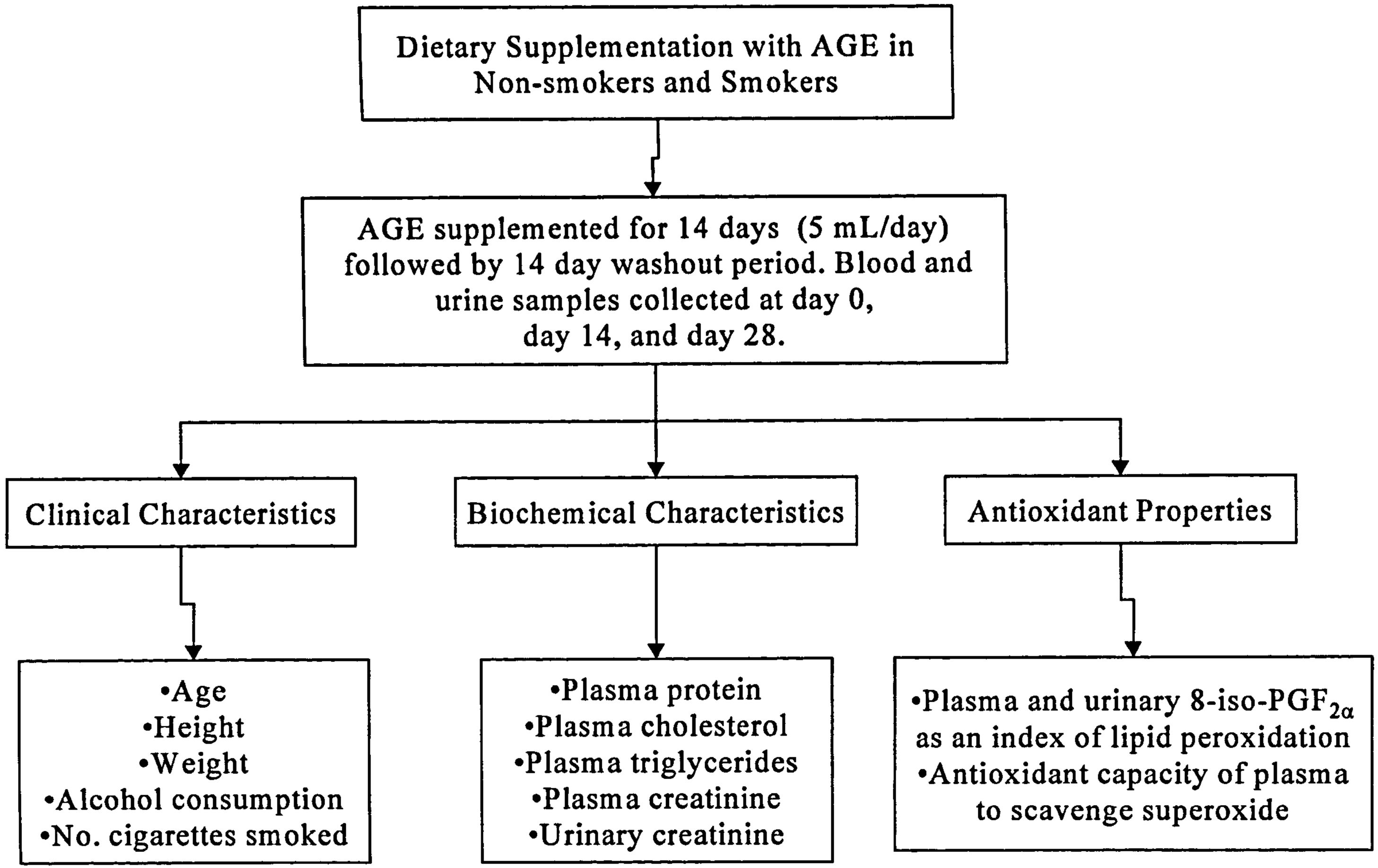


Figure 5.1. Overview of Study.
Dietary supplementation study to assess the antioxidant properties of AGE *in vivo* in non-smoking and smoking men and women.

5.2. RESULTS

5.2.1. The Clinical and Biochemical Characteristics of Subjects

All non-smoking and smoking subjects were asked to complete a simple questionnaire (Appendix IV), which provided information relating to age, height, weight, alcohol consumption, and smoking habits. Both groups consumed similar amounts of alcohol and were similar in age, height and weight. In fact the only difference observed between the two groups of subjects was in smoking habits (Table 5.1).

The biochemical characteristics of non-smoking and smoking groups were monitored throughout the study and are given in Table 5.2. Plasma protein concentration was measured using the Bradford assay (Bradford, 1976) and HSA as the standard (Appendix VIII). Total plasma cholesterol and triglycerides were measured using enzymatic methods (Katterman et al., 1984, Nagele et al., 1984) and relevant cholesterol and triglyceride standards (Appendices IX & X). Plasma and urinary creatinine were monitored using a colourimetric assay (Heinegard & Tiderstrom, 1973) and creatinine standards (Appendix XI). Plasma creatinine concentrations were subsequently used to determine creatinine clearance; a measure of kidney function (Cockcroft & Gault, 1976). This latter measurement was performed to ensure dietary supplementation with AGE had no effect on kidney function.

All subjects had normal plasma protein concentrations, were normolipidaemic, and had normal kidney function as assessed by creatinine clearance (Table 5.2). These perameters did not differ significantly between non-smokers and smokers. No significant differences were observed before and after supplementation with AGE or after the washout period. All individual subject's clinical and biochemical characteristics are presented in Appendix XII.

Table 5.1. Clinical Characteristics of Non-Smoking and Smoking Volunteers. All values are the means ± SEM. Analysis for significance was performed using the two-tailed unpaired t-test; significant differences ($p<0.05$) are indicated by *.

Characteristic	Non-smokers (n=10)	Smokers (n=10)	T-test (non-smokers vs. smokers)
Gender (male:female)	5:5	5:5	
Age (years)	41.60±4.01	41.00±4.08	p=0.57
Weight (kg)	72.76±5.07	67.51±4.05	p=0.37
Height (m)	1.69±0.04	1.68±0.03	p=0.75
Body Mass Index (BMI, kg/m^2)	25.20±1.54	23.71±0.72	p=0.42
Alcohol Consumption (units/week)	14.05±4.01	17.60±6.12	p=0.65
Number of Cigarettes smoked/day	0	18.50±1.83	p=0.00*

Table 5.2. Biochemical Characteristics of Non-Smoking and Smoking Volunteers. All values are the means ± SEM. These biochemical perameters were monitored throughout the clinical trial (days 0, 14, and 28) and did not significantly differ between non-smokers and smokers and before and after supplementation with AGE and after the two week period.

Characteristic	Non-Smokers (n=10)			Smokers (n=10)		
	0	14	28	0	14	28
Plasma protein (g/L)	73.50 ± 1.94	70.15 ± 2.58	72.01 ± 0.95	73.45 ± 2.19	71.65 ± 2.12	76.55 ± 3.03
Total plasma cholesterol (mmol/L)	4.55 ± 0.28	4.63 ± 0.24	4.61 ± 0.32	4.89 ± 0.31	4.90 ± 0.34	5.01 ± 0.26
Total plasma triglycerides (mmol/L)	1.01 ± 0.23	0.93 ± 0.18	0.97 ± 0.21	0.73 ± 0.08	0.81 ± 0.13	0.91 ± 0.10
Plasma creatinine (μmol/L)	85.75 ± 7.14	88.40 ± 7.32	90.17 ± 5.31	85.73 ± 6.20	87.50 ± 4.42	91.10 ± 6.18
Creatinine clearance (mL/min)	99.41 ± 11.12	96.19 ± 9.03	92.70 ± 9.51	92.04 ± 7.12	88.76 ±6.70	86.37 ± 7.85
Urinary creatinine (mg/mL)	0.82 ± 0.23	0.87 ± 0.31	1.03 ± 0.31	1.25 ± 0.30	1.26 ± 0.19	1.35 ± 0.30

5.2.2. The Effect of AGE Supplementation in Non-smokers and Smokers on Plasma and Urinary Levels of 8-iso-$PGF_{2\alpha}$

Plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ were determined using a competitive enzyme-linked immunoassay and sample values were interpolated from a standard curve using 8-iso-$PGF_{2\alpha}$ (Appendices V & VI). Whereas plasma concentrations of 8-iso-$PGF_{2\alpha}$ were given as nmol/L plasma urinary concentrations were standardised to urinary creatinine concentrations and were given as pmol/mmol creatinine.

The level of total 8-iso-$PGF_{2\alpha}$ in plasma from smokers was significantly higher when compared to that measured in non-smokers (1.25 ± 0.19 nmol/L vs. 1.98 ± 0.18 nmol/L, p=0.04) (Figure 5.2). After 14 days of aged garlic extract supplementation plasma levels of 8-iso-$PGF_{2\alpha}$ were significantly reduced by 29% in non-smokers (1.25 ± 0.19 nmol/L vs. 0.88 ± 0.12 nmol/L, p=0.03) and 35% in smokers (1.98 ± 0.18 nmol/L vs. 1.28 ± 0.19 nmol/L, p=0.0001) (Figure 5.2). Interestingly, the significant difference seen in the concentration of plasma total 8-iso-$PGF_{2\alpha}$ between non-smokers and smokers prior to the ingestion of AGE was reduced after two-weeks of dietary supplementation (p=0.04 vs. p=0.07, Figure 5.2). After the two-week washout period plasma concentrations of total 8-iso-$PGF_{2\alpha}$ increased by 26% in non-smokers (0.88 ± 0.12 nmol/L vs. 1.12 ± 0.18 nmol/L) and 34% in smokers (1.28 ± 0.19 nmol/L vs. 1.71 ± 0.19 nmol/L). In fact, plasma total 8-iso-$PGF_{2\alpha}$ concentrations were now similar to those prior to ingestion of AGE (Figure 5.2).

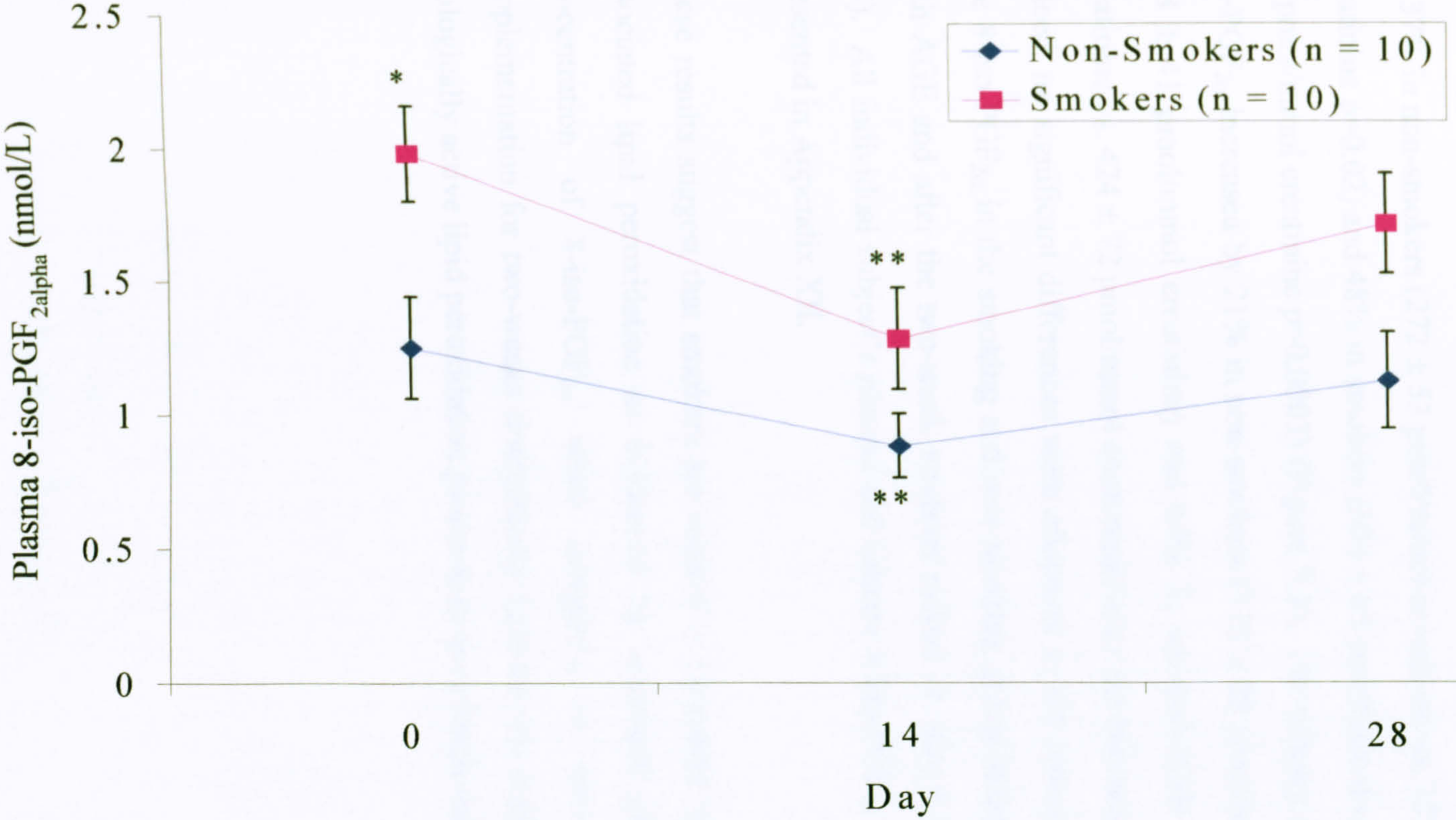


Figure 5.2. Total Plasma Concentrations of 8-iso-$PGF_{2\alpha}$ (nmol/L). Day 0 represents initial levels, day 14 represents levels after supplementation with AGE, day 28 represents levels following a washout period. Values are presented as means ± SEM. Significant differences ($p<0.05$) from control (non-smokers) are indicated by *, from control (Day 0) are indicated by **.

The concentration of free 8-iso-$PGF_{2\alpha}$ in the urine of smokers was approximately twice that of non-smokers (272 ± 53 pmol/mmol creatinine vs. 504 ± 85 pmol/mmol creatinine, p=0.06, Figure 5.3). The changes observed in the urinary concentrations of 8-iso-$PGF_{2\alpha}$ in non-smokers and smokers after dietary supplementation with AGE followed a similar pattern to those observed with plasma total 8-iso-$PGF_{2\alpha}$ concentrations. Thus, after 14 days of aged garlic extract supplementation urinary levels of 8-iso-$PGF_{2\alpha}$ were significantly reduced by 37% in non-smokers (272 ± 53 pmol/mmol creatinine vs. 172 ± 28 pmol/mmol creatinine p=0.02) and 48% in smokers (504 ± 85 pmol/mmol creatinine vs. 265 ± 57 pmol/mmol creatinine p=0.0003) (Figure 5.3). The urinary concentration of 8-iso-$PGF_{2\alpha}$ increased by 21% in non-smokers (172 ± 28 pmol/mmol creatinine vs. 208 ± 41 pmol/mmol creatinine) and 60% in smokers (265 ± 57 pmol/mmol creatinine vs. 424 ± 72 pmol/mmol creatinine) after the two-week washout period. Indeed, no significant differences were observed in the concentration of urinary free 8-iso-$PGF_{2\alpha}$ in the smoking and non-smoking group before supplementation with AGE and after the two-week washout period i.e. day 0 vs. day 28 (Figure 5.3). All individual subject's plasma and urinary 8-iso-$PGF_{2\alpha}$ concentrations are presented in Appendix XII.

These results suggest that smokers are subject to increased oxidative stress and associated lipid peroxidation as evidenced by increased plasma and urinary concentration of 8-iso-$PGF_{2\alpha}$ when compared to non-smokers. AGE supplementation for two-weeks dramatically reduced the concentration of these biologically active lipid peroxidation products in the plasma and urine of smokers.

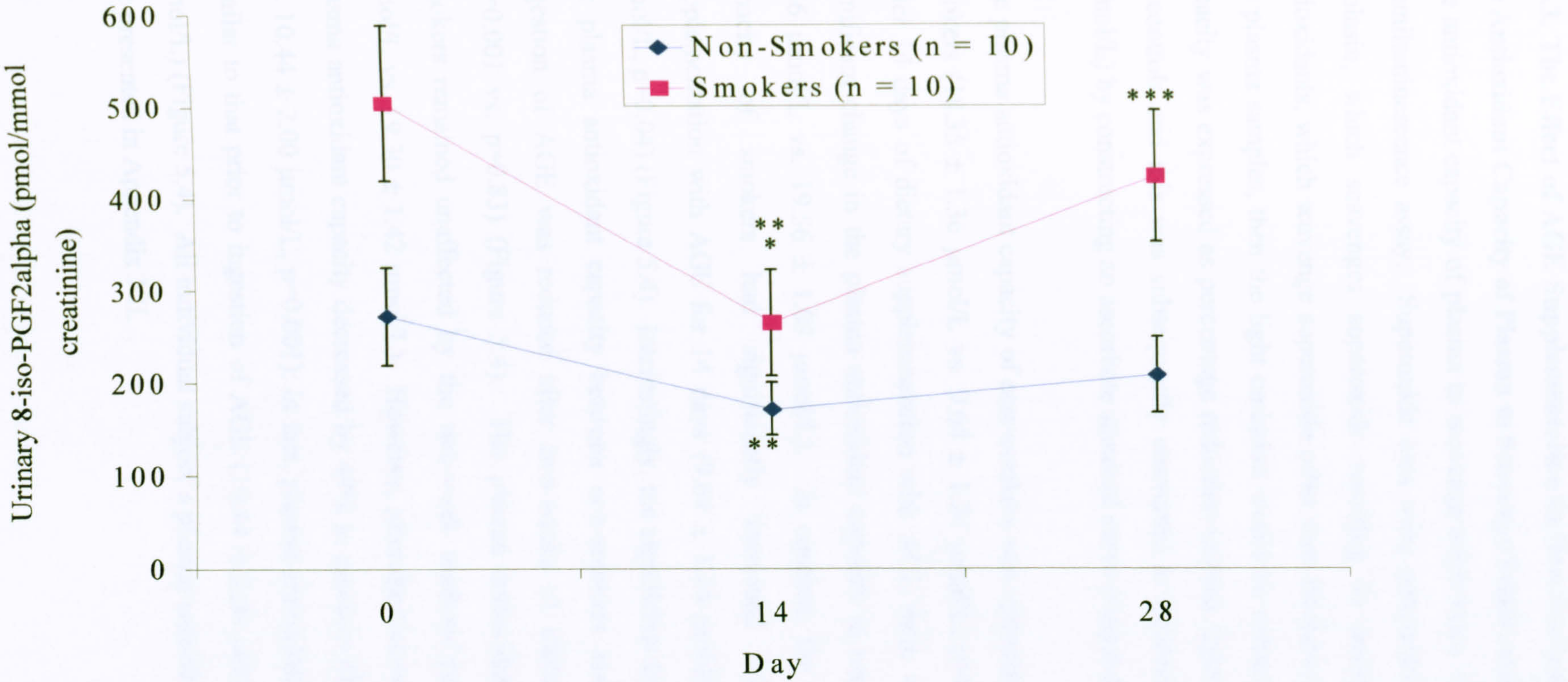


Figure 5.3. Urinary Concentrations of 8-iso-$PGF_{2\alpha}$ (pmol/mmol creatinine). Day 0 represents initial levels, day 14 represents levels after supplementation with AGE, day 28 represents levels following a washout period. Values are presented as means ± SEM. Significant differences ($p<0.05$) from control (non-smokers) are indicated by *, from control (Day 0) are indicated by **, and from control (day 14) are indicated by ***.

5.2.3. The Effect of AGE Supplementation in Non-smokers and Smokers on the Antioxidant Capacity of Plasma to Scavenge Superoxide

The antioxidant capacity of plasma to scavenge superoxide was assessed using a chemiluminescence assay. Superoxide ions were generated in the presence of Pholasin, which scavenges superoxide resulting in emission of light. If antioxidants, which scavenge superoxide other than Pholasin were present i.e. in the plasma samples, then the light emission would be reduced. The antioxidant capacity was expressed as percentage reduction in peak light emission compared to controls and this was subsequently converted to equivalent ascorbate units (μmol/L) by constructing an ascorbate standard curve (Appendix VII).

The plasma antioxidant capacity of non-smokers was approximately twice that of smokers (18.35 ± 1.36 μmol/L vs. 9.64 ± 1.24 μmol/L, p=0.001) (Figure 5.4). After 14 days of dietary supplementation with AGE there was a minimal non-significant change in the plasma antioxidant capacity in non-smokers (18.35 ± 1.36 μmol/L vs. 19.56 ± 1.03 μmol/L). In contrast, the plasma antioxidant capacity of smokers had significantly increased by 53% following supplementation with AGE for 14 days (9.64 ± 1.24 μmol/L vs. 20.48 ± 4.22 μmol/L, p=0.04) (Figure 5.4). Interestingly, the significant difference observed in the plasma antioxidant capacity between non-smokers and smokers prior to ingestion of AGE was reduced after two-weeks of dietary supplementation (p=0.001 vs. p=0.83) (Figure 5.4). The plasma antioxidant capacity of non-smokers remained unaffected by the two-week washout period (19.56 ± 1.03 μmol/L vs. 19.30 ± 1.42 μmol/L). However, after the two-week washout period plasma antioxidant capacity decreased by 49% in smokers (20.48 ± 4.22 μmol/L vs. 10.44 ± 2.00 μmol/L, p=0.001); in fact, plasma antioxidant capacity was now similar to that prior to ingestion of AGE (10.44 ± 2.00 μmol/L vs. 9.64 ± 1.24 μmol/L) (Figure 5.4). All individual subject's plasma antioxidant capacity values are presented in Appendix XII.

These results suggest that smokers have a reduced plasma antioxidant capacity, which was alleviated following two-week dietary supplementation with AGE. In fact, the plasma antioxidant capacity was increased to those observed in non-smokers.

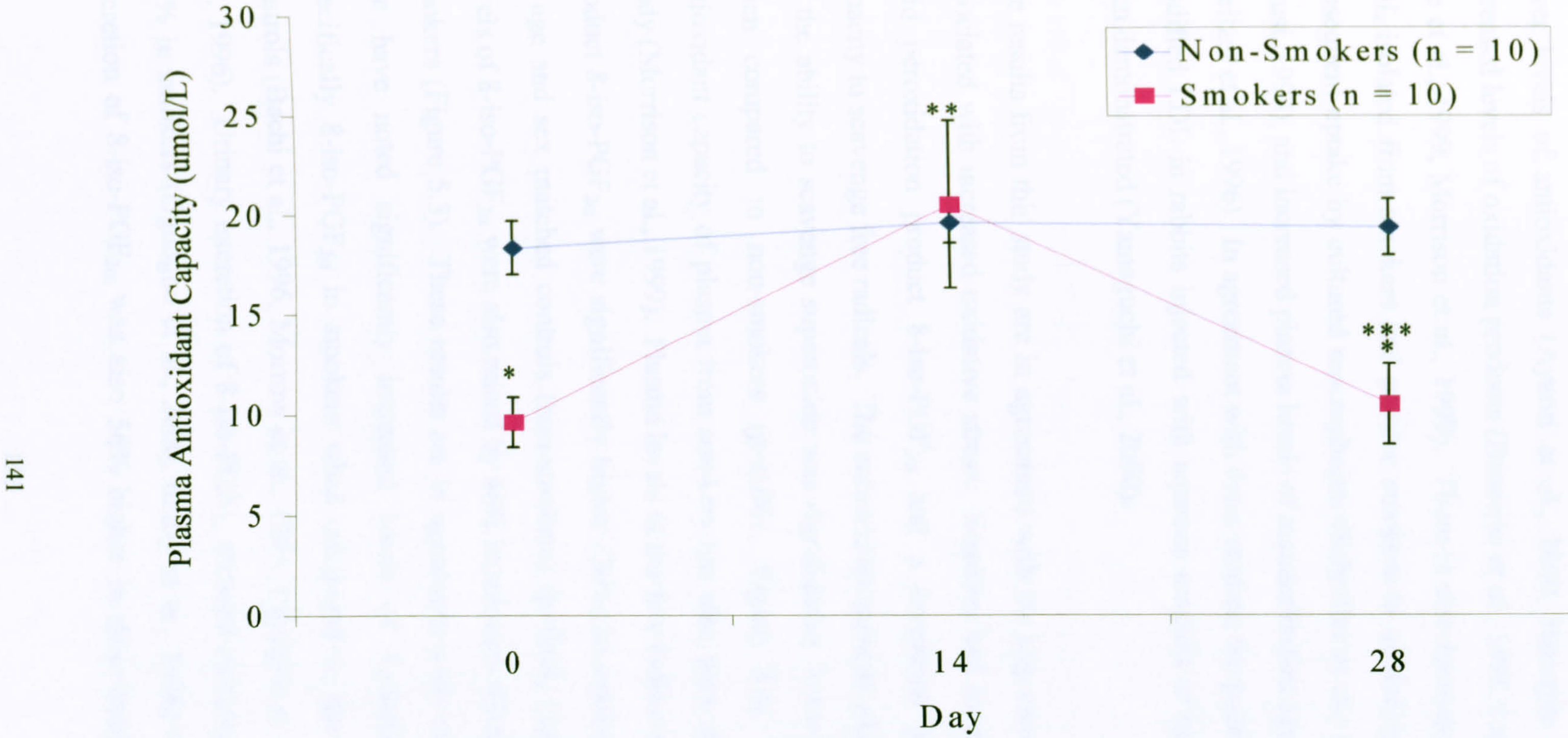


Figure 5.4. The Antioxidant Capacity of Plasma to Scavenge Superoxide Expressed as Ascorbate Equivalent Antioxidant Units (μmol/L). Day 0 represents initial levels, day 14 represents levels after supplementation with AGE, day 28 represents levels following a washout period. Values are presented as means ± SEM. Significant differences ($p<0.05$) from control (non-smokers) are indicated by *, from control (Day 0) are indicated by **, and from control (day 14) are indicated by ***.

5.3. DISCUSSION

Smoking is a risk factor for cardiovascular disease (Feeman, 1999, Tierney et al., 2000) and although the mechanisms behind this association are unclear it is thought to be primarily due to the increased oxidative stress associated with cigarette smoking. This increased oxidative stress is evidenced in smokers by lower levels of antioxidants (Ayaori et al., 2000, Banerjee et al., 1998) and increased levels of oxidation products (Banerjee et al., 1998, Lapenna et al., 1995, Lee et al., 1999, Morrison et al., 1999). There is also increased susceptibility of LDL isolated from smokers and passive smokers to oxidative modification and subsequent uptake by cultured macrophages (Scheffler et al., 1992, Valkonen & Kuusi, 1998), and increased plasma levels of autoantibodies against oxidised LDL (Heitzer et al., 1996). In agreement with these studies, the presence of oxidatively modified LDL in rabbits injected with aqueous extracts of cigarette smoke has been demonstrated (Yamaguchi et al., 2000).

The results from this study are in agreement with the hypothesis that smoking is associated with increased oxidative stress. Smokers had increased levels of the lipid peroxidation product 8-iso-$PGF_{2\alpha}$ and a decreased plasma antioxidant capacity to scavenge free radicals. The antioxidant capacity of plasma as assessed by the ability to scavenge superoxide was significantly lower (48%) in smokers when compared to non-smokers ($p=0.001$, Figure 5.4). This decreased antioxidant capacity of plasma from smokers has also been observed in another study (Morrison et al., 1999). Plasma levels of the free radical mediated oxidation product 8-iso-$PGF_{2\alpha}$ were significantly higher (36%) in smokers when compared to age and sex matched controls (non-smokers) ($p=0.04$, Figure 5.2). Urinary levels of 8-iso-$PGF_{2\alpha}$ were also raised by 48% in smokers when compared to non-smokers (Figure 5.3). These results are in agreement with other similar studies that have noted significantly increased levels of F_2-isoprostanes or more specifically 8-iso-$PGF_{2\alpha}$ in smokers when compared to age and sex matched controls (Bachi et al., 1996, Morrow et al., 1995, Oguogho et al., 2000, Reilly et al., 1996). Urinary excretion of 8-iso-$PGF_{2\alpha}$ showed elevations between 42% - 69% in smokers (Oguogho et al., 2000, Reilly et al., 1996) and rate of urinary excretion of 8-iso-$PGF_{2\alpha}$ was also 56% higher in these subjects (Bachi et al.,

1996). Plasma levels of 8-iso-$PGF_{2\alpha}$ were 50% higher and elevations of 57% and 39% have been demonstrated for free and esterified F_2-isoprostanes, respectively in the plasma of smokers (Morrow et al., 1995, Oguogho et al., 2000). The observation in these previous studies that smokers have elevated levels of F_2-isoprostanes esterified to plasma lipids supports the hypothesis that the link between and risk of coronary artery disease may be attributed to enhanced LDL oxidation.

After 14 days of aged garlic extract supplementation, plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ decreased significantly by 29% and 37% respectively, in non-smokers ($p=0.03$ and $p=0.02$, respectively, Figures 5.2 & 5.3). In comparison, this observation was significantly greater in smokers following 14 days of aged garlic extract supplementation. Plasma levels of 8-iso-$PGF_{2\alpha}$ significantly decreased by 35% ($p=0.0001$, Figure 5.2) and urinary levels significantly decreased by 48% ($p=0.0003$, Figure 5.3) in conjunction with a significantly increased antioxidant capacity of plasma to scavenge superoxide ($p=0.04$, Figure 5.4). Plasma and urinary 8-iso-$PGF_{2\alpha}$ levels in both non-smokers and smokers following the 14 day washout period when aged garlic extract supplementation had ceased, increased and were not significantly different from 8-iso-$PGF_{2\alpha}$ levels observed at the beginning of the study (day 0, Figure 5.2 & 5.3). The increased antioxidant capacity of plasma observed in smokers after 14 days of AGE supplementation also decreased to that observed at the beginning of the clinical trial (day 0, Figure 5.4).

A similar human volunteer supplementation study also demonstrated that dietary intervention with the antioxidant vitamin C alone or in conjunction with vitamin E reduces urinary 8-iso-$PGF_{2\alpha}$ excretion by 30% in smokers (Reilly et al., 1996). Interestingly, this study noted that supplementation with vitamin E alone had no effect. Vitamin C and/or vitamin E supplementation has also been shown to reduce 8-iso-$PGF_{2\alpha}$ levels in other syndromes associated with increased oxidative stress such as diabetes, hypercholesterolemia, and alcoholic liver disease (Davi et al., 1997, 1999, Gopaul et al., 1995, Meagher et al., 1999, Reilly et al., 1998). 50%-80% higher levels of urinary 8-iso-$PGF_{2\alpha}$ were reported in these syndromes

when compared to age and sex matched controls which were reduced by 37%-58% following supplementation with vitamin E or vitamin C. Levels of 8-iso-$PGF_{2\alpha}$ esterified to LDL were 63% higher in hypercholesterolemic patients when compared to normocholesterolemics (Reilly et al., 1998). Consistent with 8-iso-$PGF_{2\alpha}$ formation as a consequence of oxidative stress *in vivo* this study and others have demonstrated increased levels of 8-iso-$PGF_{2\alpha}$ in syndromes associated with increased oxidative stress with a reduction observed following dietary intervention with antioxidant supplements. Due to time constrictions and number of samples analysed an enzyme immunoassay was used in this study, although the most popular method for analysing isoprostane levels is a gas chromatography-mass spectrometry method. While both of these methods provide quantitatively distinct results, qualitatively the information provided is similar (Pratico et al., 1999). This study provides the first direct evidence to suggest that AGE acts as an antioxidant *in vivo* by reducing the formation and excretion of these free radical-catalyzed lipid peroxidation products and increasing the antioxidant capacity of plasma to scavenge free radicals.

8-iso-$PGF_{2\alpha}$ has been shown to enhance platelet activation, vasoconstriction and smooth cell proliferation and has also been shown to be present in increased amounts in human atherosclerotic vascular tissue when compared to healthy vascular tissue (Fukunaga et al., 1993, Hoffman et al., 1997, Kang et al., 1993, Kromer & Tippins, 1996, Lahaie et al., 1998, Mehrabi et al., 1999, Minuz et al., 1998, Oguogho et al., 1999, Pratico et al., 1996). These pro-atherogenic properties of 8-iso-$PGF_{2\alpha}$ together with the findings that elevated levels of 8-iso-$PGF_{2\alpha}$ are associated with traditional risk factors associated with the development of cardiovascular disease suggests that dietary supplementation with a preparation such as AGE that reduces the levels of these free radical catalysed products possibly by enhancing the antioxidant capacity of plasma to scavenge free radicals and hence prevent initiation of oxidative events may be beneficial in reducing the development and progression of atherosclerosis.

CHAPTER 6

GENERAL DISCUSSION

6. GENERAL DISCUSSION

Atherosclerosis associated cardiovascular disease is a major cause of mortality and morbidity in the western society. Many epidemiological studies have identified certain risk factors, which are associated with the development of cardiovascular disease. These traditional risk factors are numerous and include elevated plasma lipids (cholesterol and triglycerides), alterations in glucose metabolism (insulin-dependent and non-insulin-dependent diabetes), hypertension, increased platelet aggregation and decreased fibrinolytic activity in blood, advanced age, male gender, obesity, and smoking (Frishman, 1998, Wood, 2001). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been strongly implicated in the pathogenesis of atherosclerosis in that they initiate the oxidative modification of low-density lipoprotein. It is this oxidised LDL that is recognised by scavenger receptors expressed by macrophages and smooth muscle cells present in the sub-endothelial space, which subsequently leads to unregulated uptake by these cells (Keaney, 2000).

ROS/RNS-mediated oxidative modification of LDL is also an important risk factor for the development and progression of atherosclerotic disease and many of the more traditional risks factors associated with this disease are linked to the oxidative modification of LDL hypothesis. Oxidation of LDL is central to this hypothesis because as well as facilitating foam cell formation oxidised LDL elicits numerous atherogenic properties, which enhance development and progression of the atherosclerotic lesion (Keaney, 2000). One of the associations between increased risk of cardiovascular disease and alterations in glucose metabolism is the atherogenic glycation of LDL that occurs in the presence of elevated glucose levels (Menzel et al., 1997, Moro et al., 1999, Napoli et al., 1997). This *in vitro* or *in vivo* glycated LDL is more susceptible to *in vitro* oxidative modification. The strong implication of ROS/RNS in the pathogenesis of atherosclerosis supports the link between smoking and an increased risk for the development of cardiovascular disease as cigarette smoke is a rich source of potentially damaging reactive species which are capable of modifying LDL (Halliwell & Gutteridge, 1999). Increased plasma cholesterol and the concomitant increase in LDL cholesterol is also a risk factor because increased

circulating plasma levels of LDL increase the risk of LDL becoming either minimally oxidised in the plasma and/or trapped in the sub-endothelial spaces of arteries where it becomes progressively oxidised and ultimately progresses to foam cell formation and atherosclerotic lesion development.

The protective effects of garlic with regards to promoting a healthy cardiovascular system have long been recognised but it is only recently that interest has focused on identifying the active components of garlic responsible for its beneficial effects. Garlic in its numerous commercial forms has been shown to attenuate many of the traditional risk factors associated with cardiovascular disease (Agarwal, 1996, Rahman, 2001). These have included lowering of plasma lipids and the atherogenic lipoprotein LDL, increasing fibrinolytic activity, decreasing platelet aggregation and reducing blood pressure. Although, some dietary supplementation studies have suggested that some garlic preparations have no beneficial effects the general consensus of opinion is that garlic and its preparations are effective in attenuating many of the risk factors associated with cardiovascular disease. In contrast, the evidence supporting the role of garlic as an antioxidant is less convincing. Many studies have identified the antioxidant potential of garlic and its commercial preparations *in vitro* and a few studies have shown antioxidant activity *in vivo* using animal models. To date, evidence demonstrating the antioxidant activity of garlic and its preparations *in vivo* in humans has yet to be presented.

The mechanisms and evaluation of antioxidant action are varied and include scavenging of reactive species, inhibiting the formation of reactive species, recycling of other antioxidants, upregulation of endogenous antioxidant defences, and binding catalytic metal ions (Halliwell et al., 1995, Halliwell, 1997). With reference to identifying a potential antioxidant or an anti-atherosclerotic preparation, the ability of the potential compound or plant extract to prevent oxidative modification of LDL is a routinely used methodology to assess its antioxidant and anti-atherogenic potential. In the work presented here an attempt was made to define the antioxidant potential and the antioxidant mechanisms of AGE using cell free assays, *in vitro* LDL oxidation studies, and

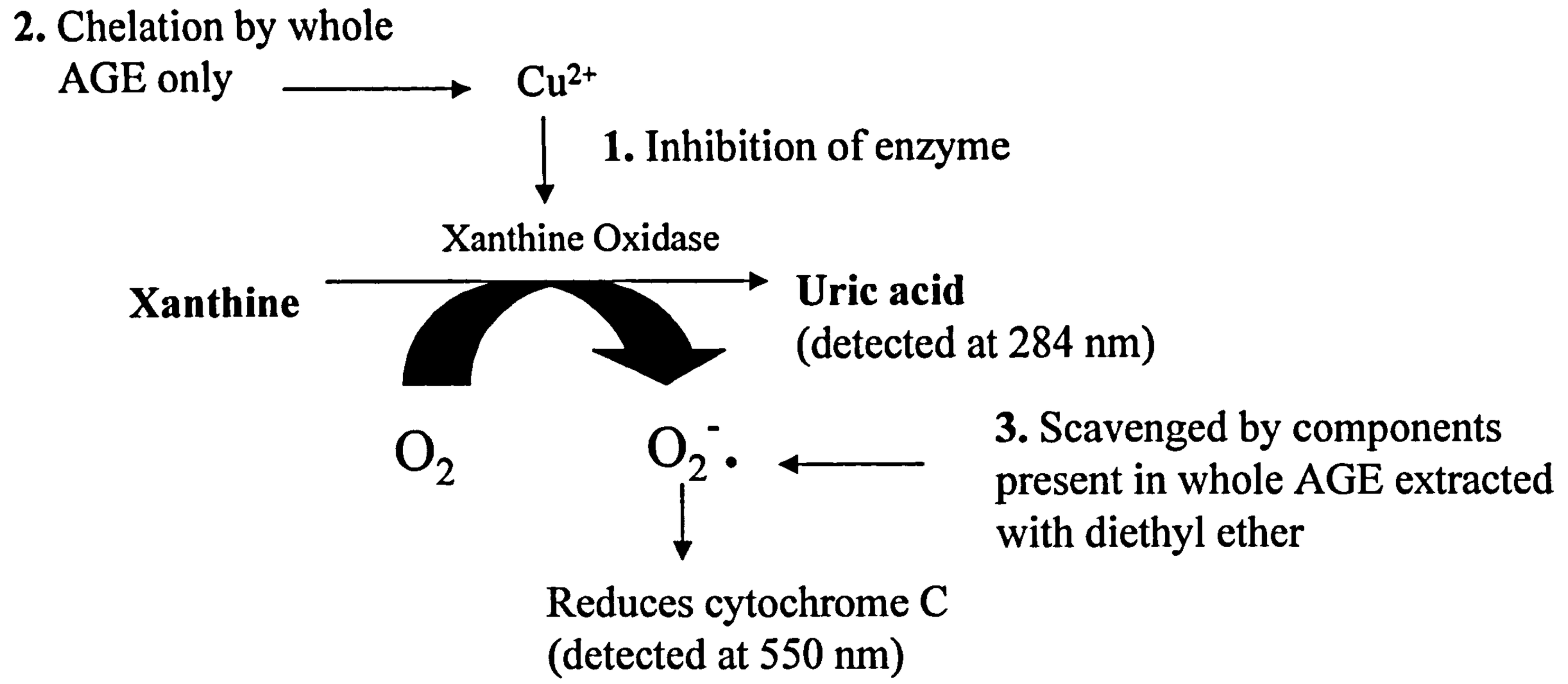
chromatographical analyses, and finally an attempt was made to observe the antioxidant effects of AGE supplementation *in vivo* in humans.

Superoxide is a ROS and enhanced production of this free radical due to increased activity of xanthine oxidase has been implicated in the pathogenesis of atherosclerosis (Houston et al., 1999, Swain & Gutteridge, 1995). Using a cell free system AGE was assessed for its ability to scavenge or inhibit the generation of superoxide generated during the xanthine oxidase-mediated breakdown of xanthine to uric acid (Chapter 3). Whole AGE was capable of reducing the formation of superoxide in a concentration dependent manner using this system. A diethyl ether extract of AGE also demonstrated the ability to reduce the formation of superoxide in a concentration dependant manner and in addition the mechanism of action of this extract was confirmed to be superoxide scavenging. The demonstration of superoxide scavenging ability observed with the diethyl ether extract of AGE could not be determined with whole AGE due to interference with the particular part of the assay that monitors xanthine oxidase activity. Therefore, although compounds present in AGE extracted with diethyl ether displayed the ability to scavenge superoxide the possibility that other compounds present in whole AGE may act to directly inhibit xanthine oxidase activity could not be excluded (Figure 6.1). The role of superoxide in mediating LDL oxidation in the presence and absence of AGE was also examined but it was concluded that in this study superoxide alone was not sufficient to induce oxidation of LDL (Chapter 3). This is in agreement with another study which also concluded that superoxide alone cannot induce lipid peroxidation of LDL (Garner et al., 1994).

Other studies that have examined the ability of garlic to scavenge reactive species have shown that fresh garlic and garlic powder extracts scavenge hydroxyl and peroxyl radicals using cell free systems (Aruoma et al., 1997, Prasad et al., 1995). AGE has also been shown to scavenge the ROS hydrogen peroxide and *t*-butyl hydroperoxide (Ide & Lau, 1999a, Ide et al., 1996, Imai et al., 1994). In contrast to results presented here, Ide et al (1996) did not observe any significant superoxide scavenging by AGE. The conflict between these two results may be due to different methodologies used, although the methods cannot be compared

due to insufficient information provided by Ide et al (1996). As well as the ROS scavenging observed in this study and others, AGE and other garlic preparations have been shown to enhance the activity of the antioxidant enzymes superoxide dismutase and catalase, which act to scavenge superoxide and hydrogen peroxide (Geng & Lau, 1997, Helen et al., 1999, Pedraza-Chaverri et al., 2000). Superoxide is a reactive species that may be involved in oxidation of lipids and proteins either directly or by interacting with other reactive species to generate more potent radicals, thus the ability of AGE to scavenge or reduce the formation of superoxide and hence prevent any oxidative events would be advantageous in human diseases associated with increased oxidative stress such as atherosclerosis.

Figure 6.1 Metal Chelation and Superoxide Scavenging by AGE



1. Cu^{2+} inhibits xanthine oxidase
2. Whole AGE binds Cu^{2+} and relieves inhibition of xanthine oxidase
3. Whole AGE scavenges superoxide ions

15-lipoxygenase is an enzyme that oxygenates polyunsaturated fatty acids (PUFA) resulting in the initiation and subsequent propagation of lipid peroxidation. This then results in the decomposition of lipid hydroperoxides into products capable of modifying proteins (Yamamoto, 1992). Evidence has now been accumulated that strongly implicates 15-lipoxygenase in the pathogenesis of atherosclerosis (Bocan et al., 1998, Cyrus et al., 1999, Harats et al., 2000, Hiltunen et al., 1995, Yla-Herttuala et al., 1990). One study has demonstrated increased 15-lipoxygenase mRNA expression in early atherosclerotic lesions of hyperlipidaemic rabbits (Hiltunen et al., 1995), whilst another study demonstrated colocalisation of 15-lipoxygenase mRNA and protein with oxidised LDL in the atherosclerotic lesions of these hyperlipidaemic rabbits (Yla-Herttuala et al., 1990). The most convincing of these studies are recent publications that have demonstrated that 15-lipoxygenase inhibitors or disruption of 15-lipoxygenase gene expression attenuates lesion development in atherosclerosis-prone rabbits and mice (Bocan et al., 1998, Cyrus et al., 1999), and overexpression of 15-lipoxygenase in the vascular endothelium accelerates early atherosclerosis in mice (Harats et al., 2000).

The work presented here shows that compounds present in whole AGE extracted with diethyl ether reduced 15-lipoxygenase-mediated peroxidation of linoleic acid in a cell-free system (Chapter 3). In addition, 15-lipoxygenase was able to induce significant lipid peroxidation of isolated human LDL, which was reduced in the presence of the diethyl ether extract of AGE (Chapter 3). In contrast, protein oxidation of LDL was not observed in this study. This was not unexpected as 15-lipoxygenase is directly involved in the peroxidation of lipids, not the oxidation of proteins. Due to interference with these assays the effect of whole AGE could not be observed. The reduction of lipid peroxidation observed in the presence of AGE may be due to direct inhibition of 15-lipoxygenase activity or scavenging of lipid hydroperoxides and hence prevention of the chain propagating process of lipid peroxidation. Although both mechanisms seem plausible the precise mechanism responsible could not be determined and was beyond the scope of this study. The mechanism could be clarified using a method described by Robak et al., (1988). This study investigated the effect of numerous plant flavonoids on oxygen consumption during 15-lipoxygenase-mediated peroxidation of

arachidonic acid. Any inhibition of oxygen consumption would suggest that direct inhibition of 15-lipoxygenase itself was taking place.

This is one of the first studies to demonstrate that a garlic preparation such as AGE reduces 15-lipoxygenase-mediated peroxidation of linoleic acid and isolated human LDL and hence may reduce the involvement of the enzyme in the lipid peroxidation process. AGE and other garlic preparations may also reduce lipid peroxidation by modulating the glutathione redox cycle. *In vitro* studies with AGE and *in vivo* supplementation studies in animals with other garlic preparations have shown that in response to an oxidative stress stimulus these garlic extracts increase the activity of glutathione peroxidase and glutathione disulphide reductase and the levels of glutathione (Geng & Lau, 1997, Helen et al., 1999, Ide & Lau, 1999a, Pedraza-Chaverri et al., 2000). This would result in an increased capacity to reduce hydrogen peroxide and damaging lipid hydroperoxides to harmless alcohols and water.

Although there is little evidence supporting a role for Cu^{2+} in the development and progression of atherosclerosis, increased amounts of Cu^{2+} have been identified in atherosclerotic tissue when compared to normal arterial tissue (Evans et al., 1995, Smith et al., 1992, Swain & Gutteridge, 1995). The results presented in this study demonstrate that Cu^{2+} was able to induce significant oxidation of both the lipid and protein moiety of isolated human LDL (Chapter 3). The presence of the diethyl ether extract of AGE reduced both Cu^{2+}-mediated protein oxidation and lipid peroxidation of LDL. Whilst the effect of whole AGE on Cu^{2+}-mediated lipid peroxidation could not be assessed due to interference with the TBARS assay, it was observed that Cu^{2+}-mediated protein oxidation of LDL was completely inhibited in the presence of whole AGE. These results demonstrated that whilst the diethyl ether extract of AGE exerted an appreciable protective effect against Cu^{2+}-induced oxidation of LDL this protection was greater when whole AGE was employed. In addition, the protection AGE exerted against Cu^{2+}-mediated protein oxidation of LDL has not been demonstrated before with AGE or any other garlic preparation, which is surprising as it is the oxidation of the protein moiety in LDL that facilitates recognition by scavenger receptors expressed on macrophages (Hunt et al., 1994).

Cu^{2+}-mediated oxidation is a well-used and traditional method of assessing the ability of possible antioxidant compounds or plant extracts for their protective effect with regards to reducing lipid peroxidation. Esterbauer extensively studied the characteristics of Cu^{2+}-mediated lipid peroxidation and observed that the process is characterised by three phases, which begins with the lag phase when endogenous LDL antioxidants become depleted. Once antioxidant depletion has occurred the propagation phase follows when rapid lipid peroxidation occurs, this is then followed by the decomposition phase when lipid hydroperoxides decompose and/or rearrange to form a wide range of products (e.g. aldehydes, ketones, alcohols) capable of modifying proteins (Esterbauer & Ramos, 1995, Esterbauer et al., 1991a, 1993, Klatt & Esterbauer, 1996). The ability of an antioxidant plant extract to extend the lag phase of Cu^{2+}-mediated lipid peroxidation i.e. the time before rapid lipid peroxidation occurs, suggests that preservation of endogenous antioxidants may be happening. In fact, the results presented here clearly demonstrate that compounds present in AGE extracted with diethyl ether extend this lag time to such an extent that there was no observable propagation phase (Chapter 3). A similar *in vitro* study has shown that AGE protects against Cu^{2+}-mediated lipid peroxidation of LDL (Ide et al., 1997). In addition, dietary supplementation with AGE in humans has been shown to extend the lag time of Cu^{2+}-mediated lipid peroxidation *in vitro* (Munday et al., 1999), with similar observations demonstrated for garlic powder extracts (Orekhov et al., 1994, Phelps & Harris, 1993), although more recent observations with regards to garlic powder extracts have been conflicting (Byrne et al., 1999, Simons et al., 1995).

Although many other studies have shown that AGE and other garlic and plant extracts are able to reduce Cu^{2+}-mediated oxidative modification of LDL these studies have failed to address the mechanism of this reduction (Fuhrman et al., 1997d, Hodgson et al., 1999b, Ide et al., 1997, Orekhov et al., 1994). Multiple mechanisms could be involved in the protection of LDL against Cu^{2+}-induced oxidation of LDL and these could include chelation of Cu^{2+}, scavenging of lipid hydroperoxides, and recycling of endogenous LDL antioxidants. In this study the Cu^{2+} chelation properties of AGE were identified using a novel approach based on the inhibition of xanthine oxidase activity by Cu^{2+} (Lowe et al., 1998) and the

restoration of this activity in the presence of a Cu^{2+} chelator (Chapter 4). This approach was confirmed using a common Cu^{2+} chelating agent, namely EDTA. While whole AGE was shown to be an efficient chelator of Cu^{2+}, the diethyl ether extract of AGE displayed no such properties (Figure 6.1). In conjunction with earlier studies this suggests that at least two mechanisms might be responsible for the protection of LDL against Cu^{2+}-mediated oxidation. One of these mechanisms being the chelation of Cu^{2+} exhibited by whole AGE, and hence prevention of any initiation events resulting in LDL oxidation. This is supported by the observation that whole AGE completely inhibited Cu^{2+}-mediated protein oxidation of LDL. The diethyl ether extract of AGE did not display any Cu^{2+} chelating properties and hence was not as efficient in preventing protein oxidation of LDL induced by Cu^{2+} suggesting another mechanism such as recycling of endogenous antioxidants may be involved in this situation.

Earlier results demonstrated that the diethyl ether extract of AGE prevented Cu^{2+}-mediated lipid peroxidation of LDL by extending the lag phase of this process when endogenous LDL antioxidants become depleted (Chapter 3). This observation and the demonstration of no significant chelation properties suggest that the mechanism of protection against Cu^{2+}-mediated oxidation of LDL might be due to preservation of endogenous LDL antioxidants. To test this hypothesis the depletion of the LDL antioxidants α-tocopherol, β-carotene, and lycopene were observed during Cu^{2+}-mediated oxidation of LDL in the presence and absence of the diethyl ether extract of AGE (Chapter 4). This study confirmed that rapid depletion of these antioxidants occurs in the presence of Cu^{2+} with a concomitant increase in lipid peroxidation. In this part of the study rapid freezing of samples was used to terminate the oxidative modification of LDL induced by Cu^{2+} in the presence or absence of the diethyl ether extract of AGE. The significant depletion of α-tocopherol, β-carotene, and lycopene that occurred at 0 hours in all samples containing Cu^{2+} when compared to control (LDL only) suggested that rapid freezing was not sufficient to halt this process and a more efficient approach may have been the use of a Cu^{2+} chelator such as EDTA. This may be of importance when considering any future studies of this kind. In this study to overcome this problem all arbitrary values obtained from the antioxidant

depletion studies were also expressed as a percentage of their respective controls at 0 hours (Chapter 4).

The diethyl ether extract of AGE did not act to preserve α-tocopherol levels and in fact appeared to slightly enhance its depletion even though lipid peroxidation was reduced in the same experiments. In contrast, Cu^{2+}-induced depletion of lycopene was unaffected and depletion of β-carotene was significantly reduced in the presence of the diethyl ether extract of AGE (Chapter 4). One possible explanation for the partial preservation of β-carotene during Cu^{2+}-mediated oxidation of LDL is that compounds present in AGE may act as chain-breaking antioxidants, which scavenge lipid hydroperoxides and hence prevent the propagation of lipid peroxidation, a similar role to that of α-tocopherol. In this situation preservation of the carotenoids may be observed. Interestingly, other studies utilising plant polyphenols have observed similar effects on α-tocopherol levels even though the isolated LDL was more resistant to oxidation (Belinky et al., 1998, Hayek et al., 1997). These studies showed that the presence or consumption of the licorice and red wine polyphenols glabridin, catechin, and quercetin failed to protect or increase the depletion of α-tocopherol during oxidation of LDL isolated from atherosclerotic mice. Belinky et al (1998) also observed that glabridin partially preserved β-carotene and lycopene levels during oxidation of LDL. A more recent study has shown that oxidation of human LDL isolated from plasma previously incubated with lemon oil is more resistant to oxidation and this was attributed to the partial preservation of β-carotene and lycopene (Grassmann et al., 2001). These observations are similar to the findings of the study presented here which demonstrated that Cu^{2+}-induced lipid peroxidation of LDL was reduced in the presence of components of AGE and this reduction was not due to preservation of α-tocopherol levels but may in fact be due to partial preservation of β-carotene levels in LDL.

Garlic is a rich source of polyphenolic compounds and there is currently a large amount of interest in the antioxidant properties of these plant compounds, although few studies have investigated the phenolic content of commercial garlic preparations. This study prevents evidence to suggest that AGE has a high

phenolic content with different types of phenolic compounds mainly present in their conjugated form (Chapter 4). Whole AGE as well as the diethyl ether extract of AGE contained phenolic compounds although the phenolic content was considerably greater in whole AGE and it may be that the antioxidant effects of the diethyl ether extract of AGE may be attributed to the presence of these plant antioxidants. This high and varied phenolic nature of AGE is also true for fresh garlic (Miean & Mohamed, 2001, Vinson et al., 1998), but would not be necessarily be true for all commercial garlic preparations due to extensive processing and extraction methods although this has yet to be determined. Plant extracts, and plant polyphenols such as the flavonoids have been shown to exhibit lipoprotein-bound activity, which may explain their efficacy in preventing oxidation of LDL (Belinky et al., 1998, Grassmann et al., 2001, Hayek et al., 1997, Ivanov et al., 2001, Vinson et al., 1995, 1998). The results presented here also suggest that compounds present in AGE may also bind to or become incorporated within LDL and hence reduce the pro-atherogenic oxidation of LDL (Chapter 4). A more detailed analysis of how AGE binds to or becomes incorporated within LDL may help to elucidate the earlier finding of partial preservation of β-carotene and not α-tocopherol during Cu^{2+}-induced lipid peroxidation of LDL.

Monitoring lipid peroxidation *in vivo* has been problematic which has led to inconsistent results with regards to monitoring the effects of dietary intervention with antioxidant nutrients. In this study, the F_2-isoprostane 8-iso-$PGF_{2\alpha}$ was used as a biomarker of lipid peroxidation *in vivo* as it has been shown to be reliable and specific endpoint of the non-enzymatic free radical-catalysed oxidation of arachidonic acid (Pratico, 1999). Another method of assessing the efficacy of antioxidant supplementation is to measure the capacity of plasma to scavenge reactive species in comparison with known antioxidants such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E). This present study utilised a novel index of lipid peroxidation, namely 8-iso-$PGF_{2\alpha}$ and the capacity of plasma to scavenge superoxide compared to ascorbic acid to assess the effects of AGE supplementation. These analyses were performed in smokers and non-smokers before and after supplementation with AGE, and after a two-week washout period

(Chapter 5). Smokers were subject to increased oxidative stress as they had increased total plasma and free urinary 8-iso-$PGF_{2\alpha}$ concentrations and a decreased antioxidant capacity to scavenge superoxide when compared to non-smokers.

Dietary supplementation with AGE significantly reduced plasma and urinary 8-iso-$PGF_{2\alpha}$ concentrations in both smokers and non-smokers although the reduction was more pronounced in smokers, and also increased the antioxidant capacity of plasma in smokers. After the two-week washout period plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ in smokers and smokers and the antioxidant capacity of plasma in smokers returned to that observed prior to AGE supplementation. Numerous studies have also demonstrated that smoking is associated with increased oxidative stress by measuring 8-iso-$PGF_{2\alpha}$ or total F_2-isoprostanes and the antioxidant capacity of plasma; with many of these studies demonstrating that antioxidant vitamin supplementation attenuates these factors (Bachi et al., 1995, Morrison et al., 1999, Morrow et al., 1995, Oguogho et al., 2000, Reilly et al., 1996). A few studies have also examined the effect of dietary supplementation with plant extracts such as soy, black tea, onion, olive oil on plasma and/or urinary 8-iso-$PGF_{2\alpha}$ or F_2-isoprostane concentrations in healthy human subjects with only olive oil consumption showing a beneficial effect (Djuric et al., 2001, Hodgson et al., 1999a, O'Reilly et al., 2001, Visioli et al., 2000). The results presented in this study suggest that AGE possesses antioxidant activity *in vivo* and may be useful in reducing the increased oxidative stress associated with smoking and atherosclerosis. 8-iso-$PGF_{2\alpha}$ is also a pro-atherogenic biologically active isoprostane that has been shown to enhance vasoconstriction, smooth muscle cell proliferation, and platelet activation, and hence any reduction in the circulation of this product of lipid peroxidation by AGE would also reduce its potentially pro-atherogenic properties (Fukunaga et al., 1993, Hoffman et al., 1997, Kang et al., 1993, Kromer & Tippins, 1996, Lahaie et al., 1998, Minuz et al., 1998, Pratico et al., 1996).

8-iso-$PGF_{2\alpha}$ is formed in situ from free radical attack on arachidonic acid in membranes and lipoproteins. It is presumably cleaved by phospholipases and

circulates in the plasma where it may exert its pro-atherogenic biological effects prior to excretion in the urine (Morrow et al., 1990a, 1990b, 1992, Pratico et al., 1998). This study has shown that AGE supplementation decreases total plasma and free urinary 8-iso-$PGF_{2\alpha}$ concentrations which may initially suggest that AGE prevents the formation of this isoprostane. Another scenario may be that AGE affects phospholipase activity and hence the release of 8-iso-$PGF_{2\alpha}$ from membranes and lipoproteins. The large decreases observed in total plasma 8-iso-$PGF_{2\alpha}$ concentrations in non-smokers and smokers (29% & 35%, respectively) suggest that AGE supplementation is reducing the formation of these compounds. This remains to be confirmed and may be clarified by measuring free and total 8-iso-$PGF_{2\alpha}$ in the plasma and examining the effect of AGE on phospholipase activity directly.

In this study it has been demonstrated that AGE is an effective antioxidant plant extract with numerous mechanisms of action that reduce free radical-mediated modification of lipids and proteins *in vitro* and *in vivo*. One of the main limitations of this study is that no comparison can be made with other garlic extracts because the chemical composition of commercially available garlic preparations varies widely (Lawson, 1996). In this study a commercial aged garlic extract (AGE, Kyolic) was used which contains high concentrations of water-soluble compounds and is standardised to SAC, its' major organosulphur compound. In contrast, garlic powder extracts contain mainly oil-soluble compounds and are standardised to allicin. Garlic oil preparations are rich in other sulphur compounds such as the allyl sulphides, ajoenes, and vinyldithiins. Many studies also utilise raw garlic, the composition of which cannot be standardised because growth and storage conditions affect garlic bulb composition. In this study the phenolic content of AGE has been investigated and related to the antioxidant effects of this extract (Chapter 4). Few studies have investigated the antioxidant potential of non-sulphur constituents in garlic preparations and this needs to be explored further. Many of the reported beneficial effects of these garlic preparations are attributed to the major sulphur compounds in these preparations but few human dietary intervention studies have been performed using the presumed active constituents. More human intervention

studies are needed using standardised garlic preparations and their major sulphur and non-sulphur constituents. Until this time confusion will persist when trying to interpret results from studies using different garlic preparations.

In summary, AGE possesses superoxide-scavenging ability, reduces 15-lipoxygenase-mediated lipid peroxidation of linoleic acid and isolated human LDL and Cu^{2+}-mediated lipid peroxidation and protein oxidation of isolated human LDL. Numerous properties were also identified that may be responsible for these antioxidant effects. AGE has a substantial and varied phenolic content, which may in part be responsible for the antioxidant mechanisms observed, such as chelation of Cu^{2+} and preservation of the LDL antioxidant β-carotene. The efficacy of AGE in preventing oxidation of LDL may also be attributed to its ability to bind to or become incorporated within isolated human LDL. These antioxidant effects, properties and mechanisms were all observed using cell-free assays, *in vitro* LDL oxidation studies, and chromatographical analyses. The final aim of this study was to identify the antioxidant properties of AGE *in vivo*. The dietary supplementation study confirmed that smoking is a syndrome of increased oxidative stress as evidenced by a decreased antioxidant capacity of plasma to scavenge superoxide and increased plasma and urinary levels of 8-iso-$PGF_{2\alpha}$, a novel marker for oxidative stress *in vivo*. Dietary supplementation with AGE alleviated oxidative stress in smokers by significantly increasing the antioxidant capacity of plasma to scavenge superoxide and significantly decreasing plasma and urinary 8-iso-$PGF_{2\alpha}$. Plasma and urinary 8-iso-$PGF_{2\alpha}$ was also significantly decreased in non-smokers but to a lesser extent. The collective results presented in this study suggest that AGE exerts numerous antioxidant effects *in vitro* and *in vivo* and if taken as a dietary supplement may reduce the development and progression of atherosclerosis.

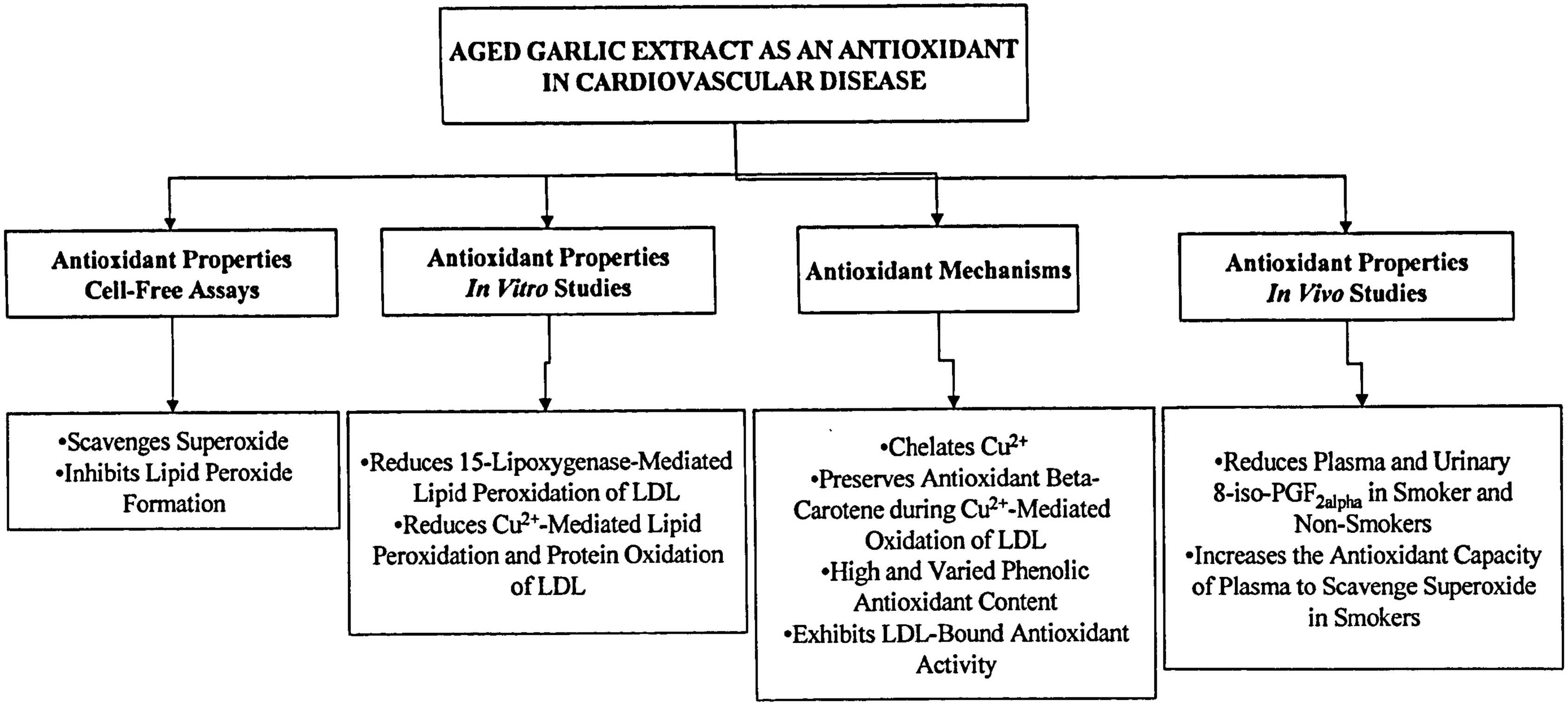


Figure 6.2. Summary of the Antioxidant Properties of Aged Garlic Extract

6.1. Future Work

This study provides evidence to suggest that one of the mechanisms behind the efficacy of AGE in reducing the oxidation of LDL is that it may bind to or become incorporated within the LDL particle. This can be supported by further studies involving extraction and isolation of these compounds bound to LDL followed by chromatographical analysis to identify these compounds. The nature of the compounds in AGE that become associated with the LDL particle might also provide some insight into why AGE may act to preserve β-carotene levels.

This study has shown that AGE is a rich and varied source of polyphenolic compounds. Specific identification of these compounds would be advantageous. Because whole AGE is a complex phytochemical, the diethyl ether extract of AGE would be more suitable for these analyses which could be performed using a gas chromatography-mass spectrometry and should enable identification of any phenolic compounds present. A comparative study with other commercial garlic preparations may also be useful.

A small dietary supplementation study in smokers and non-smokers confirmed the *in vivo* antioxidant properties of AGE. This supplementation study ideally needs to be expanded to include more volunteers, which would provide more significant evidence for the role of AGE in reducing oxidative stress *in vivo*. A comparative dietary supplementation study with other commercial garlic preparations would also be interesting. Total plasma 8-iso-$PGF_{2\alpha}$ concentrations (free + esterified) are elevated in smokers. Analysis of these lipid peroxidation products esterified to LDL isolated from smokers and non-smokers would specifically identify whether LDL from smokers is subject to increased lipid peroxidation *in vivo* and would lend credence to the hypothesis that the link between and risk of atherosclerosis may be attributed to enhanced LDL oxidation. AGE supplementation could then be assessed by its ability to reduce the amount of 8-iso-$PGF_{2\alpha}$ esterified to LDL. This may be a more direct, specific, and sensitive method of measuring oxidised lipids in LDL and the efficacy of antioxidant supplementation on reducing the content of these oxidised lipids in LDL *in vivo*.

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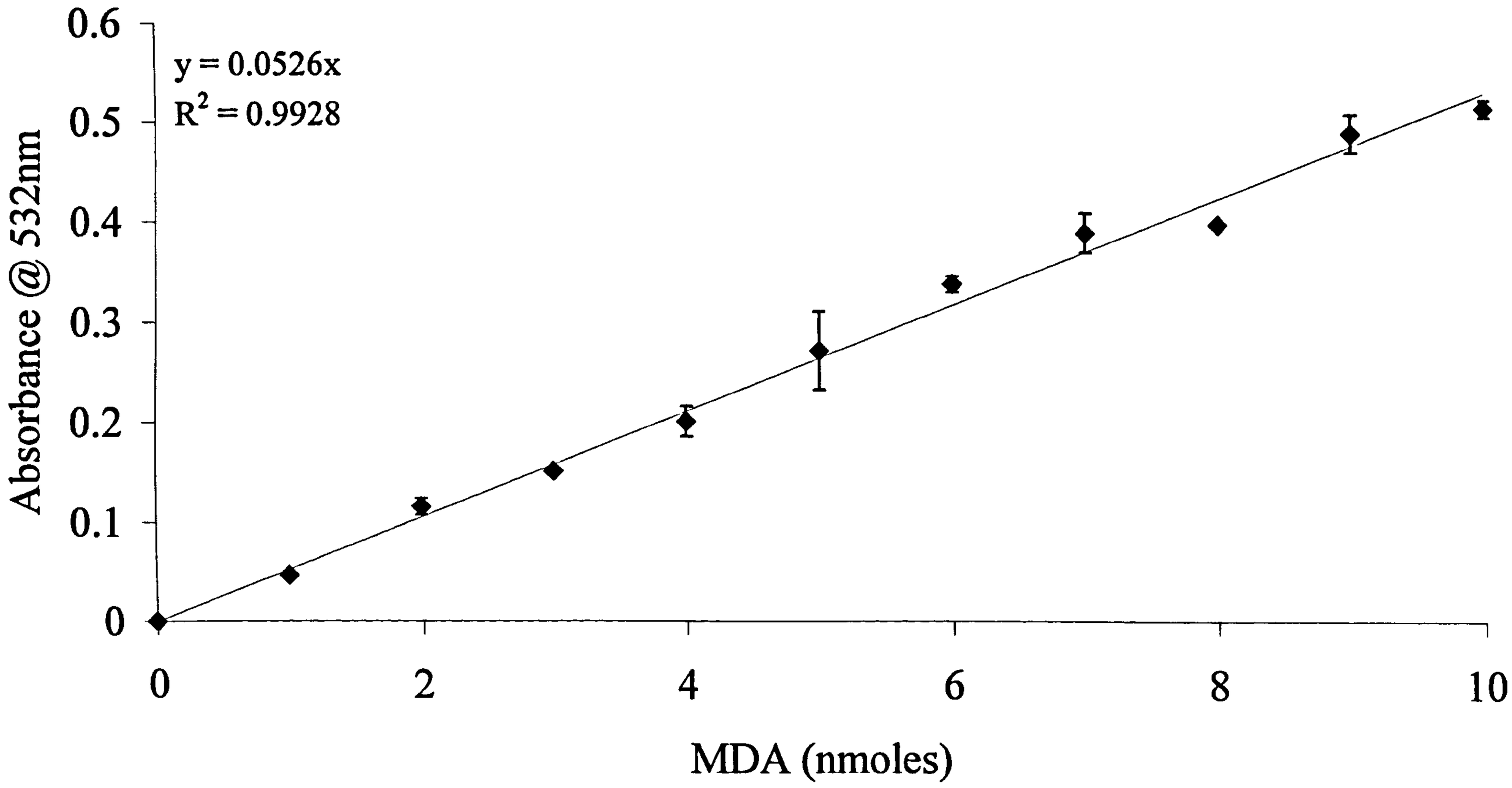
APPENDICES

APPENDIX I

STANDARD CURVE FOR TBARS ASSAY

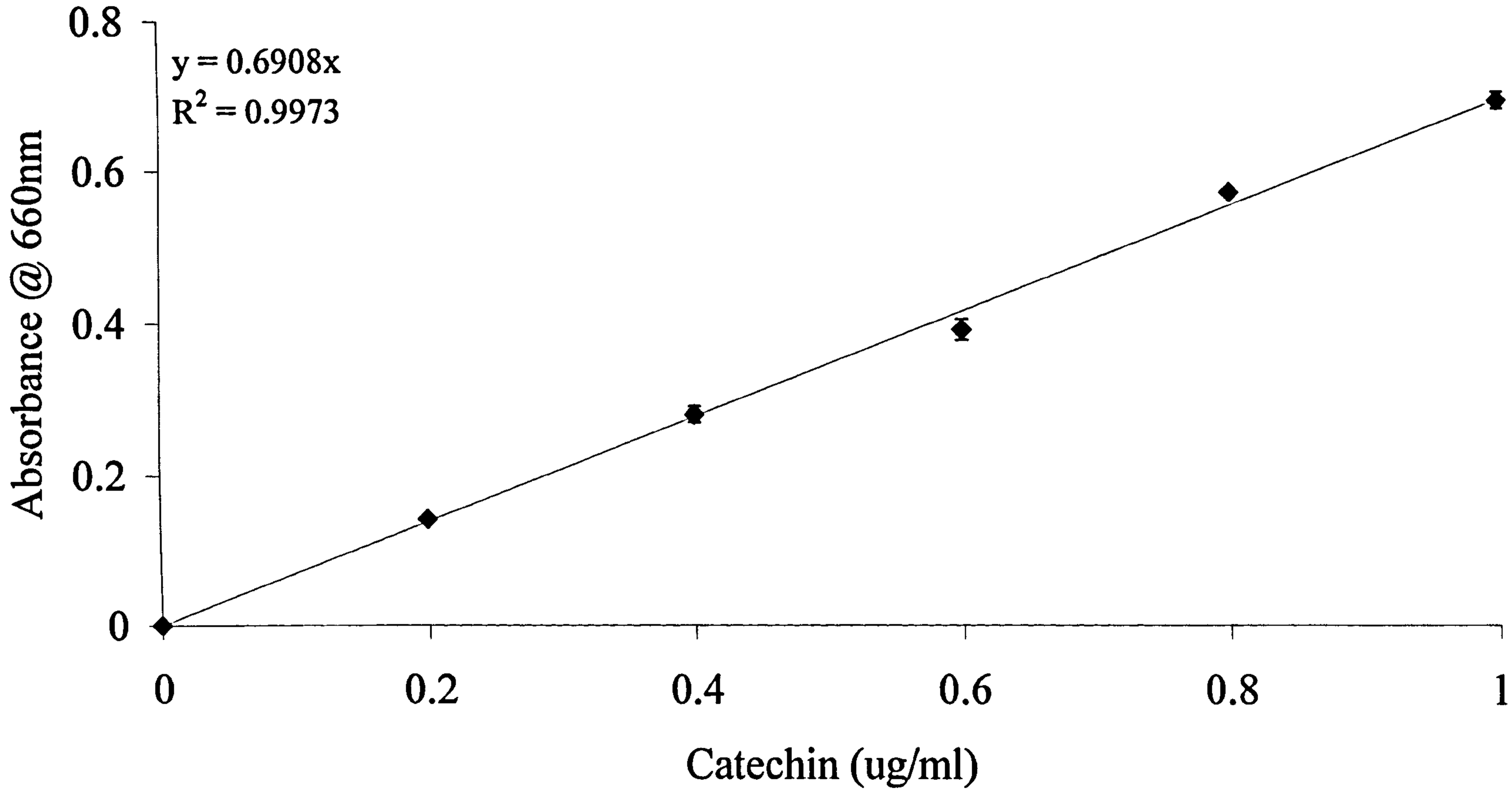
MDA Standard Curve for TBARS Assay



APPENDIX II

STANDARD CURVE FOR FOLIN-CIOCALTEU ASSAY

Catechin Standard Curve for Folin Ciocalteu Assay



APPENDIX III

PARTICIPANT INFORMATION SHEET FOR DIETARY SUPPLEMENTATION STUDY

SUBJECT INFORMATION SHEET

Aged Garlic Extract as an Antioxidant in Cardiovascular Disease.

Background information:

There are various risk factors associated with cardiovascular disease such as increased serum cholesterol and triglyceride concentrations, increased susceptibility of blood platelets to aggregate, decreased fibrinolytic (clot dissolution) activity and increased susceptibility of low density lipoprotein (LDL) to oxidation. One of the most cost-effective ways of controlling this disease is to supplement the diet with phytochemicals known to have anti-cardiovascular and antioxidant properties. One such phytochemical is Aged Garlic Extract (AGE). Recent in *vitro* studies have shown AGE to have antioxidant properties. This antioxidant property of AGE will be investigated further in this clinical trial.

About the study:

Hypothesis: It is likely that once AGE is consumed it modifies the LDL particle making it less prone to oxidation and reduces the oxidative stress the LDL particle is subjected to, hence reducing the risk of cardiovascular disease. A novel family of markers of oxidative stress occurring within the body is the F_2 isoprostanes. Increased levels of F_2 isoprostanes have been reported in humans with conditions thought to be associated with oxidative stress such as smoking, diabetes, hypercholesterolemia, and atherosclerosis. These oxidation products circulate in the plasma and are excreted in urine. This project will investigate the levels of this marker in both urine and plasma samples before and after ingestion of AGE and will provide further information on the antioxidative mechanisms by which AGE may prevent or delay cardiovascular disease.

Your part in the study:

Both smokers and non-smokers are required for this small clinical trial. You will be required to give a blood sample (10-15mls) and a urine sample at the beginning of the trial. You will then be required to take a specified daily dose of AGE (5mls) for a period of two weeks. A blood and urine sample will again be taken. You will then be required to stop taking AGE for two weeks and a final blood and urine sample will be taken. The levels of F_2 isoprostanes in these blood and urine samples will then be measured.

You will not have to alter your diet or life style and there is minimum pain involved in giving a venous blood sample.

Contact information:

Stephanie Ann Dillon (PhD researcher): Ex 2483
Dr. Khalid Rahman (Supervisor): Ex 2087
Dr. Gordon M. Lowe (Supervisor): Ex 2142

APPENDIX IV

PARTICIPANT QUESTIONNAIRE FOR DIETARY SUPPLEMENTATION STUDY

QUESTIONNAIRE FOR DIETARY SUPPLEMENTATION STUDY VOLUNTEERS

Name: _____

Sex (male/female):_____

Age (years):_____

Weight (kilograms/pounds):_____

Height (metres/inches):_____

Average weekly alcohol consumption:

Please provide as much information as possible on type of alcohol i.e. red/white wine, premium/normal strength beer, bottles/pints of beer, types of spirits/ liqueurs, alcopops.

_____

_____

_____

_____

Average number of cigarettes smoked per day:_____

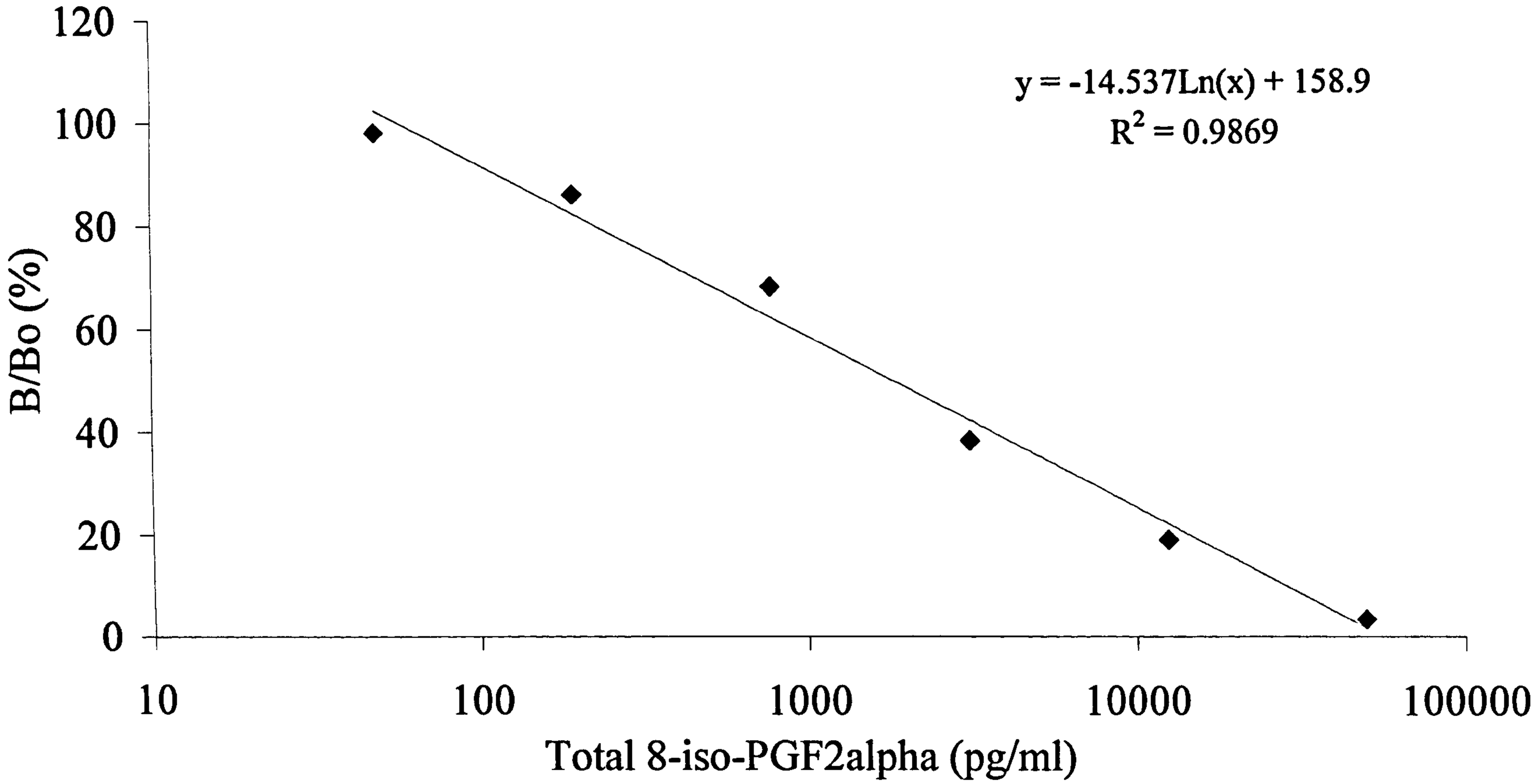
Do you have any known medical conditions (yes/no):_____

If yes to above are you taking any prescribed medication (yes/no):_____

APPENDIX V

STANDARD CURVE FOR TOTAL PLASMA 8-ISO-$PGF_{2\alpha}$ ELISA

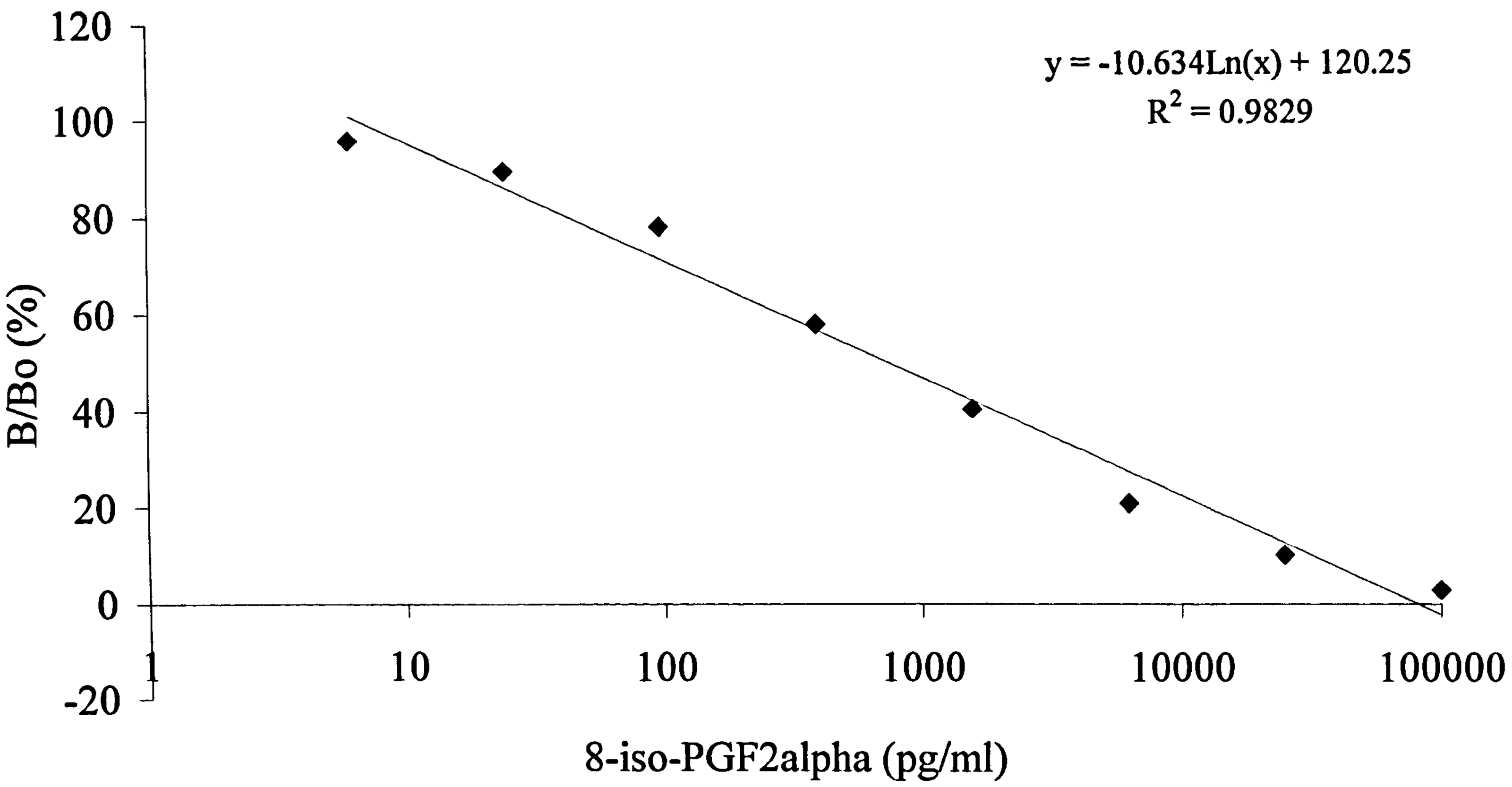
Standard Curve for Total Plasma 8-iso-PGF_{2alpha}



APPENDIX VI

STANDARD CURVE FOR FREE URINARY 8-ISO-$PGF_{2\alpha}$ ELISA

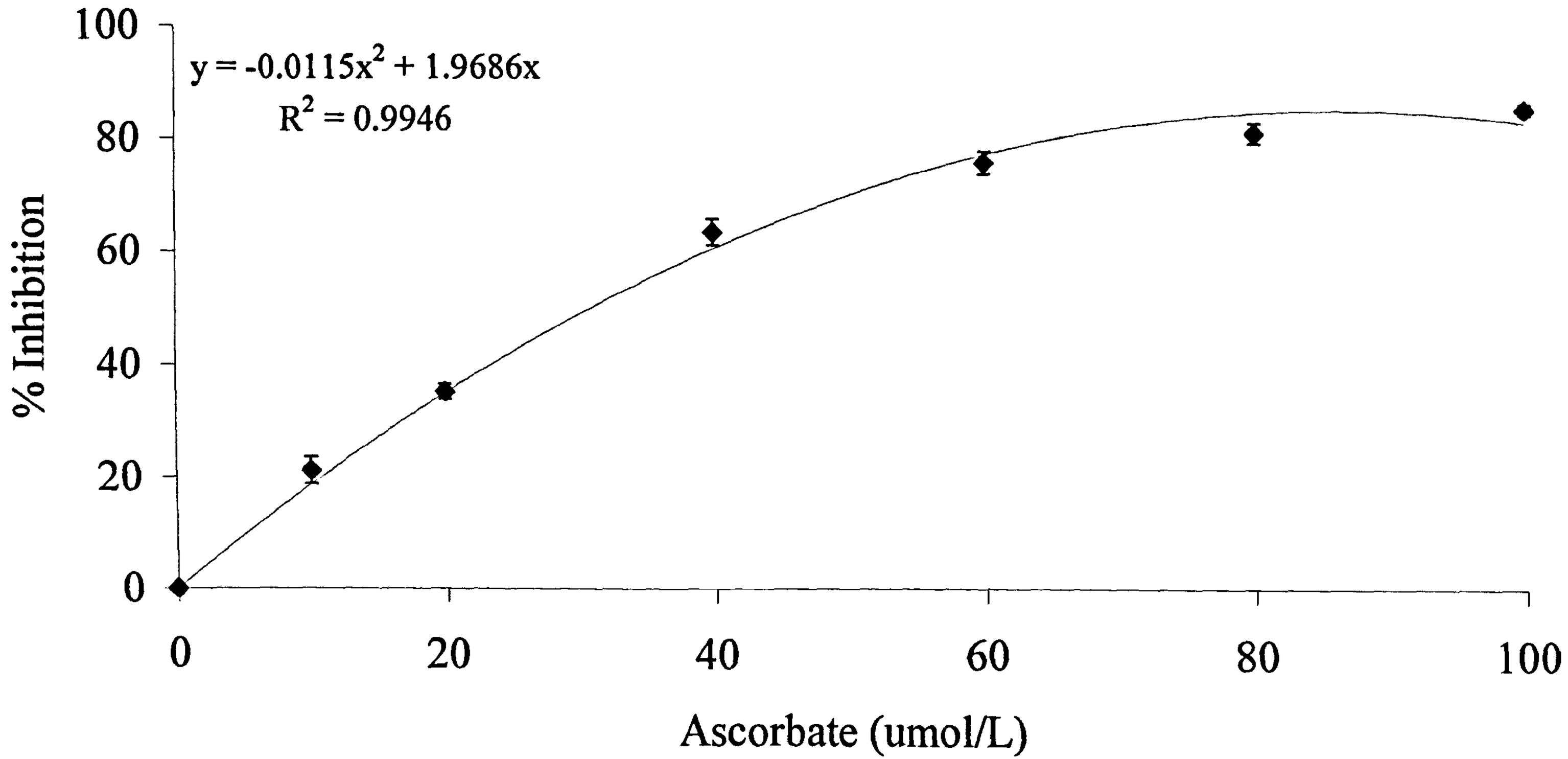
Standard Curve for Free Urinary 8-iso-PGF_{2alpha}



APPENDIX VII

STANDARD CURVE FOR ANTIOXIDANT CAPACITY OF PLASMA TO SCAVENGE SUPEROXIDE ASSAY

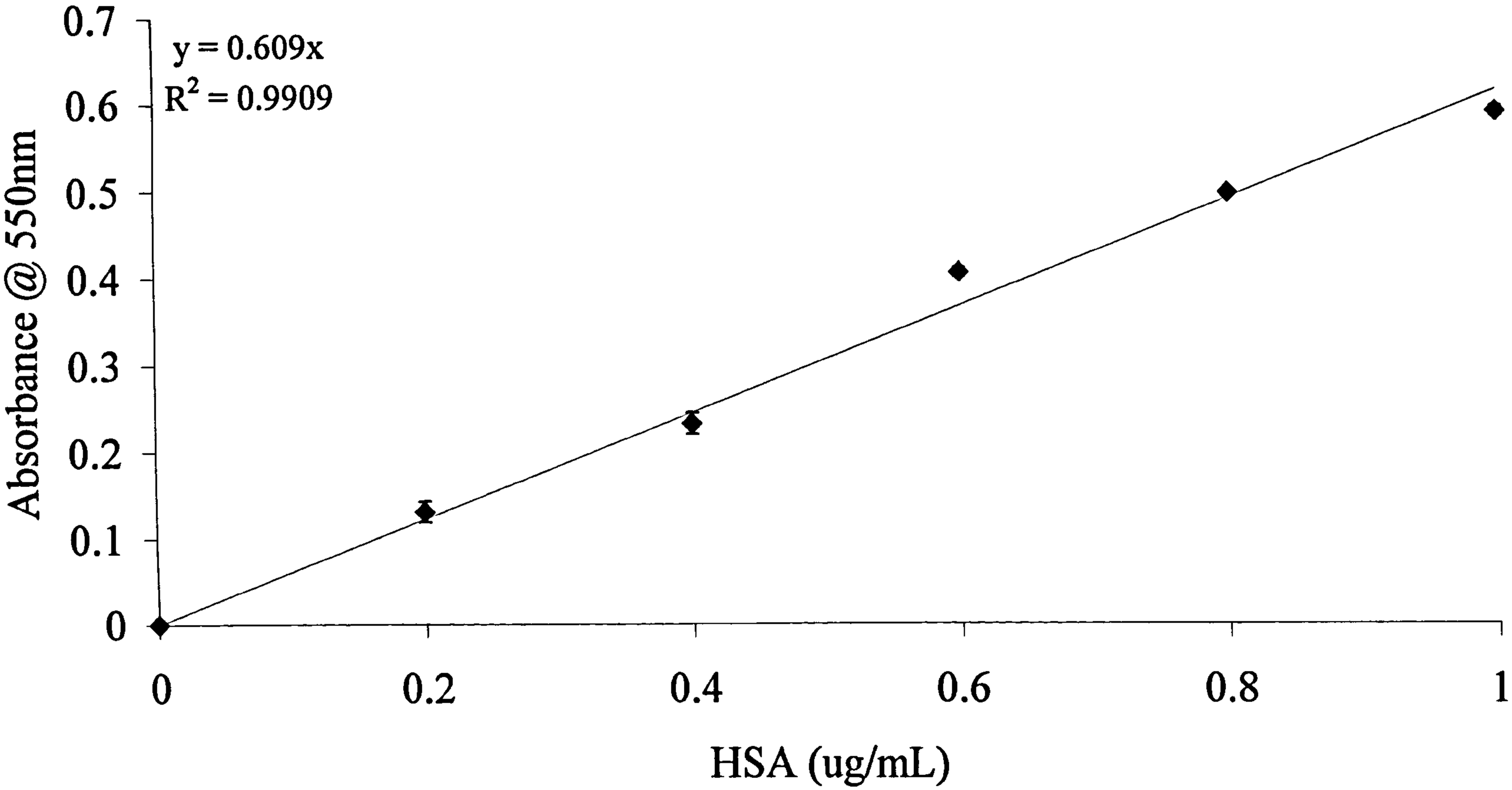
Ascorbate Standard Curve for Determining Antioxidant Capacity of Plasma



APPENDIX VIII

STANDARD CURVE FOR BRADFORD PROTEIN ASSAY

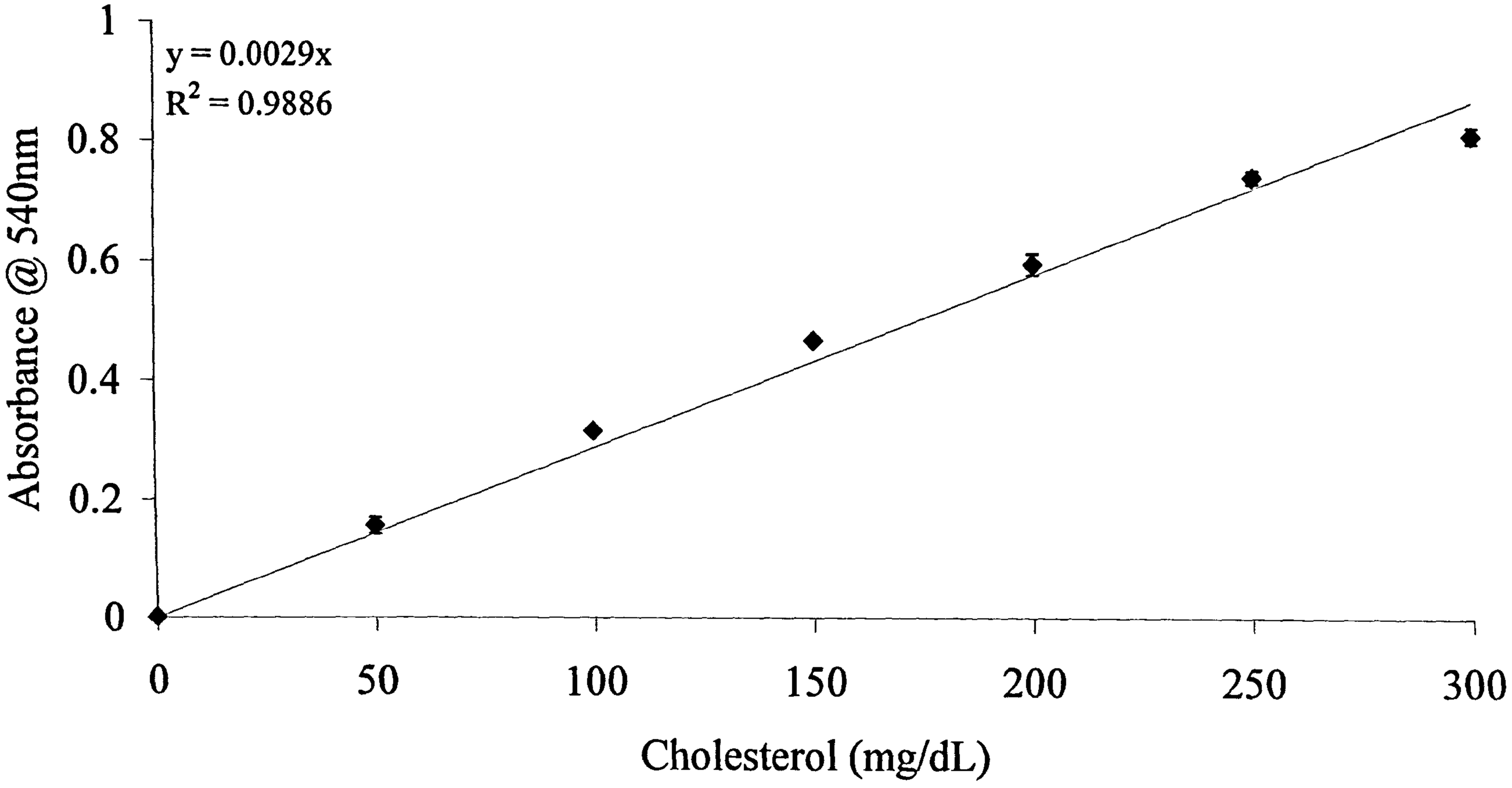
HSA Standard Curve for Bradford Assay



APPENDIX IX

STANDARD CURVE FOR CHOLESTEROL ASSAY

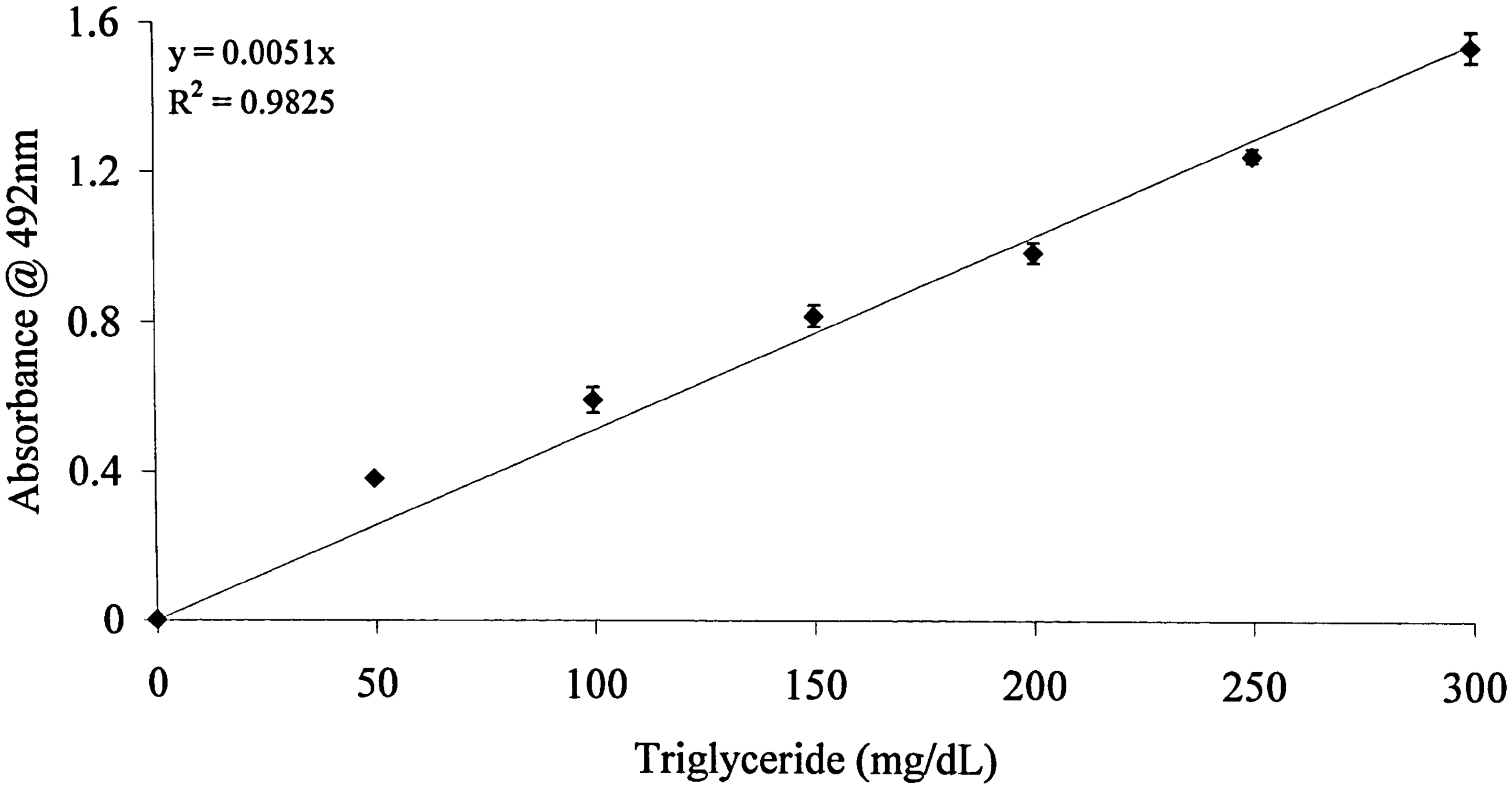
Standard Curve for Cholesterol Assay



APPENDIX X

STANDARD CURVE FOR TRIGLYCERIDE ASSAY

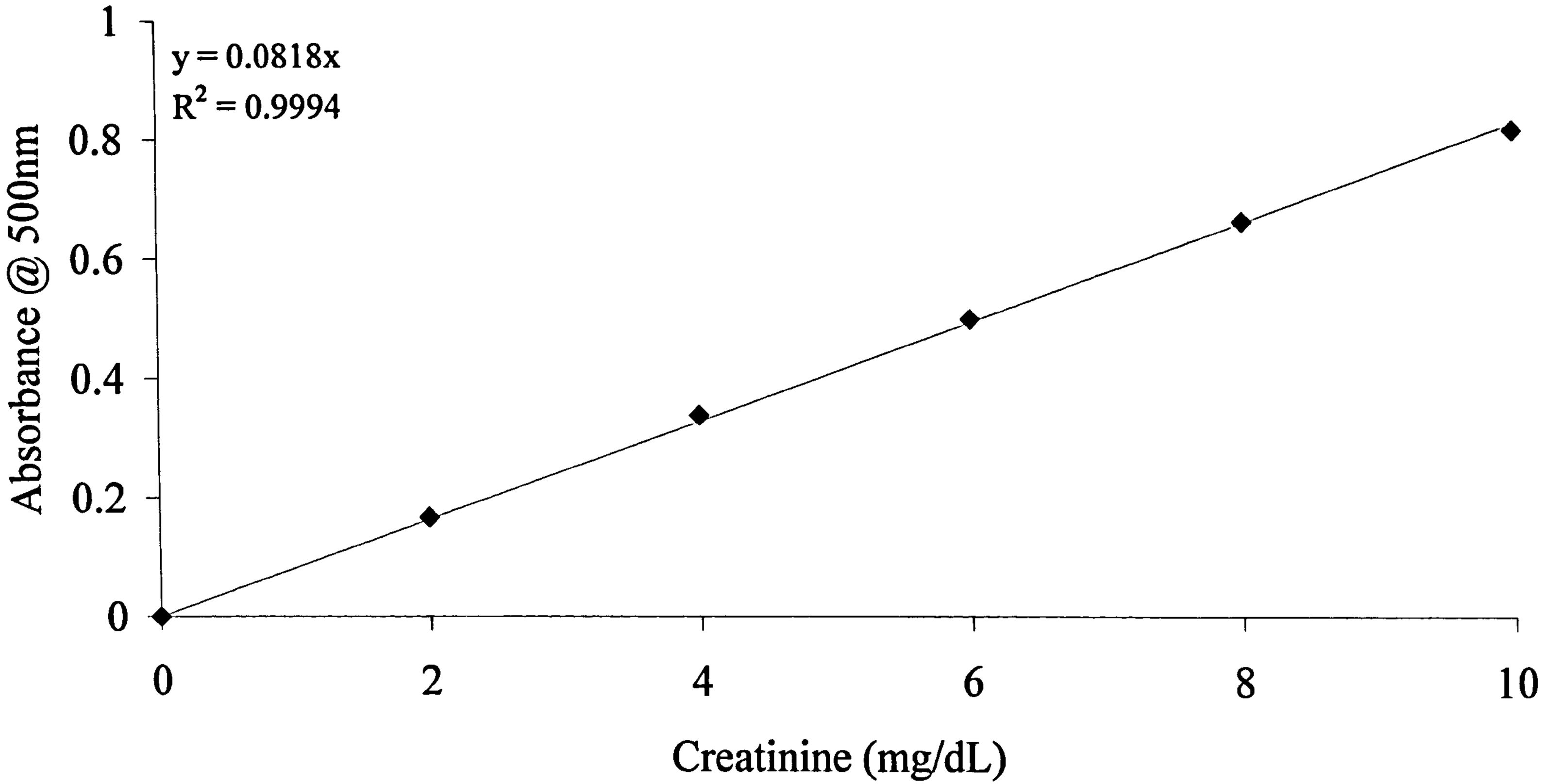
Standard Curve for Triglyceride Assay



APPENDIX XI

STANDARD CURVE FOR CREATININE ASSAY

Standard Curve for Creatinine Assay



APPENDIX XII

INDIVIDUAL SUBJECT DATA FROM DIETARY SUPPLEMENTATION STUDY

Aged Garlic Extract as an Antioxidant in Cardiovascular Disease – Dietary Supplementation Study

Individual Clinical and Biochemical Characteristics of Clinical Trial Subjects

Non-Smoker 1 (Female):

Age	28		
Weight (kilograms)	44.54		
Height (metres)	1.58		
Body Mass Index	18		
Alcohol(units/week)	9		
Cigarettes/day	0		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	69.68	59.09	75.15
Plasma Cholesterol (mmol/L)	3.5	4.14	3.97
Plasma Triglycerides (mmol/L)	0.61	0.48	0.64
Plasma Creatinine (mg/dL)	0.65	0.74	0.8
Creatinine Clearance (mL/min)	88.79	79.14	73.34
Urinary Creatinine (mg/mL)	0.15	0.73	0.45
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	687.67	474.65	553.93
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	297.36	215.28	601.05
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	15.12	15.12	14.2

Non-Smoker 2 (Female):

Age	34		
Weight (kilograms)	61		
Height (metres)	1.69		
Body Mass Index	21		
Alcohol(units/week)	18		
Cigarettes/day	0		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	62.31	67.96	73.21
Plasma Cholesterol (mmol/L)	4.31	4.66	4.78
Plasma Triglycerides (mmol/L)	0.43	0.46	0.57
Plasma Creatinine (mg/dL)	1.15	1.33	1.2
Creatinine Clearance (mL/min)	65.36	66.08	62.41
Urinary Creatinine (mg/mL)	1.83	3.3	1.23
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	173.52	135.45	152.96
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	168.81	102.89	136.11
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	23.81	23.85	26.97

Non-Smoker 3 (Male):

Age	22		
Weight (kilograms)	89		
Height (metres)	1.89		
Body Mass Index	24		
Alcohol(units/week)	36		
Cigarettes/day	0		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	79.85	74.62	72.52
Plasma Cholesterol (mmol/L)	4.69	4.7	3.98
Plasma Triglycerides (mmol/L)	0.71	0.62	0.5
Plasma Creatinine (mg/dL)	1.34	1.39	1.38
Creatinine Clearance (mL/min)	108.76	105.12	105.71
Urinary Creatinine (mg/mL)	1.19	1.93	3.46
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	356.91	187.93	207.42
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1613.38	785.48	1316.97
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	14.53	18.70	18.60

Non-Smoker 4 (Female):

Age	40		
Weight (kilograms)	89.09		
Height (metres)	1.61		
Body Mass Index	35		
Alcohol(units/week)	3.5		
Cigarettes/day	0		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	76.33	79.85	75.6
Plasma Cholesterol (mmol/L)	5.98	5.82	6.2
Plasma Triglycerides (mmol/L)	1.41	1.56	1.16
Plasma Creatinine (mg/dL)	0.61	0.7	0.71
Creatinine Clearance (mL/min)	172.31	150.65	148.95
Urinary Creatinine (mg/mL)	2.12	0.39	1.37
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	530.99	292.99	661.31
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	708.47	567.72	821.56
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	15.87	19.58	20.60

Non-Smoker 5 (Male):

Age	48		
Weight (kilograms)	72.54		
Height (metres)	1.68		
Body Mass Index	25.9		
Alcohol(units/week)	4		
Cigarettes/day	0		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	70.71	63.19	68.86
Plasma Cholesterol (mmol/L)	5.45	5.35	5.69
Plasma Triglycerides (mmol/L)	1.16	0.89	1.67
Plasma Creatinine (mg/dL)	0.85	0.93	0.92
Creatinine Clearance (mL/min)	109.5	100.21	100.66
Urinary Creatinine (mg/mL)	1.13	0.61	1.86
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	666.75	484.53	701.97
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	853.44	683.08	760
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	20.45	21.86	21.85

Non-Smoker 6 (Male):

Age	38		
Weight (kilograms)	82.7		
Height (metres)	1.77		
Body Mass Index	26.5		
Alcohol(units/week)	35		
Cigarettes/day	0		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	71.31	79.64	68.86
Plasma Cholesterol (mmol/L)	3.02	3.22	2.97
Plasma Triglycerides (mmol/L)	0.27	0.28	0.35
Plasma Creatinine (mg/dL)	0.88	0.93	0.91
Creatinine Clearance (mL/min)	133.49	125.5	128.81
Urinary Creatinine (mg/mL)	0.29	0.28	1.37
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	304.03	440.18	268.75
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	720.88	679.54	241.9
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	25.84	22.80	25.15

Non-Smoker 7 (Female):

Age	56		
Weight (kilograms)	76.36		
Height (metres)	1.63		
Body Mass Index	29		
Alcohol(units/week)	12		
Cigarettes/day	0		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	76.33	79.85	75.6
Plasma Cholesterol (mmol/L)	5.07	4.84	4.73
Plasma Triglycerides (mmol/L)	2.7	1.86	1.77
Plasma Creatinine (mg/dL)	0.88	0.76	0.94
Creatinine Clearance (mL/min)	86.28	99.38	80.77
Urinary Creatinine (mg/mL)	0.29	0.53	0.67
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	776.33	343.95	445.64
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1449.3	483.35	279.11
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	15.92	17.63	16.58

Non-Smoker 8 (Female):

Age	38		
Weight (kilograms)	63.63		
Height (metres)	1.5		
Body Mass Index	26.5		
Alcohol(units/week)	6		
Cigarettes/day	0		
	Day 0	Day 14	Day 28
Plasma Protein (g/L)	69.6	65.55	72.94
Plasma Cholesterol (mmol/L)	3.97	3.72	3.74
Plasma Triglycerides (mmol/L)	0.74	0.68	0.4
Plasma Creatinine (mg/dL)	0.93	0.93	1.03
Creatinine Clearance (mL/min)	82.83	82.83	74.45
Urinary Creatinine (mg/mL)	0.24	0.2	0.22
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	441.64	382.34	446.21
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1266.41	957.91	1087.02
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (µmol/L)	18.23	17.68	15.46

Non-Smoker 9 (Male):

Age	64		
Weight (kilograms)	56.4		
Height (metres)	1.68		
Body Mass Index	20		
Alcohol(units/week)	0		
Cigarettes/day	0		
	Day 0	Day 14	Day 28
Plasma Protein (g/L)	75.03	60.14	69.2
Plasma Cholesterol (mmol/L)	4.96	5.2	5.58
Plasma Triglycerides (mmol/L)	1.6	1.7	2.1
Plasma Creatinine (mg/dL)	1.26	1.16	1.14
Creatinine Clearance (mL/min)	47.12	51.54	52.42
Urinary Creatinine (mg/mL)	0.22	0.19	0.22
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	278.88	119.07	361.34
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	216.7	218.08	298.41
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	20.97	23.58	19.84

Non-Smoker 10 (Male):

Age	48		
Weight (kilograms)	92.3		
Height (metres)	1.88		
Body Mass Index	26.1		
Alcohol(units/week)	17		
Cigarettes/day	0		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	84.04	71.56	68.16
Plasma Cholesterol (mmol/L)	4.58	4.69	4.5
Plasma Triglycerides (mmol/L)	0.47	0.72	0.53
Plasma Creatinine (mg/dL)	1.18	1.16	1.19
Creatinine Clearance (mL/min)	100.09	101.43	99.44
Urinary Creatinine (mg/mL)	0.75	0.5	1.49
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	220.62	271.52	162.08
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1187.21	646.03	950.36
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (µmol/L)	12.78	15.12	13.78

Smoker 1 (Female):

Age	25		
Weight (kilograms)	70		
Height (metres)	1.68		
Body Mass Index	25		
Alcohol(units/week)	11		
Cigarettes/day	20		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	76	71.96	64.8
Plasma Cholesterol (mmol/L)	3.34	4.13	4.14
Plasma Triglycerides (mmol/L)	0.36	0.44	0.91
Plasma Creatinine (mg/dL)	1	1.01	1.06
Creatinine Clearance (mL/min)	95.41	94.1	90.1
Urinary Creatinine (mg/mL)	1.42	2.12	2.37
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	583.2	337.9	531.58
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1248.43	632.03	910.47
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	14.1	15.12	5.76

Smoker 2 (Female):

Age	30		
Weight (kilograms)	53		
Height (metres)	1.58		
Body Mass Index	21		
Alcohol(units/week)	0		
Cigarettes/day	25		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	63.91	63.4	79.92
Plasma Cholesterol (mmol/L)	5.95	6.13	6.08
Plasma Triglycerides (mmol/L)	0.82	1.81	1.38
Plasma Creatinine (mg/dL)	0.62	0.72	0.82
Creatinine Clearance (mL/min)	110.74	95.59	84.27
Urinary Creatinine (mg/mL)	1.81	1.37	2.09
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	889.58	701.97	542.64
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	2241.61	1243.38	1695.89
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (µmol/L)	7.33	52.11	25.98

Smoker 3 (Male):

Age	25		
Weight (kilograms)	70		
Height (metres)	1.73		
Body Mass Index	23		
Alcohol(units/week)	47		
Cigarettes/day	10		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	68.49	71.96	66.09
Plasma Cholesterol (mmol/L)	3.22	3.22	3.7
Plasma Triglycerides (mmol/L)	0.44	0.54	0.87
Plasma Creatinine (mg/dL)	0.91	0.89	0.81
Creatinine Clearance (mL/min)	122.92	125.62	137.38
Urinary Creatinine (mg/mL)	1.23	0.87	0.57
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	716.58	355.75	746.72
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1270.34	861.56	1379.59
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	9.22	18.10	11.34

Smoker 4 (Female):

Age	37		
Weight (kilograms)	63.63		
Height (metres)	1.68		
Body Mass Index	22.6		
Alcohol(units/week)	49		
Cigarettes/day	25		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	70	67.9	72.36
Plasma Cholesterol (mmol/L)	4.65	5.13	5.11
Plasma Triglycerides (mmol/L)	0.59	1.13	1.15
Plasma Creatinine (mg/dL)	0.81	0.86	0.94
Creatinine Clearance (mL/min)	103.08	94.04	87.14
Urinary Creatinine (mg/mL)	1.7	1.75	2.23
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	736.39	519.94	537.11
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1745.02	937.71	1303.18
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	7.71	9.19	5.71

Smoker 5 (Male):

Age	48		
Weight (kilograms)	70		
Height (metres)	1.76		
Body Mass Index	23		
Alcohol(units/week)	10		
Cigarettes/day	10		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	78.46	76.66	79.14
Plasma Cholesterol (mmol/L)	5.59	5.57	5.06
Plasma Triglycerides (mmol/L)	1.02	0.92	0.73
Plasma Creatinine (mg/dL)	1.22	1.23	1.44
Creatinine Clearance (mL/min)	73.03	72.71	62.11
Urinary Creatinine (mg/mL)	0.87	0.91	1.74
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	956.91	868.16	819.41
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	3537.43	2124.72	3128.84
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	6.46	34.03	5.22

Smoker 6 (Male):

Age	35		
Weight (kilograms)	97		
Height (metres)	1.88		
Body Mass Index	28		
Alcohol(units/week)	2		
Cigarettes/day	20		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	88.2	67.82	97.09
Plasma Cholesterol (mmol/L)	4.46	3.82	4.65
Plasma Triglycerides (mmol/L)	0.52	0.79	1.03
Plasma Creatinine (mg/dL)	1.36	1.34	1.36
Creatinine Clearance (mL/min)	111.36	112.6	111.03
Urinary Creatinine (mg/mL)	3.45	2.23	2.52
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	859.46	563.78	598.56
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1519.56	958.64	1013.81
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	6.77	15.94	6.46

Smoker 7 (Female):

Age	57		
Weight (kilograms)	54		
Height (metres)	1.53		
Body Mass Index	22		
Alcohol(units/week)	0		
Cigarettes/day	15		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	69.23	62.39	70.18
Plasma Cholesterol (mmol/L)	4.79	3.98	4.4
Plasma Triglycerides (mmol/L)	1.04	0.6	0.39
Plasma Creatinine (mg/dL)	1.03	0.99	1.06
Creatinine Clearance (mL/min)	51.62	53.36	49.8
Urinary Creatinine (mg/mL)	0.85	0.73	0.39
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	301.25	252.56	521.48
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	697.56	552.13	804.5
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	15.92	14.66	13.19

Smoker 8 (Female):

Age	40		
Weight (kilograms)	55.5		
Height (metres)	1.6		
Body Mass Index	21.5		
Alcohol(units/week)	27		
Cigarettes/day	25		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	71.42	84.54	76.06
Plasma Cholesterol (mmol/L)	5.47	5.37	5.89
Plasma Triglycerides (mmol/L)	0.62	0.88	0.58
Plasma Creatinine (mg/dL)	0.82	0.81	0.85
Creatinine Clearance (mL/min)	73.13	72.71	69.75
Urinary Creatinine (mg/mL)	0.14	0.99	0.23
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	704.88	431.42	578.88
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	1732.66	343.53	716.84
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	10.21	13.80	13.56

Smoker 9 (Male):

Age	60		
Weight (kilograms)	68.72		
Height (metres)	1.69		
Body Mass Index	24.2		
Alcohol(units/week)	30		
Cigarettes/day	15		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	70.39	72.7	74.55
Plasma Cholesterol (mmol/L)	5.98	6.55	6.13
Plasma Triglycerides (mmol/L)	0.74	0.62	0.79
Plasma Creatinine (mg/dL)	1.05	1.07	1.01
Creatinine Clearance (mL/min)	75.75	74.04	78.56
Urinary Creatinine (mg/mL)	0.95	1.25	1.28
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	491.86	162.08	204.88
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	867.22	435.38	837.47
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	14.44	23.20	10.47

Smoker 10 (Male):

Age	53		
Weight (kilograms)	73.2		
Height (metres)	1.65		
Body Mass Index	26.8		
Alcohol(units/week)	0		
Cigarettes/day	20		
	Day 0	**Day 14**	**Day 28**
Plasma Protein (g/L)	78.41	77.12	85.31
Plasma Cholesterol (mmol/L)	5.46	5.1	4.98
Plasma Triglycerides (mmol/L)	1.12	1.39	1.23
Plasma Creatinine (mg/dL)	0.85	0.95	0.93
Creatinine Clearance (mL/min)	103.38	92.04	93.66
Urinary Creatinine (mg/mL)	0.12	0.38	0.1
Plasma 8-epi-$PGF_{2\alpha}$ (pg/mL)	758.63	323.25	989.3
Urinary 8-epi-$PGF_{2\alpha}$ (pg/mg creatinine)	870.93	161.97	1421.74
Plasma Antioxidant Capacity (Equivalent Ascorbate Units (μmol/L)	4.25	8.52	6.87

APPENDIX XIII

Dietary Supplementation with Aged Garlic Extract Reduces Plasma and Urine Concentrations of 8-iso-Prostaglandin $F_{2\alpha}$ in Smoking And Non-Smoking Men and Women

APPENDIX XIV

Preliminary Evidence to Suggest that Antioxidant Phenolics in Aged Garlic Extract Protect against *In-Vitro* Copper Oxidation of LDL

PRELIMINARY EVIDENCE TO SUGGEST THAT ANTIOXIDANT PHENOLICS IN AGED GARLIC EXTRACT PROTECT AGAINST *IN VITRO* COPPER OXIDATION OF LOW-DENSITY LIPOPROTEIN (LDL).

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Several studies suggest that aged garlic extract may protect against the development and progression of atherosclerotic disease. Aged garlic extract contains many phenolic and sulphurous components, and S-allylcysteine has been identified as a potential antioxidant. Initial work in this laboratory indicated that aged garlic extract acted in two ways to protect against LDL oxidation by copper. A novel enzyme assay based on the inhibition of xanthine oxidase activity by copper demonstrated the copper chelation properties of the whole aged garlic extract. However, a diethyl ether extract of the aged garlic extract did not exhibit any copper chelation properties but markedly reduced copper oxidation of LDL assessed by both TBA-RS (lipid peroxidation) and electrophoretic mobility (protein oxidation). This is in contrast to recent studies in which fresh garlic and other commercial garlic preparations have been shown to be pro-oxidants.

Spectrophotometric and HPLC analysis indicated that the diethyl ether extract of the aged garlic extract contained several components with a 274nm peak. Further analysis by thin layer chromatography of the diethyl ether extract using a ethylacetate : methanol : water (10:2:1) solvent system, and using detection by both UV and ferric chloride indicated that at least 4 phenolic compounds are present. Using catechin as the standard the Folin & Ciocalteu phenol assay demonstrated that the total phenolic content of the whole aged garlic extract was 22.7mg/ml, and the diethyl ether extract contained 1.1mg/ml.

Further studies are underway to determine the nature of these phenolic compounds.