The vasodilatory and antioxidant activities of polyphenolic substances

Jennifer Sarah McGinn

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Institute of Biomedical & Life Sciences
University of Glasgow

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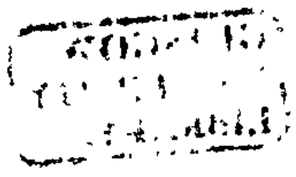

Abstract

Wine has been enjoyed by individuals for centuries. Epidemiological research has clearly demonstrated that individuals who choose to drink alcohol in moderation exhibit improved cardiovascular health and, on average, live longer. While alcohol alone can help prevent coronary heart disease (CHD) through a number of mechanisms, red wine appears to offer protection above and beyond that attributable to alcohol alone. Red wine is a rich source of biologically active phytochemicals, specifically phenolic acids and polyphenols, derived primarily from the grape skin, whose individual and summated actions may be able to provide health benefits.

Tea, like red wine is also a rich source polyphenols. Tea is also a source of bioactive molecules, an important member of the family of antioxidants and a beverage which may be able to confer beneficial health properties.

This project was designed to determine the effect of a wide range of grape and tea based extracts and green tea catechins on vascular tension *in vitro*, further determine the relationship between their vasodilatory and antioxidant activities and identify the major polyphenols present in green and black tea.

Grape and tea based extracts were examined for their vasodilator activity *in vitro* using standard organ bath pharmacology. All extracts evoked biphasic concentration-dependent relaxation in rabbit aortic vessels. Grape based extracts were significantly better vasodilators than tea based extracts. Previous work by others has shown that grape based products induce vascular relaxation via nitric oxide (NO) and endothelium-dependent mechanisms. This study demonstrated that a range of grape based extracts induced vasorelaxation responses via complex mechanisms including endothelium-dependent and independent mechanisms via vasodilating prostaglandins by way of prostacyclin and endothelial NO production. Tea based extracts on the other hand, induced vasorelaxation via the combined interactions of

vasodilating endothelium-dependent prostaglandins. The relationship between the vasodilation capacity, antioxidant activity, based on the reduction of the free Fremy's radical and ferric reducing power, and total phenolic content of each extract was also determined. In general, a significant inverse correlation was identified between the vasodilator abilities of the grape and tea extracts *in vitro* and their antioxidants activities.

This study demonstrates that grape and tea based extract induce vasorelaxation in isolated rabbit aortic vessels and are effective antioxidants *in vitro,* within a concentration range that may be reached *in vivo* by moderate wine and tea consumption. The results presented here also indicate that consumption of green tea may have greater benefits than consumption of black tea in terms of their vasodilatory activity and antioxidant capacity *in vitro.* These results do not however take into account metabolism and absorption of grape and tea components *in vivo.* Further work is required to elucidate the identification of the vaso-active components present in theses extracts and their possible effects *in vivo.*

Green tea in this study has been shown to be an antioxidant and a vasodilator *in vitro.* In a separate study the six main catechins and epicatechins derivatives present in green tea were examined for their vasodilator activity *in vitro* using standard organ bath pharmacology. (+)-Catechin gallate (CG) was identified to be the most potent derivative *in vitro,* that is inducing vasodilation at the lowest derivative concentration added to the organ bath, and (-)-epigallocatechin gallate (EGCG) to be the most effective, that is inducing the highest maximum vasodilation value of all derivatives investigated. (-)-Epicatechin (EC) and catechin were found to have no significant effect on vascular tension *in vitro.* In all cases, vasorelaxation occurred via the release of NO and is dependent upon the presence of intact functional vascular endothelium. The catechins and epicatechins examined in this study were identified to be effective antioxidants, a feature positively correlated with their vasodilatory activity. The total phenolic and total catechin content of the epicatechin derivatives was also positively correlated

with vasodilatory and antioxidant activity. The ferric reducing ability (FRAP)-derived antioxidant activity of the catechin and epicatechin derivatives examined in this study ranged in the order magnitude of EGCG>EGC>ECG≥Catechin>CG>EC.

Differences in the statistical results presented for the vasodilatory and antioxidant activities of the grape and tea based extracts as compared to that of the green tea epicatechin and catechin derivatives, may be due to genuine differences in the basic chemical composition of the compounds investigated as standard methodology was used in both studies. The grape and tea extracts investigated contained a range of active components in varying concentrations which may have interacted together to produce cumulative effects as compared to the purified epicatechin and catechin standards of a known and predetermined concentration. Contamination of the extracts is another factor which may have had an affect on the results presented and which could not be ruled out.

A novel fractionation approach was also employed to further determine the identity of the major polyphenolics present in green and black tea, based on differences in their polarities. Tea catechins were separated using a preparative HPLC system and fractionated in to 50-60 aliquots. The antioxidant activity of each fraction was determined using electron spin resonance spectroscopy (ESR) and their ferric reducing ability (FRAP). The total phenolic and catechin content of each fraction was also determined. The effect of the green tea fractions on vascular tension *in vitro* was also investigated.

Green tea fractions with high antioxidant activity and high phenolic and catechin contents were able to induce vasodilation *in vitro*. Increasing antioxidant activity was positively correlated with total phenolic and catechin content of each fraction. The presence of the main catechins and flavonols were identified in the green tea sample using HPLC. The purine alkaloid

caffeine was identified in fraction 27 and may account for the poor vasodilator effects of this specific fraction.

Black tea fractions were found to be significantly lower in both antioxidant activity and total phenolic and catechin content than the green tea fractions. The presence of the main catechins and flavonols were identified in the black tea sample. Theaflavins and thearubigens were also putatively identified.

This study demonstrates that red wine and tea have antioxidant capabilities and are able to induce vasorelaxation in vascular endothelium, optimising blood flow through the cardiovascular system. These results also indicate that anthocyanins present in red wine account for the vasodilatory activity of grape derived products and catechins are responsible for the potent antioxidant activity conferred by extracts of tea and green tea derivatives.

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McGinn, J. S., and MacLean, M. R. (1999) Grape skin extract induces both endothelium-dependent and endothelium-independent vasorelaxation in rabbit aorta. *British Journal of Pharmacology* **128,** 266P

McGinn, J. S., Crozier, A., and MacLean, M. R. (2000) Comparison of the vasodilator activities of various grape and tea extracts. *British Journal of Pharmacology* **131,** 102P

McGinn, J. S., Crozier, A., and MacLean, M. R. (2001) Mechanisms of vasodilation of green and black tea extracts and epicatechin derivatives. *British Journal of Pharmacology* **133,** 94P

McGinn, J. S., Coleman, R. A., Crozier, A., Duthie, G. G., and MacLean, M. R. (2001) Endothelium-dependent and independent- vasodilation of rabbit aorta by grape and tea extracts. *British Journal of Pharmacology* (submitted for publication)

Mullen, W., McGinn, J. S., Lean, M. E. J., MacLean, M. R., Gardner, P., Duthie, G. G., Yokota, T., and Crozier, A. (2002). Phenolics, ellagitannins and flavonoids and their contribution to the antioxidant and vasorelaxation properties of red raspberries. *Journal of Agricultural and Food Chemistry* **50,** 5191-5196

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Finally, to my mum and dad, for their love, reassurance, support and continual belief in me.

This is for you.

Author's Declaration

The composition of this thesis and the work described within it was carried out entirely by myself unless cited or acknowledged. Its contents have not previously been submitted for any other degrees. The research for this thesis was carried out between October 1998 and September 2001.

Signed. Jennifer M. McGinn.....

Jennifer Sarah McGinn

February, 2002

Definitions

ACh	acetylcholine
cAMP	cyclic Adenosine monophosphate
cGMP	cyclic Guanosine monophoshate
CCRC	cumulative concentration response curve
CHD	Coronary Heart Disease
CG	Catechin gallate
DNA	deoxyribonucleic acid
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ESR	Electron Spin Resonance
Fe (II)	Iron Sulphate ($FeSO_4$)
Fe (III)	Iron chloride ($FeCl_3$)
FRAP	Ferric reducing ability of plasma
GAE	Gallic acid equivalents
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
LDL	Low-density lipoprotein
KCl	Potassium chloride
L-NAME	Nω-nitro-arginine-methyl-ester
NO	Nitric oxide
nm	nanometers
MS	Mass spectroscopy
m/z	mass to charge ratio
PDE	Phosphodiesterase
PE	phenylephrine
ROS	reactive oxygen species
R_t	retention time
SD	Standard deviation
SEM	Standard error of the mean
TPTZ	2,4,6 Tri(2-pyr-idyl)-s-triazine

Aims of Study

Wine and tea have been part of the diet for many centuries. Epidemiological studies have indicated that moderate consumption of alcohol, especially consumption of red wine, and increased consumption of plant derived foods, such as tea, may reduce the risk of cancers and coronary heart disease, the main causes of human mortality. The possible beneficial health effects of wine and tea have mainly focused on their role as antioxidants and their ability to scavenge free radicals, but other health promoting activities have been suggested.

Hypotheses to be investigated:

1. the effects of grape and tea based extracts on vascular tension *in vitro* will be related to their antioxidant activity. The mechanism and pathways of vasorelaxation will also be determined.

2. the effects of green tea catechin and epicatechin derivatives on vascular tension *in vitro* will be related their antioxidant activity. The mechanism of vasorelaxation will also be determined.

3. the antioxidant and vasodilator activity of green and black tea fractions will be identified in samples of tea following large-scale preparative fractionation. The polyphenolics and specifically catechins will be quantified within each sample.

Chapter 1 Introduction

1.1 Introduction to wine

Wine has been an important and integral part of human culture and diet for more than 6,000 years serving dietary and socio-religious functions. Grapevine belongs to the botanical family Vitaceae, which comprises 12 to 14 different genera, possessing about 1,000 species, although this exact number remains under discussion. It is the *Vitis* and its 700 species, especially *Vitis vinifera*, that are of particular interest to the winemaker (Soleas *et al.*, 1997). *Vitis vinifera* originated and was cultivated in an area south of the Black Sea in Transcaucasis. The first recorded mention of vine is found in the Bible which repeatedly goes on to mention vineyards, wine and drunkenness in following passages. Noah after weathering the storms for 40 days and nights left the Ark and set out to "become a husbandman, and he planted a vine: he drank some of the wine, and became drunk" (Genesis IX, verses 6:9 – 10:28) and was subsequently "found by his sons in a drunken stupor". Not bad for a drunkard who was 600 years old when the rains started and lived for another 350 years after the great flood. Likewise, the Marriage Feast at Cana (John 2.1), was the first miracle of the New Testament, when all the wine was finished, Christ miraculously converted water to wine "keeping the best wine till last". This was all that was needed to approve its consumption by Christians throughout the world.

From its first reported origins, the discovery and development of grape growing and winemaking in the southern Caucasus region, travelled south to Palestine, Syria and Egypt. From this base, wine consumption, and its socio-religious connections, distributed winemaking throughout the Mediterranean (Soleas *et al.*, 1997). Although not enjoyed by the general public at this stage, the royal priests made use of its mystical and healing properties. Indeed, Hippocrates, the father of medicine, used wine as an integral part of his remedies.

Due to the extensive travels of the Romans, grape growing and winemaking spread swiftly throughout Europe. By the 18th century, *Vitis vinifera* was being transported to Australia and New Zealand, South America and South Africa. Its introduction to such countries was mainly due to the extensive travels of catholic missionaries who required a supply of wine for their religious ceremonies.

Today the production of wine takes place on every continent, and its chemical composition is profoundly influenced by enological techniques, the grape varieties from which it originates and climatic and geographical factors. In recent years wine has increased in popularity, due in part to the publicity about the beneficial effects on health. The increasing popularity of red wine today, has been attributed to the phenomenon known as the "French Paradox", the large-scale study of 18 western countries, which highlighted the ability of red wine in moderation, to confer protection against coronary heart disease (St Leger *et al.*, 1979)

Wine comes in many forms. It may be white, pink or red and range from light and fruity to dark and robust. Many of the characteristics of individual wines are due to their phenolic content. The protective effects conferred by the consumption of wine, especially that of red wine, has been attributed to these phenolic compounds

1.2 Introduction to phenolics

The majority of fruits and vegetables contain a large number of compounds that are without apparent nutritive value for humans, but which have pronounced biological activity. Flavonoids are a group of low molecular weight C_{15} polyphenolic compounds, of diverse chemical structure found in fruits, vegetables, grains, roots, flowers, tea and wine (Middleton *et al.*, 2000). Plants produce thousands of phenolic and polyphenolic compounds as secondary metabolites. They are a family of structurally related compounds which may come from the fruit (both the seeds and the skin), vine stems,

production by yeast metabolism and extraction from wood co'operage. Phenolic compounds are characterised by having an aromatic ring with more than one hydroxyl group attached. With more than 4000 naturally occurring flavonoids (Harborne, 1993) and more than 8000 phenolic structures reported to date, they are widely distributed throughout the plant kingdom. The nature and distribution of phenolics can vary depending on the plant tissue. The majority of the phenolics are synthesized from carbohydrates via the Shikimate acid pathway (Figure 1 and Table 1) or are derived from this pathway in which *p*-coumaric acid is a key component (Duthie & Crozier, 2002). This is the pathway responsible for the biosynthesis of the aromatic amino acids. These have been termed "essential" amino acids as the Shikimate pathway is limited to plants and micro-organisms and therefore must make up part of the mammalian diet (Burns *et al.,* 2000). Flavonoids are synthesized in a series of discrete steps, each step being catalysed by the appropriate oxidative or reductive enzyme. The first flavonoid intermediate is a chalcone, formed by chalcone synthase (Rice-Evans, 1998). This is immediately isomerised to the corresponding flavanone. Although chalcones and flavanones are biosynthetic intermediates, they can also be accumulated separately as end-products of the pathway (Bravo, 1998; Harborne, 1999).

Natural phenolics can range from simple low molecular weight, single ringed aromatic compounds, such as the phenolic acids, to the large highly polymerised and complex tannins (Bravo, 1998). They can be classified by the number and arrangement of their carbon atoms and the level of oxidation of their central C-ring. They occur primarily in the conjugated form, with one or more sugar residues linked to hydroxyl groups. The associated sugars can be present as monosaccharides, disaccharides, or even oligosaccharides (Bravo, 1998). Glucose is the most common sugar residue. They are commonly found conjugated to other phenolics, carbohydrates, lipids and organic acids.

The phenolics can be classified into two groups, the flavonoids and the non-flavonoids.

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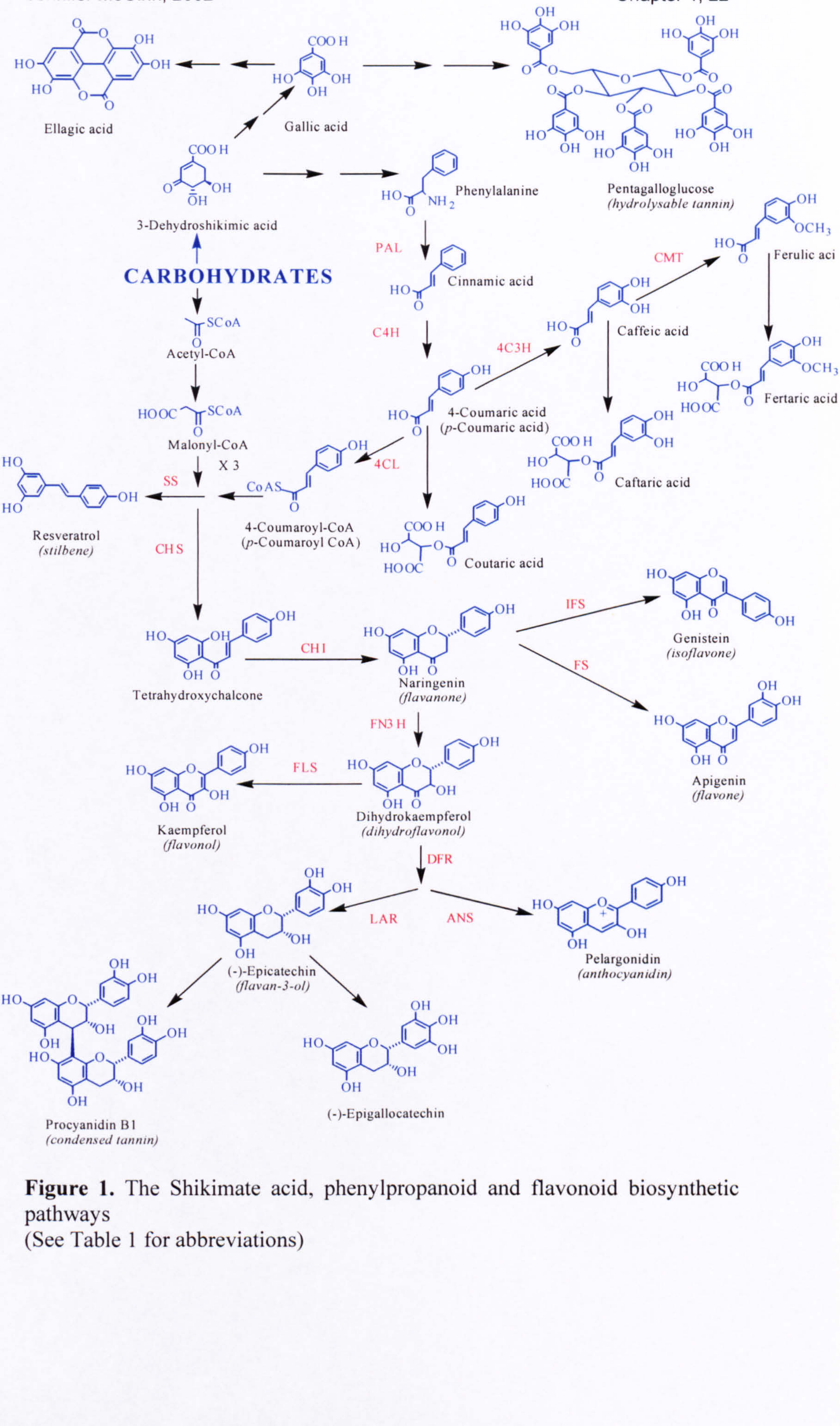


Figure 1. The Shikimate acid, phenylpropanoid and flavonoid biosynthetic pathways
(See Table 1 for abbreviations)

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

Table 1 List of enzyme acronyns for the biosynthetic pathways

1.2.1 Flavonoids

The flavonoids are an important group of substances in the diet which occur naturally in fruit, vegetables, seeds, nuts and flowers (Cook & Samann, 1996), principally as sugar conjugates. The term flavonoid encompasses the flavones, flavonols, flavan-3-ols, flavanones, isoflavones, and anthocyanins (Figure 2).

The structural basis underlying all substances of flavonoid character is the flavane nucleus or 2-phenyl-benzo-γ-pyrane, representing a system of 2 benzene rings (A & B) mutually combined by the mediation of the oxygen containing pyrane C-ring. They have the common skeleton of diphenylpropanes (C_6-C_3-C_6), which occur in a wide range of plants. The basic flavonoid structure is derived from two sources. The B-ring and part of the C-ring are derived from the Shikimate pathway, the biosynthetic route for aromatic amino acids, while the A-ring is formed from the condensation and cyclisation of 3 acetate units from malonyl Co-A (Harborne, 1999) (see Figure 1). The antioxidant activities of flavonoids and their metabolites depend on their chemical structure and orientation of the various substitution patterns in the two benzene rings (Bravo, 1998). Due to this feature allowing extensive substitution within the 3 rings a large number of flavonoids can be found in nature. There are numerous structural variations within the major flavonoid classes which depend on the level of hydrogenation, methylation, hydroxylation and sulphation of the molecules (Adhere & O'Brien, 2002).

1.2.1.1 Flavonols

The flavonols are of particular importance in this review because of their potentially protective role in carcinogenesis and coronary heart disease. Flavonols are commonly found in foods. Vegetables, fruits and beverages, such as tea and red wine (Table 2), are the main dietary sources of flavonols, primarily as glycosides of quercetin, kaempferol and myricetin (Crozier *et al.*, 1997a).

Table 2 Flavonol content of selected beverages

Beverage	Flavonol content*
Loose green tea	
Japanese	32.8 ± 0.4
Loose black tea	
Safeway	21.0 ± 0.3
Typhoo	39.1 ± 0.9
Red wines	
Cabernet Sauvignon, Chile	58.4 ± 4.0
Pinot Noir, California	30.2 ± 0.6
Merlot, Chile	25.2 ± 1.2
Beaujolais, France	9.9 ± 0.9
White wines	
Reisling, Australia	1.7 ± 0.2
Bordeau Blanc, France	n.d.
Dry White, South Africa	n.d.

All data expressed as mg L^{-1} total flavonols ± s.e.m, n=3, n.d., not Detected. Adapted from Crozier *et al.,* 2000

The variability within the flavonols is extensive, with over 380 flavonol glycosides and more than 200 different quercetin and kaempferol glycosides being described to date (Bravo, 1998). Conjugation occurs most commonly at the 3 position of the C-ring although substitutions at the 7, 4′, 3′ and 5′ also occur (Harborne, 1993). Flavonols occur in an extensive array of derivatives with extra hydroxylation and/or methylation. Their glycosides have been found in practically all types of plants but are the major components in fruits (Herrmann, 1988).

The formation of flavonol glycosides normally depends on light. In general, the highest concentrations of these compounds occur in the leaves, while only trace amount are found in the parts of the plant below the soil surface (Herrmann, 1976, 1988; Hertog, 1994). The common onion is the well-known exception to the rule. It appears that in general, UV light is the predominant factor in determining the flavonol content of the plant. However, growing plants in glasshouses reduces the flavonol content, particularly in the outer leaves, as has been shown for kale, brussel sprouts and lettuces as they are out of direct contact with UV light (Hertog, 1994). The concentrations of flavonols and flavones, like those of all secondary plant metabolites, vary within certain limits and are dependant upon a number of factors, such as the growing conditions, degree of ripeness and the size and variety of the fruit (Herrmann, 1976).

1.2.1.2 Flavones

Flavones differ from flavonols only in the absence of hydroxylation at the 3-position in the C-ring (Rice-Evans, 1998) (Figure 2). The most common flavones are apigenin and luteolin which are mainly found in vegetables, such as celery, sweet red pepper, citrus fruits and herbs. The flavone, luteolin, corresponds in the hydroxylation pattern to the flavonol quercetin. Chemically flavones are also classified as 3-hydroxyflavones (Herrmann, 1976).

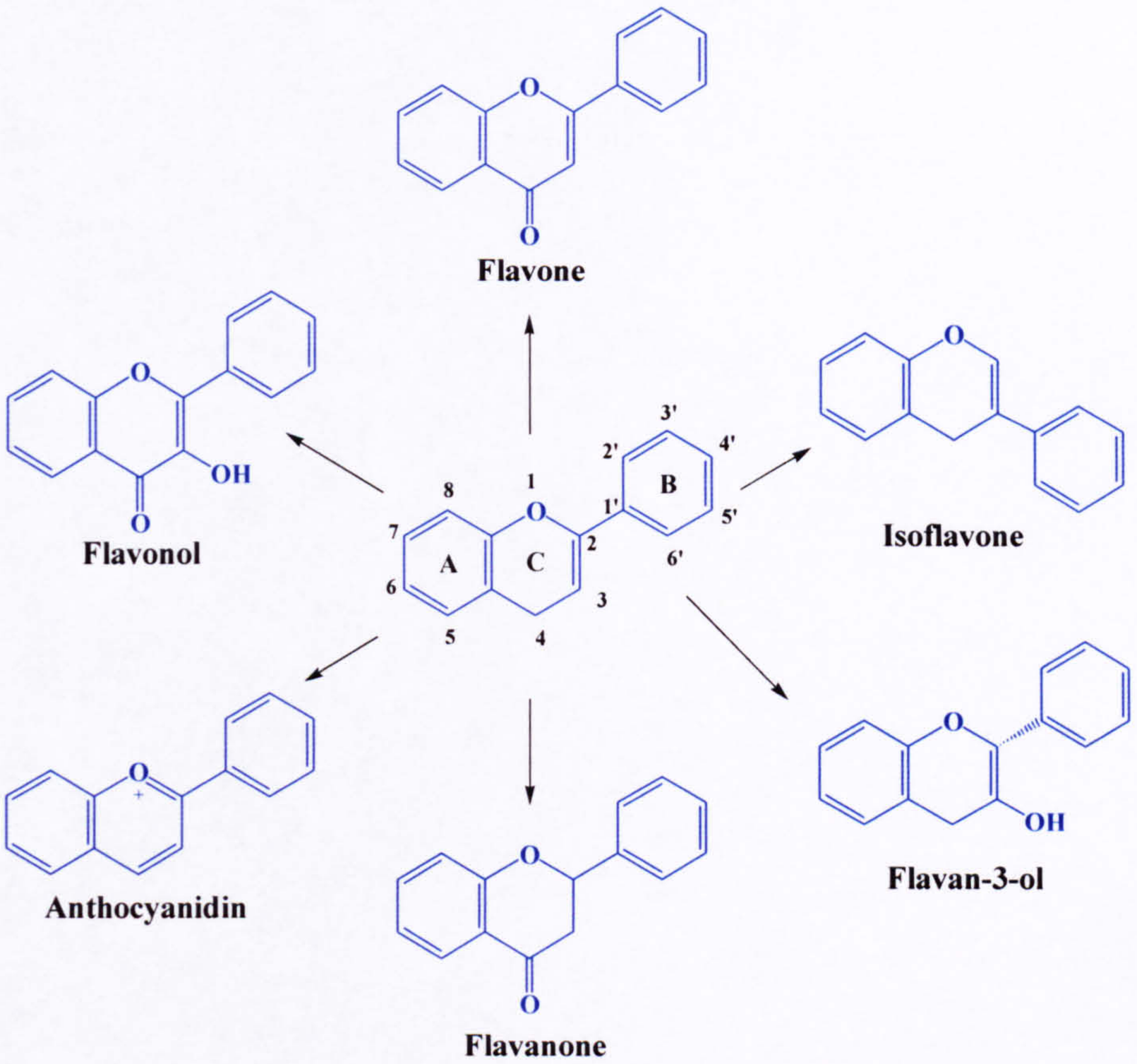


Figure 2 The generic structure of flavonoids

1.2.1.3 Flavan-3-ols

This is the most complex subclass of flavonoids ranging from the simple (+)-catechin and its isomer (-)-epicatechin, to the oligomeric and polymeric proanthocyanins, also known as condensed tannins (Balentine *et al.,* 1997). In addition to forming complexes with other flavan-3-ols, they also undergo esterification with gallic acid to form catechin gallates, and hydroxylation reactions to form gallocatechins (Robb & Brown, 2001). Proanthocyanins are a subclass of flavan-3-ols which are universally present in woody plants and are normally present in the non-glycosidic form. Flavan-3-ols are involved in the taste and texture of food and confer a bitter and astringent taste to red wine and tea (Balentine *et al.,* 1997). Catechins and epicatechins are discussed in more detail in section 1.12.1.

1.2.1.4 Flavanones

The flavanones are the first flavonoid products of the biosynthetic pathway. Two main structural features characterise the flavanones, the absence of the C2-C3 double bond, and the presence of a chiral centre at C2. In naturally occurring flavanones, the C2 phenyl group is orientated downwards, in the α-configuration (Bohm, 1998). The flavanones group is highly reactive and they have been reported to undergo hydroxylation, glycosylation and *O*-methylation. The most common flavanones, naringenin and hesperidin, and their glycosides, are abundant in citrus fruits and juices, for example in grapefruits, oranges (125 – 150 mg/L of juice) (Scalbert & Williamson, 2000) and lemons, where they contribute to the bitter flavour, and in prunes, liquorice and peppermint. In citrus fruits, flavanones are usually found conjugated to rutinosides, that have no taste (Scalbert & Williamson, 2000).

1.2.1.5 Anthocyanins

Anthocyanins are widely distributed throughout the plant kingdom and are responsible for most floral, fruit and leaf colours in nature, apart from the green chlorophyll and yellow carotenoids (Rice-Evans, 1999). In nature they are responsible for blue, pink, red and purple colours. They play an important role in attracting insects to flowers for pollination but are also involved in protecting the plant against UV light. Anthocyanins consist of an anthocyanidin bound to one or more sugar moieties, with a sugar always present at C3 and frequently on carbons 5, 7, 3′ and 5′ (Rice-Evans, 1998). The term anthocyanin refers to the glycosides of anthocyanidin, for example pelargonidin, malvidin and cyanidin (Bravo, 1998). In addition to glycosylation, common linkages with aromatic acids and methyl ester derivatives also occur. Anthocyanins also readily form loose, hydrogen-bonded complexes with flavones and metal ions, acting as co-pigments which help to stabilise pigments in the hostile acidic environment of the plant cell sap (Rice-Evans, 1998). Anthocyanin colour is pH dependent with anthocyanins usually red at acid pH's becoming colourless then shifting to blue as the pH increases. Fruit anthocyanin content usually increases as the fruit matures (Peterson & Dwyer, 1998). Anthocyanins are present in cherries, strawberries, grapes, red currents, raspberries and plums.

1.2.1.6 Isoflavonoids

Isoflavonoids are a distinct class of flavonoids best known for their estrongenic activity and their suggested role in the prevention of breast cancer and osteoporosis (Scalbert & Williamson, 2000). Structurally they differ from the common flavonoid in the orientation of the B ring. They are characterised by having the B ring attached at the C3 of the phenylchromane structure. Isoflavonoids are derived from the biosynthetic pathway and can be converted into a wide variety of different isoflavonoids, the isoflavanones, isoflavones and isoflavonols (Harborne, 1993).

The best known isoflavonoids are daidzein and genistein. They are found predominately in legumes. Soybeans are the major source of daidzein (~1 mg/g dry bean), while genisten is found in black beans, green split peas and sprouts (Peterson & Dwyer, 1998). As with the rest of the flavonoid family they also undergo hydroxylation and methylation reactions.

1.2.2 Non-flavonoid phenolics

The main non-flavonoid phenolics are derivatives of benzoic and cinnamic acids and their respective aldehydes (Soleas *et al.*, 1997). Along with the other phenylpropanes they are synthesized by the phenylpropanoid pathway (Figure 1). These non-flavonoid phenolics are structurally simpler and are stored within the cell vacuoles of the grape. They are easily extracted on crushing and commonly occur esterified to sugars, organic acids, or various alcohols. They are susceptible to substitution of the aromatic ring and also to oxidative modifications, thereby giving rise to a large number of related compounds (Singleton, 1995).

1.2.3 Function & distribution in plants

Phenolics and polyphenolics are essential to the plant's physiology. Several functions of compounds in plants have been proposed or demonstrated. Plants have evolved to produce flavonoids to protect against damage by UV-B light, fungal parasites, herbivores, pathogens and oxidative cell injury. They are also plant hormone secretion controllers and enzyme action inhibitors (Markham, 1989; Harborne, 1993). Flavonoids also produce stimuli to assist in pollination and are involved in the regulation of pollen tube growth in the stigma, and are able to produce stimuli to help guide insects to their food source (Cook & Samman, 1996). In foods, phenolics may play important roles as natural colorants and in their flavouring of compounds. They have been reported to exhibit a wide range of clinically relevant biological effects, which will be discussed in more detail in this chapter.

1.2.4 Dietary intake of flavonoids

The structural diversity of polyphenols makes the estimation of their content in food difficult due to the wide variation in phenolic content of certain common foods in the diet. Until recently our knowledge of flavonoid intakes was limited to the data of Kühnau, 1976, who estimated the total flavonoid intake in the USA to be approximately 1000 mg/day (expressed as glycosides) or 650 mg/day (expressed as aglycones) consisting of primarily of flavonols, flavanones and flavones. These values have been widely quoted in the literature, however they are based on food analysis techniques now considered inappropriate and inaccurate leaving the estimated dietary intake of phenolics open to interpretation. A range of methods have been used to quantify the phenolics present in the diet but few studies have examined more than one food group. In a more recent study of Dutch people, using data from the Dutch National Food Consumption Survey, the average dietary flavonoid intake was estimated to be approximately 23 mg/day, as expressed as aglycones (Hertog *et al.,* 1992), with tea and onions being the major sources at 48% and 29% of total intake respectively. In fact, the Dutch investigators measured not flavonoids but selected flavonols and flavones. These intakes of flavonoid intake are, therefore, almost certainly a severe underestimate. Red wine and apples are the other main dietary sources of phenolics in the diet. Calculated flavonol/flavone intakes from other studies have ranged from 3 mg/day in Finland to 65 mg/day in Japan (Duthie *et al.,* 2000) (Table 3). In general, dietary intakes of flavonols and flavones are quantitatively similar to those of many well recognised micro-nutrients, such as vitamins E and C, and the overall flavonoid intake may be well in excess of the traditional vitamins (Duthie & Crozier, 2002). It should be recognised however that there is substantial potential for error and bias in the methods employed to estimated dietary intake. These are based on subjects' memories, competence and honesty. Under-reporting is common and the methods do not provide details on the varieties of plant foods which may have widely varying nutrient contents.

Table 3 Estimated flavonol and flavone intake of different countries

Country	flavonol & flavone intake (mg/day mean value)
The Netherlands	3 - 33
Finland	3 - 6
USA	13
Greece	16
Italy	27
Japan	16 - 65
Scotland	17
Wales	26

Adapted from Duthie & Duthie, 2000

1.3 Bioavailability of polyphenols

An understanding of the absorption and bioavailability of phenolic compounds is critical before biological activity and nutritional potential value can be estimated. In general, the absorption, distribution, metabolism and excretion of dietary phenolics in humans is poorly understood.

The rate and extent of intestinal absorption and metabolism of food phenolics are determined by their chemical structure, which depends on several factors including the degree of glycosylation and polymerisation, conjugation with other phenolics, solubility and their basic structure, whether benzene or flavone derivative (Bravo, 1998). Glycosylation influences the chemical, physical and biological properties of the polyphenol, all factors which influence its bioavailability *in vivo*.

Indirect evidence of their absorption through the gut barrier has focused on the increase in the antioxidant capacity of the plasma after the consumption of polyphenol rich food. This increase in the antioxidant capacity of plasma has been shown following repeated consumption of tea (Serafini *et al.*, 1996; van het Hof *et al.*, 1999) and whiskey (Duthie *et al.*, 1998) and red wine (Fuhrman *et al.*, 1995; Serafini *et al.*, 1998; Duthie *et al.*, 1998). On the other

hand, direct evidence on the bioavailability of a small number of polyphenols has been obtained by measuring their concentrations in plasma and urine of human volunteers following consumption of pure compounds and polyphenol rich extracts and beverages, such as onions (Hollman *et al.,* 1995, 1996; Aziz *et al.,* 1998), red wine (Donovan *et al.,* 1999; Caccetta *et al.,* 2000), and green and black tea (He & Kies, 1994; Kivitis *et al.,* 1997) and flavonol rich diets (Noroozi *et al.,* 2000), with evidence suggesting that flavonol conjugates are absorbed to a greater extent than the parent aglycone. However, increases in the concentrations of polyphenols and associated metabolites in plasma and urine do not inevitably mean that they have significant effects *in vivo* (Duthie & Crozier, 2002). While many aspects of phenolic and flavonoid absorption and metabolism remain unknown at present, there is adequate evidence to suggest that a number of these compounds will be absorbed in sufficiently high concentrations to have physiological effects.

1.4 From grape to glass

A number of factors have been shown to affect the level of phenolics in grapes and their corresponding wines (McDonald *et al.,* 1998). Processing and vinification are the series of unit operations leading from the initial crushing of the grape to the bottling of the finished wine.

1.4.1 Viticulture

Grape variety is the first important stage in the production of wine. Phenolics and polyphenols are derived from the skin of the grape where they function as UV protectors. Thick skinned grape have the potential to release more polyphenols than thin skinned grapes. Today, wines are produced from numerous grape varieties of grapes, including Cabernet Sauvignon, Merlot, Pinot Noir, Rondinella, Sangiovese, Grenache, Tempranillo and Carignan (Soleas *et al.,* 1997). The type of grape used is crucial to the characteristics and taste of the finished wine.

Sunlight is the most important climatic factor affecting grape development (Soleas *et al.,* 1997). High sunlight encourages the synthesis and accumulation of polyphenols. Grapes grown in the shade been shown to have a 7-fold lower flavonol content than those grown in direct sunlight (Price *et al.,* 1995). Changes also occur in anthocyanin composition during ripening which are significant to the overall wine colour stability.

Temperature has a pronounced effect on anthocyanin synthesis and stability in grapes, and influences enzyme function and cell membrane permeability (Soleas *et al.,* 1997). Warm conditions also increase sugar accumulation and amino acid content of the maturating grape. High nitrogen levels have been shown to suppress anthocyanin production levels with high potassium levels affecting grape pH thereby altering the stability of colour. Over irrigation can double fruit yield and increase fruit size, however it can also reduce sugar content and increase acidity (Soleas *et al.,* 1997).

1.4.2 Vinification

Red wine is today produced on every continent. Its chemical composition is profoundly influenced by enological techniques employed by the winery, the country in which it originates and climatic and geographical factors influencing its development (McDonald *et al.,* 1998).

Immediately after the harvest of the grapes from the vine, the stems, leaves and grape stalks are removed. The grapes are then crushed to avoid microbial contamination and better control of enzymatic oxidation. The fruit is then passed through a set of rollers, the juice (must), pulp, seeds and skins are collected in vats ready for maceration and alcoholic fermentation which allows the rupture and release of enzymes from grape cells bound in the skin, flesh and seeds (Soleas *et al.,* 1997). In red wine production, the style of the wine can be altered dramatically by the duration and conditions of maceration and fermentation. It can be quick, lasting only 24 hours to produce a light rose wine, or up to several days to produce a wine for early consumption with good

colouration and low tannin levels. Wines for long aging are usually fermented for up to 21 days, which may decrease free anthocyanin content but enhance colour stability and aging potential (Soleas *et al.*, 1997).

Following maceration and fermentation, wines are pressed and heated to improve the colour of the wine. The temperature used depends on the variety of the grape and strength of the wine. The wine is then fined prior to blending. Fining agents (fine clays, silica gels, albumin) are then added to the wine to remove or reduce any large particles leaving the wine clear (Soleas *et al.*, 1997). This process helps remove tannins and proteins and help prevent the formation of sediment in the bottle. Formation of sediment is usually the sign of a high tannin content and a high quality wine. The wines are then matured in stainless steel vats, or in the case of higher quality vintages in oak barrels, for varying periods before being filtered and bottled.

1.5 Grape & wine phenolics

Red wine is a complex fluid containing grape, yeast and oak derived products, which may influence the aroma and texture of the wine. The major contributors to taste, aroma and texture are those compounds found in higher quantities, such as water, ethanol, sugars, organic acids, glycerol and tannins (Soleas *et al.*, 1997). Wine contains 8 – 15% ethanol (v/v) (German, 2000). Although red wine contains wood and yeast derived phenolics, the greater amount of phenolics are derived from the grape. Red wines are produced from the seeds, flesh and skins of black grapes and are therefore an extremely rich source of phenolics (Singleton, 1982). Red wines, in contrast to white wines which contain few components derived from the skin and seeds of grapes, contain significant amounts of free and conjugated grape-skin derived phenols, principally quercetin and myricetin (Table 4), as a consequence of the different variety of grapes used, climatic and cultural conditions and vinification technique employed (Burns *et al.*, 2000).

Table 4 Typical composition of wines (% weight)

Component	Red wine	White wine
Water	87	87
Ethanol	10	10
Other volatiles	0.04	0.04
Sugars	0.05	0.05
Pectins	0.3	0.3
Glycerol	1.1	1.1
Acids	0.6	0.7
Phenols	0.2	0.01
Amino acids	0.25	0.25
Lipids	0.02	0.01

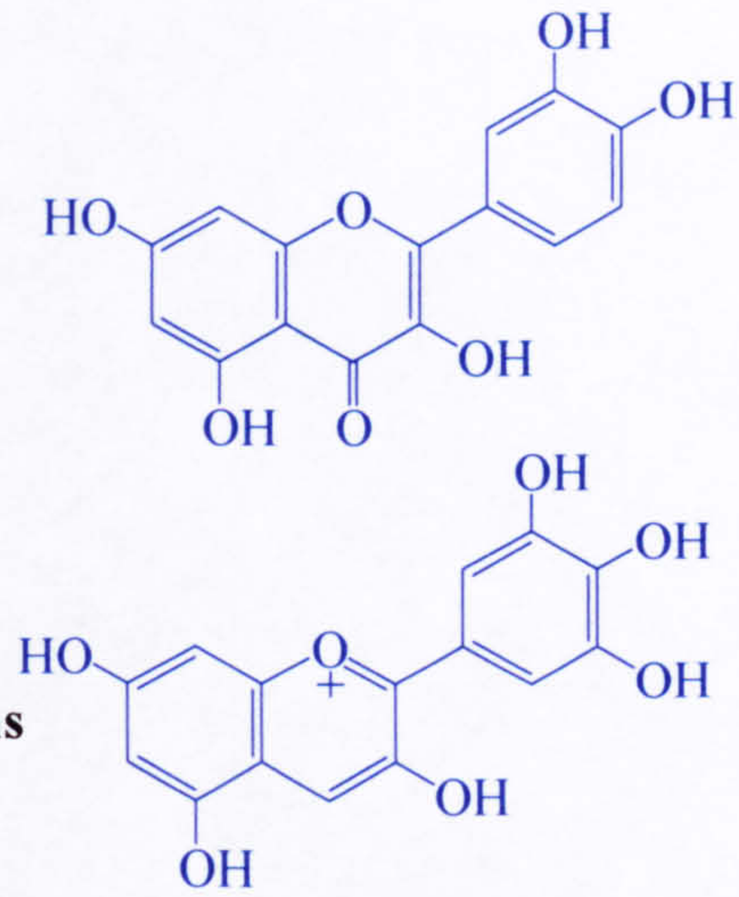
(Adapted from Singleton, 1982)

The most common flavonoids in red wine are the flavonols, flavan-3-ols and the anthocyanins, (Figure 3) with smaller amounts of free leuco-anthocyanins (flavan-3,4-diols) also occurring (Kinsella *et al.*, 1993). The flavonols and anthocyanins are derived from the grape skin and the flavan-3-ols, primarily from the seed and stems. Flavonoids may occur in the free form or polymerised to other flavonoids, sugars, non-flavonoids or to a combination of these compounds (Bravo, 1998). In red wine, flavonoids commonly constitute more than 85% of the total phenol content (Soleas *et al.*, 1997), compared to less than 20% in white wines.

Wines contain grape-skin derived flavonols, quercetin, myricetin, kaempferol and isorhamnetin (Burns *et al.*, 2000). Flavonols are found mainly as sugar conjugates which aids their solubility and subsequent transport and storage within a tissue, but up to 50% of the flavonols present in red wines can be in the free form, due to the enzymatic hydrolysis of the conjugates during fermentation releasing the glycone. In grapes and wine they occur as *O*-glucosides and glucuronides, typically bound to glucose or rhamnose. The total overall flavonol content of red wines can range from 4.5 to 42 mg/L (McDonald *et al.*, 1998). Flavan-3-ols or catechins are found in high concentrations in the grape seeds and grape stem tissue. However, the grape skins are the primary source of catechins due to the ease of transfer of

Flavonoids

Flavonols

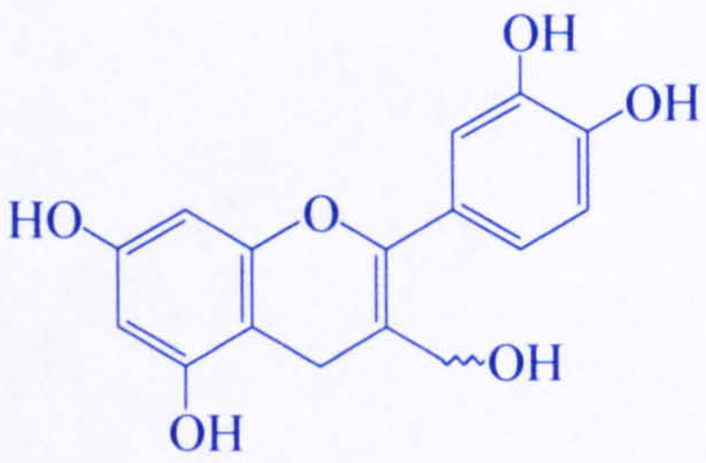


quercetin
myricetin
kaempferol
isorhamnetin

Anthocyanidins

cyanidin
delphinidin
petunidin
peonidin
malvidin

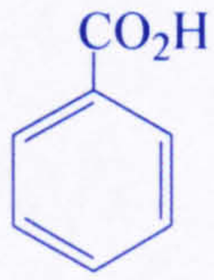
Flavan-3-ols



(+)-catechin
(-)-epicatechin
epigallocatechin
procyanidins (condensed tannins)

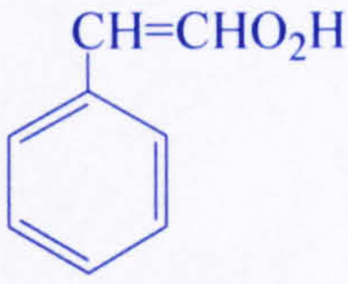
Non-Flavonoids

Hydroxybenzoates



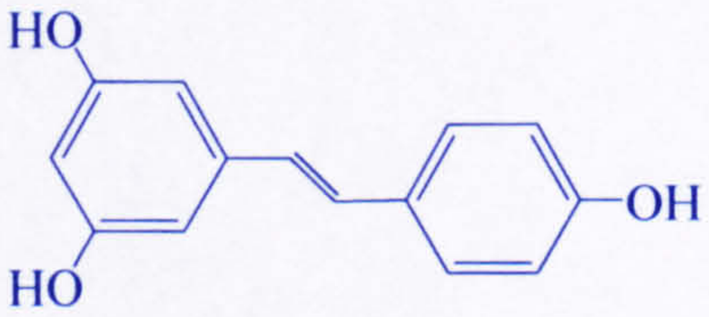
gallic acid
ellagic acid
hydrolysable tannins

Hydroxycinnamates



p-coumaric acid
caffeic acid
ferulic acid
sinapic acid

Stilbenes



trans-resveratrol
cis-resveratrol
trans-resveratrol glucoside

Figure 3 Structure of the major phenolics present in red wine

phenolics from skins into wines during manufacturing. Catechins are also found as gallate esters or as oligomers and polymers also known as condensed tannins. Additional hydroxylation of the catechin structure leads to the formation of gallocatechins. Both the gallate esters and gallocatechins are found in very low levels in grapes, which decrease further during ripening and wine maturation (Singleton, 1982).

Anthocyanins are responsible for the colouring of black grapes and red wine. They are absent in white wine due to a synthetic blockage in the Shikimate pathway (Soleas *et al.,* 1997) and due to the process of removing the skin of the grapes during the manufacturing of white wine. The amount and concentration of anthocyanins in red grapes depends on the variety of grape, climate, grape maturity and geographical factors (McDonald *et al.,* 1998). The anthocyanins reported in red grapes are primarily malvidin and the 3-*O*-glucosides of cyanidin, peonidin, delphinidin and petunidin (Burns *et al.,* 2000). During the aging of red wine the levels of anthocyanins decline as they undergo condensation reactions being converted to more stable, darker oligomeric and polymeric pigments.

The major non-flavonoid phenolics found in red wine are the hydroxybenzoic acid derivates and phenolic acids (Figure 3), especially gallic acid and its ellagic acid dimer, hydroxycinnamates and stilbenes, all which have similar biosynthetic origins (Soleas *et al.,* 1997). The hydroxycinnamates, caffeic acid and ferulic acid, are stored in the cell vacuoles of the grape flesh and are easily extracted during the manufacture of both white and red wine. They are usually found in wines esterified to sugars, organic acids and various alcohols. Gallic acid is present in the flesh of both white and black grapes and is therefore a constituent of both white and red wines, although it is present in a 5- to 10-fold higher concentrations in red wine (Frankel *et al.,* 1995). The stilbene, *trans*-resveratrol (3,5,4′-trihydroxystilbene) is also present in red wine. Over 30 stilbene and stilbene glycosides are known to occur in a restricted distribution in the plant kingdom (Soleas *et al,* 1997b), playing an important role in disease resistance,

functioning as a phytoalexin in grapes where it accumulates in the skin in response to response to fungal infection (Kopp, 1998). Within the cells of grape skins resveratrol is principally found conjugated to glucose and is known as *trans*-resveratrol-3-*O*-β-glucoside (Soleas *et al.,* 1997b). The presence of the *cis* isomer of both free and conjugated resveratrol has also been reported in red wine. Although present in red wine, *trans*-resveratrol is found in lower levels than many other phenolics (Soleas *et al.,* 1997b).

The concentration of phenolics in wine increases during skin fermentation and consequently begins to fall as the phenols bond and precipitate with proteins and yeast cell remnants, not removed during filtration. In the making of red wine, with prolonged extraction, the fermented grape juice can contain up to 40% of the phenolics originally present in the grapes (Duthie & Crozier, 2002). During fining and maturation of the wine, the phenol content continues to decrease, with the ageing of the wine having a further dramatic effect on the final phenolic content (Soleas *et al.,* 1997). Both these enological techniques and climatic and geographical factors therefore not only influence the taste, fragrance and texture of the wine but also the phenolic content which may in the long-term lead to benefits in health.

1.6 Alcohol & health

Alcohol has been consumed through the ages because of its perceived benefits as a social lubricant and for relaxation, mood alteration and sensory pleasure (Friedman & Klatsky, 1993), but long-term consumption of large amounts of alcohol is harmful, and may lead to addiction, aggressive behaviour and suicide. Today there is much controversy surrounding the use of alcohol in society. The public health issue of limiting alcohol use is not questioned.

The ever-increasing human and economic cost of coronary heart disease (CHD) has promoted extensive investigation into the associated risk factors such as diet, exercise, lifestyle and alcohol consumption. Epidemiological

studies have consistently shown an apparent protective association between light to moderate alcohol consumption, and the incidence of atherosclerosis CHD, but the interpretation of these findings remain a subject of much debate.

In the last decade, a number of large scale epidemiological studies have indicated that moderate alcohol consumption is associated with a reduced risk of coronary heart disease (CHD) and overall mortality (Jackson *et al.,* 1991; Rimm *et al.,* 1991; Cullen *et al.,* 1993; Woodward & Tunstall-Pedoe, 1995; McElduff & Dobson, 1997; Thun *et al.,* 1997), while a very small majority others have shown little or no association (Kaufman *et al.,* 1985). Evidence has been provided by socio-economic studies as well as retrospective or prospective observation studies. It is widely accepted that it is difficult to collect accurate data on alcohol consumption. The methods of assessing alcohol intake vary widely in different studies (Marmot & Brunner, 1991). Methods for analysing alcohol intake and CHD and mortality include standard qualitative questionnaires of the frequency, quantity and type of alcohol consumed, smoking, medical history and lifestyle, 7-day alcohol and food recall questionnaires and 24 hour recall studies. These studies are therefore open to potential substantial bias and error in the methods employed to estimate alcohol consumption. Under-reporting of alcohol intake is common and is based on subject's honesty, competence and memories. The evidence suggests that ingestion of two alcoholic drinks per day (15-20 g/day) is associated with reduced mortality and maybe protective against coronary heart disease. The consumption of more than four drinks per day (>34 g/day) may be associated with an increase in overall mortality risk (Marmot *et al.,* 1981). Analysis of the relationship between reported alcohol consumption and subsequent mortality persists after the removal of the confounding factors, age, cigarette smoking, blood pressure, plasma cholesterol and grade of employment (Marmot *et al.,* 1981) and social class (Shaper *et al.,* 1988).

The well-known U-shaped curve of mortality indicates that moderate alcohol intake may confer protection against cardiovascular disease in general and particularly against heart attacks (Sharper *et al.,* 1988). This curve

describes the relationship between alcohol consumption and risk of death from all cause mortality. Heavy drinkers are seen to have an increased risk of death compared with moderate drinkers, but moderate drinkers to have a lower mortality than that of abstainers (non-drinkers) (Sharper *et al.,* 1988). However the classification of abstainers and non-drinkers includes those who have stopped drinking due to ill health and therefore have an increased rate of disease, those who may die soon and those who are at higher risk of mortality due to other reasons (Klatsky, 2001). Either of these groups may account for the higher risk among non-drinkers and therefore give the CHD and alcohol mortality curve its distinctive shape. The higher risk of CHD in non-drinkers could also be due to inaccurate alcohol histories if drinkers at high risk declare themselves to be non-drinkers or life long abstainers (Marmot & Brunner, 1991). In the relationship between alcohol consumption and total mortality, a number of potential confounders could influence the shape of the curve. These include the characteristics and potential misclassification of the study population, misclassification of alcohol intake and the level of alcohol consumption associated with the lowest risk of mortality for the study population (Marmot & Brunner, 1991). Alcohol is believed to confer protection against CHD through a number of mechanisms working in synergy. These cardio-protective mechanisms include the ability to inhibit lipid peroxidation and increase the levels of "protective" high density lipoprotein cholesterol (HDL), to reduce platelet aggregation, to decrease the permeability and increase the stability of capillaries and to interact with the pathway that generates nitric oxide (NO) from vascular endothelium.

A reduction in risk of coronary heart disease has been attributed separately to beer (Yano *et al.,* 1977), to spirits (Hennekens *et al.,* 1979), and to most notably wine (Rosenberg *et al.,* 1981; Rimm *et al.,* 1996; Gronbaek, 1997; Criqui, 1998). Protective effects have also been ascribed to ethanol itself rather than other substances found in each type of drink. However, a weak inverse relationships between red wine and CHD mortality may indicate that with its abundant content of polyphenols and phenolic acids red wine confers additional health benefits, over and above ethanol and other alcoholic drinks.

1.7 Physiological activity of grape polyphenols

1.7.1 Antioxidants: mechanism of action

Oxygen is essential to maintain life, but it can also have adverse effects if the number of highly reactive oxygen containing radicals exceeds the needs of the cell or are not trapped adequately (Halliwell, 1997). Metabolic activities produce free radicals, unstable molecules able to react with electron donors to equilibrate its charge (Rice-Evans, 2001). This reaction is essential for the synthesis of nucleic acids, hormones and proteins. Free radicals are also produced to intercept cell invaders like microbes and viruses.

The oxidation/antioxidation balance is highly regulated. Oxidation is the transfer of electrons from one atom to another and represents an essential part of aerobic life and metabolism. Oxidative stress induced by an overproduction of reactive oxygen species (ROS) and leads to the disruption of cellular functions (Halliwell, 1997). Reactive oxygen species or free radicals include the hydroxyl radical (OH), superoxide radical ($O_2^{\cdot-}$), peroxyl radical (ROO·) and nitric oxide (NO) (Pietta, 2001). These ROS are produced from endogenous sources, such as electron transport chains, peroxisomes and the cytochrome P-450 system (Wiseman *et al.,* 1997). ROS can reactive together with other molecules to form more or less reactive molecules. Free radicals can also be generated by the external action of toxic substances, ozone, UV radiation, cigarette smoke or intensive exercise (Dufresne & Farnworth, 2001).

ROS have different functions *in vivo* (Pietta, 2000). They are identified to be involved in energy production, phagocytosis, regulation of cell growth and the synthesis of biologically important compounds (Halliwell, 1997). However, ROS may be damaging, altering lipids in cell membranes, decreasing their capacity to protect cell contents, proteins in tissues and enzymes, carbohydrates and DNA, inducing oxidations which cause membrane damage, protein and enzyme modifications and DNA damage (Pietta, 2000). This oxidative damage is considered to play a causative role in

aging and degenerative diseases (Halliwell & Gutterridge, 1998). The oxidation of nucleic acids bases in DNA induces mutations and heightens the risk of cancer occurrence. Although cells possess enzymatic and cellular protection against ROS, antioxidants from nutrients obtained from the diet, both vitamin and mineral, contribute highly to the overall protection of cell integrity and immune function (Pietta, 2000).

Humans have evolved antioxidant systems to protect against free radicals. There are a number of antioxidant defence mechanisms that can decrease the adverse effects of ROS. These mechanisms produced in the body and obtained from the diet are able to either suppress free radical formation and chain initiation or to scavenge free radical and chain propagation (Pietta, 2000). These include the binding of metal ions needed for catalysis of ROS generation, the scavenging of ROS and their precursors, the up-regulation of endogenous antioxidant enzymes and the repair of oxidative damage to biological molecules (Morton *et al*, 2000). Some antioxidants react readily with free radicals, quenching them before they can react with other cell components (free radical scavenging). Antioxidant defence mechanisms also include the enzymes, superoxide dismutase, which removes superoxide radicals, gluthathione peroxidase, which converts hydrogen peroxide to water, and catalase, which breaks down hydrogen peroxide (Morton *et al*., 2000). These defence mechanisms are complementary to each other since they act against different species at different cellular components (Pietta, 2000).

1.7.2 Red wine as an antioxidant

Polyphenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants, singlet oxygen quenchers (Rice-Evans *et al.*, 1996) and chelators of metal ions, preventing metal-catalysed formation of initiating radical species. The efficiency of polyphenols as antioxidants depends greatly on their chemical structure. For a polyphenol to be classed as an antioxidant, two basic conditions must be met. Firstly, when present in low concentrations relative to the substrate which is to be oxidised, they must

delay, retard or prevent the autoxidation or free radical-mediated oxidation of the substrate (Salah *et al.*, 1995). Secondly, the free radical formed after scavenging must be stable, either through intramolecular hydrogen bonding or by further oxidation (Shahidi & Wanasundara, 1992). The position and number of free hydroxyl groups (OH) in flavonoids are responsible for their antioxidant activity. In general, optimal antioxidant activity is associated with multiple phenol groups, especially at 3' and 4' positions, a carbonyl group at C4, and a free C2 and C3 double bond. If the 3'-OH group is bound glycosidically or is absent, the antioxidant activity is weakened (Herrmann, 1976). The antioxidant effect increases with the number of hydroxyl groups in rings A and B (Kühnau, 1976).

The focus on phenolics is largely due to the finding that the antioxidant properties of these non-alcoholic compounds may delay the onset of atherogenesis and CHD, by reducing chemically and enzymatically mediated peroxidative reactions and being able to down-regulate thrombotic tendencies. Although there is conflicting evidence, the majority of studies have shown that the consumption of red wine inhibits low-density lipoprotein (LDL) oxidation *in vitro* (Frankel *et al.*, 1993; Kanner *et al.*, 1994; Kerry & Abbey, 1997) and *in vivo* (Furhman *et al.*, 1995; Day *et al.*, 1997; Nigdikar *et al.*, 1998). It also promotes an overall increase in plasma levels of apoliprotein A-I and high-density lipoprotein cholesterol (HDL) which is an important anti-atherogenic factor, which inhibits the uptake of LDL and is involved in the normal clearance of cholesterol from the tissues. The antioxidant activity of red wines has been attributed to its phenolic components, as research has shown that wines devoid of phenolics do not inhibit LDL oxidation *in vitro* (Maxwell *et al.*, 1994).

It has also been shown that red wine ingestion increases human serum antioxidant capacity and activity (Maxwell *et al.*, 1994; Day *et al.*, 1997). Whitehead *et al.*, (1995), reported that after the ingestion of 300mL of red wine, the mean serum antioxidant capacity is increased by 18% after 1 hour and by 11% after 2 hours. The same amount of white wine only produces 4%

and 7% increases respectively. Alcohol-free red wine has also been shown to display a stronger *in vitro* antioxidant capacity than alcohol-free white wine, reaching peak levels between 30 and 90 minutes and remaining above basal levels even after 2 – 4 hours. The only consistent difference between red and white wines being the phenolic content, which was 20 times higher in red wine (Serafini *et al.,* 1998). It is therefore evident that the changes in plasma antioxidant capacity may be entirely attributable to the phenolic fraction of the wine. Based on the calculations of Panganga *et al.,* (1999), the antioxidant activity in a glass of red wine (150 mL) is equivalent to the antioxidant activity of 12 glasses of white wine, 2 cups of tea, 500 mL of beer and 7 glasses of orange juice.

A recent comparison of the effects of consumption of 100 mL of an aged malt whisky with that of the 100 mL of red wine by 9 fasted volunteers, indicated that both beverages increased plasma antioxidant capacity (Duthie *et al.,* 1998). Blood and urine samples were obtained up to 4 hours following consumption. The increase was greater with red wine consumption, but despite the 7-fold greater concentration of phenolics in the wine, both beverages produced similar increases in the total plasma phenolic content, suggesting that a proportionally greater amount of phenolics were absorbed from the whisky than from the red wine. This effect could be ascribed to the greater alcohol content of the whisky aiding phenolic absorption, suggesting a separate and specific role for the alcoholic fraction of wine (Duthie *et al.,* 1998).

1.7.3 Red wine & platelet function

Other potential mechanisms contributing to the cardio-protective effects of moderate alcohol consumption, and more specifically red wine consumption, include alterations in blood platelet function, coagulation and fibrinolysis. Alcohol appears to affect several factors involved in maintaining the balance between clot formation to protect against bleeding and clot dissolution to prevent blood clots forming in arteries, which have been

implicated as a risk factor in the development of CHD (Rotondo & de Gaetano, 2000).

Alcohol consumption has been associated with increased levels of tissue plasminogen activator (tPA), the clot-dissolving enzyme and lower levels of fibrinogen, anti-thrombin III and anti-atherogenic fraction apoAI (Renaud & Ruf, 1996). The reduce susceptibility of platelets to aggregation has been reported after alcohol consumption. The anti-aggregation action of daily alcohol consumption was the mechanism originally proposed to account for the low incidence of CHD mortality in French populations (Renuad & de Lorgeril, 1992) prior to the involvement of LDL oxidation and subsequent increases in HDL, which are thought to account for only 50% of the protective effect of red wine consumption and reduced CHD. The remaining 50% being attributed to the effects of alcohol on platelet function (Renaud & Ruf, 1996).

Polyphenols from grapes and those present in red wine contains compounds that may slow the process of platelet aggregation. The platelets of both dogs and monkeys exhibited significantly reduced ability to aggregate after being fed grape juice, whereas the same volumes of orange and grapefruit juice had no effect (Osman *et al.,* 1998). Similarly, rats fed red wine or red wine phenolics showed increased bleeding times *in vivo* and *in vitro* and decreased platelet aggregation as compared to rats fed either white wine or ethyl alcohol (Wollyn *et al.,* 1999). Likewise *in vitro* incubation and oral supplementation in healthy human subjects with purple grape juice and selected derived flavonoids have also shown to decrease platelet aggregation, increase platelet-derived NO release and decrease superoxide production (Freedman *et al.,* 2001). The administration of red wine and grape juice in stenosed dog coronary arteries was found to inhibit *in vivo* platelet activity and thrombosis when administered both intravenously (1.6 mg/mL, 13% alcohol in 200 mL saline) and intragastrically (4 mL/Kg) (Demrow *et al.,* 1995) using the Folts coronary thrombosis model of platelet aggregation (Antiplatelet Trialists' Collaboration, 1988). This model allowed the direct administration

of the red wine and grape juice to the bloodstream where they were able to remove the effects of metabolism and absorption of the red wine and grape products. The results therefore cannot be regarded as being completely relevant to standard feeding studies. Administration of white wine at the same concentrations had no effect on platelet activity, suggesting that there are compounds present in grape juice and red wines which are not present in white wine, which are anti-thrombotic and platelet inhibitory. Both grape juice and red wine components were also effective as anti-thrombotic and antiplatelet agents when administered directly to the stomach of the dog, indicating that the active ingredients are absorbed and transported to the bloodstream (Demrow *et al.*, 1995).

The mechanism by which polyphenols, from red wine and grape juice, exert these actions appear to be multiple and poorly understood. Polyphenols have been shown to inhibit the production of thromboxane A_2 (TXA_2), which is formed in platelet by means of the cyclo-oxygenase (COX) pathway and is a powerful proaggregatory agent and vasoconstrictor of smooth muscle cells (Vanhoutte, 1989) (Figure 4). On the other hand, nitric oxide (NO) activates guanylyl cyclase in platelets, increasing the levels of the intracellular messenger, cGMP. This increases platelet aggregation inhibition, by a reduction in the intracellular free calcium (Ca^{2+}) concentration. Prostacyclin (PGI_2) causes the same effect through adenylyl cyclase activation, which increases intracellular levels of cAMP, which as before lowers the platelet intracellular calcium levels and decreases *in vivo* platelet activity (Vanhoutte, 1989; Marin & Rodriguez-Martinez, 1995) (Figure 4). Increases in intracellular calcium concentration from extracellular sources and the mobilisation of intracellular stores, is the crucial step for the activation of the cyclo-oxygenase pathway and endothelial nitric oxide synthase (eNOS) (Martin *et al.*, 2002).

The structural feature required for polyphenols to inhibit platelet aggregation are similar to those associated with their antioxidant function.

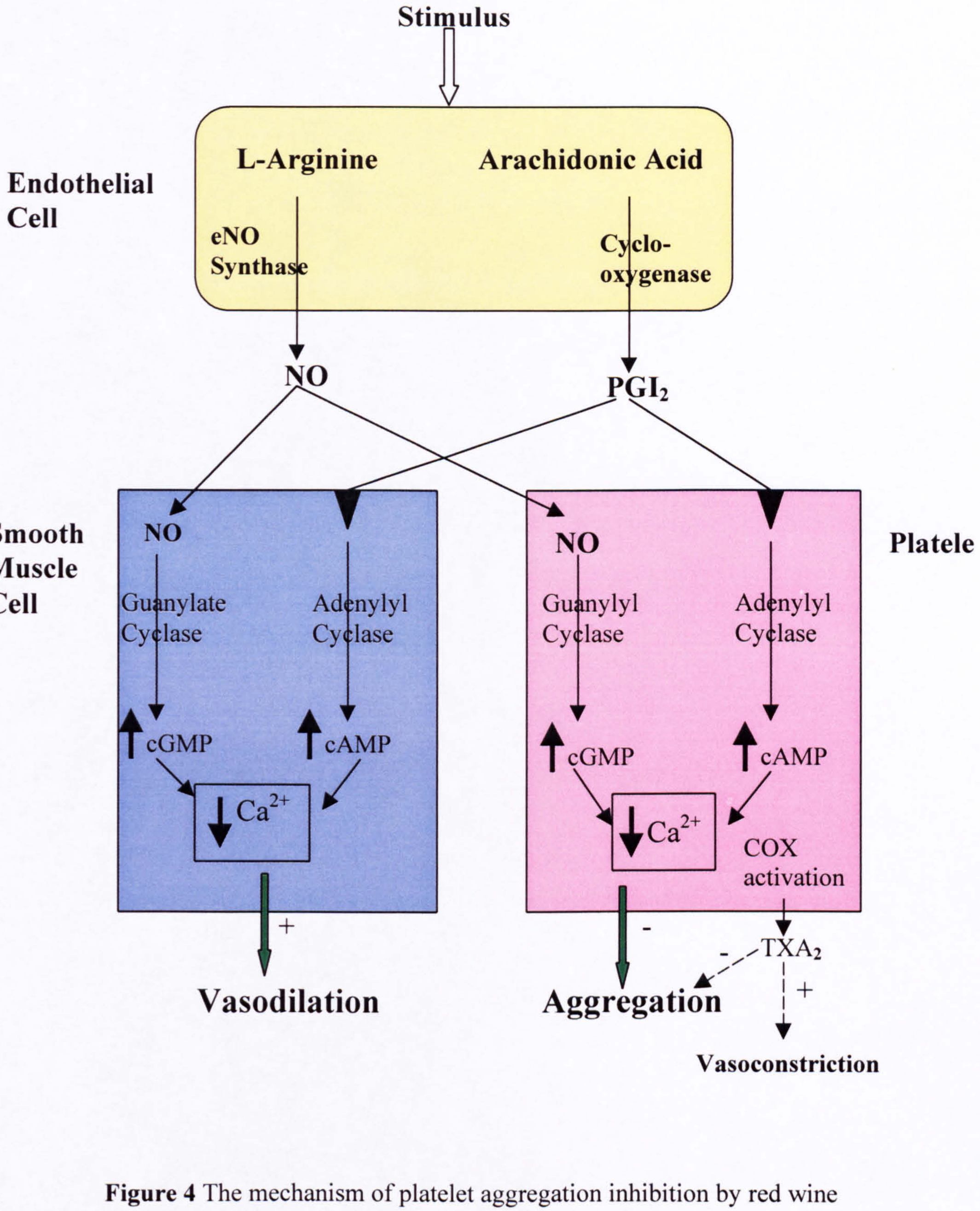


Figure 4 The mechanism of platelet aggregation inhibition by red wine Polyphenols stimulate increases in the $[(Ca^{2+})_i]$ concentration which leads to activation of eNOS and the cyclo-oxgenase pathway.

They include a double bond between C2 and C3, a 3'-OH group and a carbonyl group on C4 (Rotondo & de Gaetano, 2000).

1.7.4 Vasorelaxation & cardiovascular protection

The relaxation of the arterial smooth muscle produced by some wines and grape products appears to involve the endothelium-derived relaxing factor (EDRF), which was first described by Furchgott & Zawadzki in 1980. The EDRF involved in the relaxation produced by plant derived products was subsequently determined to be nitric oxide (NO) or a NO derivative (Palmer *et al.*, 1987).

NO has been implicated in a wide range of physiological roles in the cardiovascular system. Endothelial NO plays an important role in regulating vascular tone and myocardial contractility, maintaining endothelial integrity, inhibiting platelet aggregation and maintaining normal functioning of the cardiovascular system (Loscalzo & Welch, 1995). Dysregulation of NO production may play a role in the pathogenesis of several cardiovascular disorders. Therefore, enhancement of NO production may be useful in preventing or blocking the progression of atherosclerosis, CHD and other diseases in which there is endothelium dependant relaxation impairment, such as hypertension and diabetes mellitus (Stoclet *et al.*, 1997; Rakhit & Marber, 2001).

The vascular endothelial cell is able to produce and release several mediators that act at different sites on the adjacent smooth muscle cell to modulate local vascular tone (Ziegler *et al.*, 1995). The release of these mediators is influenced by several factors and multiple homeostatic mechanisms, determine the overall balance between endothelial-derived vasoconstricting and vasodilating influences. NO is produced by the endothelial cell during the conversion of L-Arginine to L-Citrulline, a reaction catalysed by the enzyme by nitric oxide synthase (NOS) (Ziegler *et al.*, 1995). NO is constantly produced at basal levels in the endothelial cell where it

controls and mediates local vascular tone. Nitric oxide also plays a major role in determining the resting vascular tone of coronary resistance vessels, regulates basal pulmonary vascular resistance and is responsible for the autoregulation of blood flow in several organ systems including the brain, heart and kidney (Loscalzo & Welch, 1995). Vascular endothelial cells also produce NO in response to physiological stimuli, including changes in oxygen tension, shear stress, thrombin and the platelet products, ADP and serotonin (Stoclet *et al.,* 1997). Nitric oxide therefore plays a major role in protection of the cardiovascular system but excessive nitric oxide production may by deleterious and may cause vascular collapse (Stoclet *et al.,* 1997). Large tissue nitric oxide concentrations may be cyctotoxic. The breakdown and bioavailability of nitric oxide can be reduced and removed by its reaction with superoxide anion (O_2^-) yielding peroxynitrite and peroxynitrous acid, instable molecules which are potent oxidants (Benito *et al.,* 2002).

After formation, NO rapidly diffuses from its site of synthesis, in the endothelial cell, to neighbouring smooth muscle cells where it "activates" its target enzyme, soluble guanylate cyclase (GC), resulting in an increased smooth muscle cell concentration of cGMP. Generated cGMP then initiates a cascade of intracellular events which stimulates protein kinase G (PKG), via phosphorylation, which results in the vasodilation of the smooth muscle cell (Ziegler *et al.,* 1995) (Figure 5). cGMP is continually hydrolyzed and inactivated within the smooth muscle cell by phosphodiesterase (PDE) enzymes. Therefore the continued release of NO and production of cGMP is important in maintaining basal tone in vascular beds (Marin & Rodriguez-Martinez, 1995). In response to endothelial-dependent vasodilators, the basal production of NO is increased significantly (Ziegler *et al.,* 1995).

1.7.5 Red wine & vasorelaxation

In addition to the proposed effects of regular moderate wine consumption on blood lipid changes and platelet function, a small number of animal and human studies have shown that wine and grape derived products

may affect blood vessel function. This may be a third potential explanation for the French Paradox.

The vascular endothelium lies at the interface between the circulating blood cells and the vascular smooth muscle cells, responding to vaso-active stimuli, blood flow and stress (Stoclet *et al.,* 1997). Wine as an ethanol-containing beverage, is well known to affect blood flow in the vascular system (Fitzpatrick *et al.,* 1993). However, the non-alcoholic phenolics present in red wine affect vascular blood flow and endothelial function to a greater extent than alcohol alone indicating that the beneficial effects are independent of the ethanol component (Fitzpatrick *et al.,* 1995).

The active components responsible for this vasorelaxing activity of wine and grape products are believed to be derived from the skin of the grape (Fitzpatrick *et al.,* 1993). Therefore the findings that red wines are better vasorelaxants than white wines may be attributable to the fact that the grape skins are removed before fermentation in the production of white wines, but left intact in red wine production allowing a longer time period for the extraction of the vaso-active grape skin components into the wine must (Soleas *et al.,* 1997).

The ability of red wine polyphenols to induce NO-mediated responses was first reported by Fitzpatrick *et al,* (1993) who studied the vasorelaxant effects of wine and grape-derived polyphenols in isolated rat aortic rings. Studies have showed that certain wines, grape juices and grape skin extracts were able to relax precontracted smooth muscle of intact aortic rings, an effect which was dependent upon the presence of intact endothelium. The extracts also increased smooth muscle cell concentrations of cGMP, both relaxation and increases in cGMP levels were reversed by inhibitors of NO synthase, indicating that relaxation was mediated by the NO/cGMP pathway (Fitzpatrick *et al.,* 1995, Andriambeloson *et al.,* 1997, Cishek *et al.,* 1997).

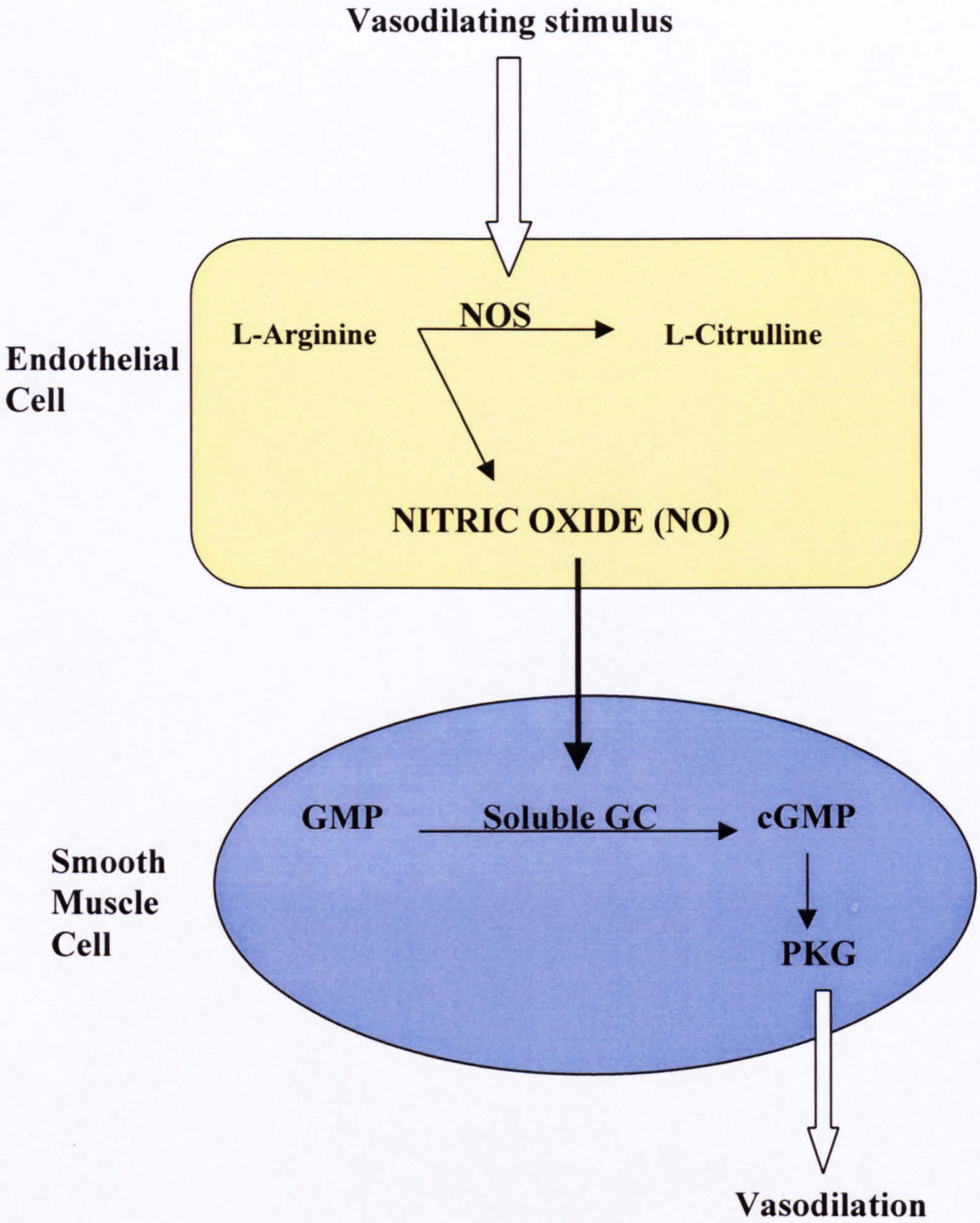


Figure 5 Pathways mediating smooth muscle vasorelaxation by NO

Recent studies have shown that the endothelium-dependent vasorelaxation of the rat aorta by red wine polyphenols is mediated by an increase in the NO aortic content, with the NO increase due to an enhancement of NO synthesis rather than increased protection against breakdown by superoxide radicals (O_2^-) (Andriambeloson *et al.*, 1997b, Benito *et al.*, 2002). It was also noted that despite their comparable antioxidant activity in red wine, leucocyanidol (flavan-3,4-ol) but not the structurally closely related catechin (flavan-3-ol) relaxed the endothelium suggesting that the antioxidant properties of polyphenols are not involved in their vasodilatory effects (Andriambeloson *et al.*, 1998). Therefore some components of red wine contribute to the prevention and treatment of cardiovascular diseases by increasing NO levels in the vascular system without increasing O_2^- production, and the subsequent formation of peroxynitrite-derived pro-oxidants (Benito *et al.*, 2002).

It is well established that increases in intracellular Ca^{2+} concentrations [$(Ca^{2+})_i$] within the endothelial cell is critical step for the activation of endothelial NO-synthase leading to the production of NO and the subsequent endothelium-dependent vasorelaxation (Andriambeloson *et al.*, 1999, Martin *et al.*, 2002). Therefore the increases in [$(Ca^{2+})_i$] can be due to either an influx of extracellular Ca^{2+}, to a release of Ca^{2+} from intracellular stores or both. Vasorelaxation was abolished after removal of extracellular Ca^{2+} from the medium and the presence of a Ca^{2+}-entry blocker, indicating that Ca^{2+} influx from extracellular stores via voltage-independent channels is the critical step involved in vasodilation with red wine and grape products (Stoclet *et al.*, 1999, Martin *et al.*, 2002). Phospholipase C (PLC) and Tyrosine Kinase (TK) are both thought to be implicated in this Ca^{2+} signalling (Martin *et al.*, 2002) with red wine and grape products. It has also been suggested that the inhibitory effect of polyphenols on PDE's involved in the breakdown of vasodilatory cyclic nucleotides may be implicated in the endothelium-independent component of the vasorelaxation induced by red wine and grape products, but this has not yet be substantiated (Andriambeloson *et al.*, 1999).

Red wine phenolics have also been shown to significantly lower blood pressure in stroke-prone spontaneously hypertensive rats (SHRSP) (Mizutani *et al.,* 1999). The phenolics were also shown to improve the aortic biomechanical properties, improving aortic elasticity and fragility following a diet rich in wine polyphenols for 8 weeks.

Studies to identify and isolate the compounds present in wine and grape products that are responsible for their effects on endothelial function and their mechanisms of action remain controversial. Purified flavonoids have been shown to induce NO-mediated vasorelaxation in pulmonary arteries. Luteolin, apigenin, myricetin, kaempferol, quercetin and isorhamnetin all induced significant relaxation in the preconstricted pulmonary vessels (MacLean *et al.,* 1997). Similarly, the polyphenolic anthocyanins and oligomeric condensed tannins have also shown to induced relaxation to levels comparable to the original red wine from which they were extracted (Andriambeloson *et al.,* 1997). Epicatechins derived from grape seed extracts have also been shown to have an effect on vascular tension *in vitro* but are considerably less active than the structurally related proanthocyanidins (Fitzpatrick *et al.,* 2000) isolated from the same extract.

1.7.6 Plant products & vasorelaxation

The previous section has shown that red wine and grape-derived products are all potent vasodilators but it has also been shown that a wide range of fruits, vegetables and nuts, herbs and spices also have an effect on vascular tension *in vitro* (Fitzpatrick *et al.,* 1995) as shown in Table 5.

Several fruits and vegetable were found to be potent vasodilators in endothelial intact aortic vessels inducing more than 50% relaxation (Table 5). Extracts of fruit such as the skin of the red apple, the skin and pulp of the plum and cranberries were also found to be strong relaxing agents. The range of vegetables examined were found to induce less vasodilation than the fruit selection. The skin of the peanut (the papery outer layer) was identified to be

an effective dilator *in vitro* as were the spices, cinnamon and garlic. Some of the extracts (strawberry, tomato, black pepper) had very little or no effect on vascular tension, while others (potato and banana) induced a vasoconstriction of the PE-contracted aortic vessels. It is surprising that the strawberry and the red cabbage which are rich in anthocyanins like red grapes did not induce significant vasorelaxation (Fitzpatrick *et al.*, 1995).

Table 5 Vasorelaxation induced by selected fruits & vegetables

Extract	**% relaxation induced**
Fruits:	
Cranberries	87 ± 6
Red apples (*skin*)	92 ± 1
Red apples (*pulp*)	81 ± 15
Plum (*skin*)	88 ± 4
Plum (*pulp*)	92 ± 2
Tomato (*skin*)	10 ± 2
Tomato (*pulp*)	5 ± 2
Strawberry	0
Banana	-21 ± 8*
Vegetables:	
Potato	-52 ± 7*
Cabbage (*green*)	26 ± 3
Cabbage (*red*)	43 ± 5
Aubergine (*skin*)	77 ± 6
Broccoli	49 ± 12
Nuts, herbs & spices:	
Peanut (*skin*)	97 ± 3
Peanut (*meat*)	60 ± 10
Pepper (*red*)	18 ± 5
Pepper (*white*)	17 ± 2
Pepper (*black*)	0
Cinnamon	98 ± 2
Garlic	65 ± 8

*Extract caused a vasoconstriction of the aortic vessel
Adapted from Fitzpatrick *et al.*, 1995

Cocoa is also a rich source of polyphenols, particularly catechins and procyanidins. It has been shown to be an effective antioxidant *in vitro* systems (Vinson *et al.*, 1999) being more potent and having a higher polyphenol

content than a wide range of fruits and vegetables with a 40 mg serving of milk chocolate containing 394 mg of polyphenol antioxidants as compared to a serving of red wine which contains 431 mg/250 mL (Vinson, 1998). However it remains to be shown whether chocolates can act as antioxidants following ingestion since fat is a well known pro-oxidant *in vivo* (Wollgast & Anklam, 2000). Further studies have also shown that cocoa polymeric procyanidins induce endothelium-dependent vasorelaxation in rabbit aortic vessels *in vitro* (Karim *et al.*, 2000) inducing up to 50% vessel vasodilation. Relaxation was mediated via the release of NO and increases in aortic cGMP content (Karim *et al.*, 2000).

1.8 Role of grape phenolics in health promotion

1.8.1 Wine & coronary heart disease

The onset of cardiovascular disease depends on numerous factors that can be modulated by components in the diet. It has been estimated that up to 20% (Weisburger, 2000) of all coronary heart disease is attributable to dietary factors.

In CHD, atherosclerotic plaques protrude from the inner surface of the arteries, narrow the lumen and reduce blood flow through the vital organs (Stanley & Mazier, 1999). At the first stage, LDL deposits at lesions sites on the arterial wall and is subjected to oxidation when antioxidant protectors, such as polyphenols or vitamins are depleted. Subsequently, oxidation of LDL induces modification in lipoproteins, stimulates inflammatory reactions, causes monocytes and monocyte-derived macrophages to accumulate large amounts of oxidised LDL and forms lipid-laden foam cells and atherosclerotic plaques by merging (Tijburg *et al.*, 1997) (Figure 6). The intake of saturated fats, smoking, excessive alcohol and little or no physical activity accelerate these events.

Epidemiological studies have led to the identification of key dietary components that are etiological factors in the pathogenesis of CHD. However the true identification of these components lies with large-scale controlled prospective intervention trials, the strength of which is dependent upon the number of people involved and the duration of study follow-up. The effect of traditional vitamins and other dietary antioxidants on atherosclerosis remain controversial.

Inverse associations with the risk of stroke have been observed for flavonols and flavones (Keli *et al.,* 1996) and carotenoids in the Health Professionals Follow-up Study (Ascherio *et al.,* 1999). However no association between dietary antioxidants and risk of stroke was reported in the American Western Electric Study (Daviglus *et al.,* 1997). In the large-scale intervention ATBC trial of 26,000 male smokers in Finland, a protective association between the consumption of fruits and vegetables and risk of stroke was confirmed (Hirvonen *et al.,* 2000). Neither flavonols, flavones or vitamin C intakes were related to a reduced risk of stroke. Vitamin E intakes were also not related to a reduced risk of CHD (Virtamo *et al.,* 1998) as assessed by dietary supplementation and dietary food recall questionaires. However high dietary β-carotene intakes were inversely associated with risk for cerebral infarction in male smokers (Hirvonen *et al.,* 2000). Likewise, the Linxian China Study is a primary prevention trial of 29,000 people testing dietary supplementation with four combinations of vitamins/micronutrients on overall mortality and cancer mortality. On analysis of heart disease, all combinations did not find evidence for reduced relative risk of cardiovascular mortality (Blot *et al.,* 1993). Methods for assessing and estimating dietary intakes in such intervention trials have included the use of dietary assessment models such as self-administered baseline food frequency and history questionnaires, 24 hour dietary recall interviews and 4 day food diaries, replacement of normal dietary intake with study diet over the specified study period, personalised dietary information and dietary surveys to ensure adhesion and compliance to experimental diets and the dietary supplementation with specific vitamin/mineral combinations. These studies

are therefore open to potential error and bias due to the methods employed and use of dietary intake information obtained from subjects.

Studies conducted *in vitro* and *in vivo* in animal models and with humans suggest that red wine and its active components may play a protective role in the development and progression of CHD.

In 1979, St Leger *et al.*, were the first to describe an association between CHD mortality and wine consumption in a large-scale epidemiological study. The findings of the MONICA study (World Health Organisation, 1989) a worldwide monitoring system of cardiovascular diseases using data from 18 western countries using 43 centres, highlighted a positive association between death from CHD and saturated fat intake while a negative correlation was identified when wine consumption was taken into account (Renaud & De Logeril, 1992). Supporting evidence for this association came from the observation that the incidence of CHD mortality in France is significantly lower (one third of the average) than that of other industrialised countries with similar high dietary fat intakes (14-15% of energy) such as the UK and the USA. This phenomenon has been referred to as the "French Paradox" (Renaud & De Logeril, 1992). It should however be recognised as stated before that due to the use of dietary intake and alcohol consumption information obtained from subjects in this study there is substantial potential for bias and error in the methods employed for the recall of information which may have affected the overall study results.

Studies have indicated that consumption of alcohol at the level of intake in France (20-30 g/day) can reduce the risk of CHD by at least 40% (German, 2000). It was also noted that other risk factors for CHD such as blood pressure, body mass index and cigarette smoking were no different from that of other industrialised countries. This phenomenon is believed to be due to the high consumption of wine together with the healthy "Mediterranean-style" diet, rich in vegetables and olive oil, consumed by the French (Renaud & De Logeril, 1992). Epidemiological studies which have studied this relationship in

specific populations have indicated that wine may be more potent than other alcoholic beverages in reducing the incidence of CHD as determined by mortality data, food disappearance rates and alcohol data from 21 developed countries between 1964 and 1988 (Criqui & Ringel, 1994). Another possibility for this relationship may be that wine is drunk in a healthier fashion than that of other alcoholic beverages. The tradition of drinking wine with meals may have a favourable effect on post-prandial hyperlipidaemia (Criqui & Ringel, 1994). Drinking with meals may also reduce some of the toxic effects of high alcohol consumption because of reduced absorption.

The phenolic components of red wine derived from the grape skin, have been identified as the primary factors responsible for this protective role against CHD, exerting their effect as antioxidants, free radical scavengers and protectors of blood vessel function. In particular, the ability of red wine to inhibit the oxidation of LDL *in vitro* has been suggested as the mechanistic explanation for the association relating red wine to a lowered risk of CHD (Frankel *et al.,* 1993, Tiessedre *et al.,* 1996, Nigdikar *et al.,* 1998). Other proposed mechanisms include the ability of red wine to inhibit platelet aggregation (Demrow *et al.,* 1995, Pace-Asciak *et al.,* 1995) and induce endothelium-dependent vasorelaxation via the release of NO (Fitzpatrick *et al.,* 1993, 1994; Andriambeloson *et al.,* 1999) in vascular tissue as previously discussed.

1.8.2 Wine & cancer

The relationship between moderate red wine intake and cancer risk are less clear and epidemiological studies give variable results. It is widely accepted that up to one-third of all human cancer cases could be related to some dietary component in the Western world (Doll & Peto, 1981). Dietary factors are now considered responsible for 40 – 60% of all cancer incidence and for 35% of cancer deaths (National Research Council, 1989).

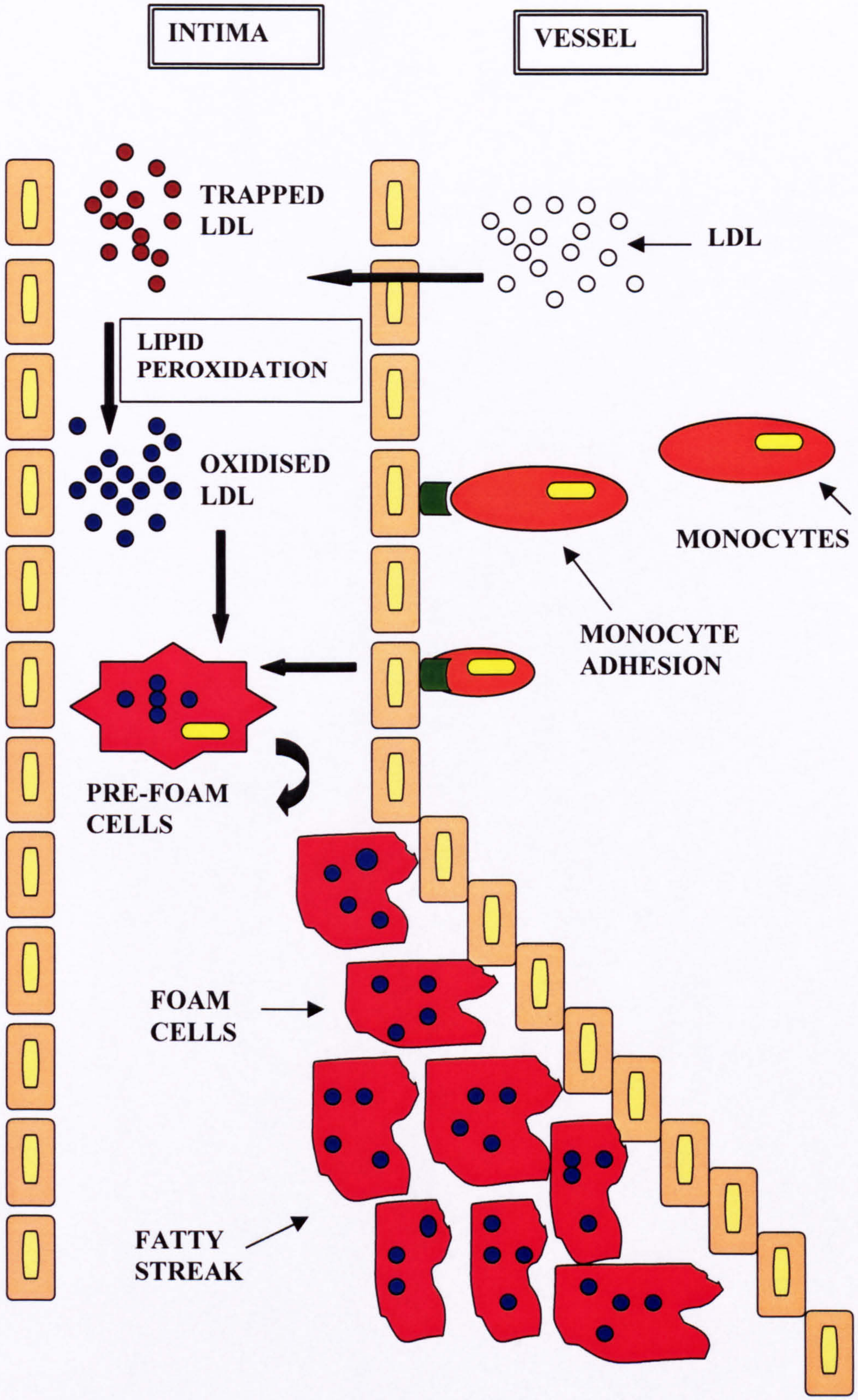


Figure 6 The development of coronary heart disease (Burns *et al.*, 2001)

Large-scale intervention trials investigating the effects of flavonols, flavones, vitamins and micronutrients on cancer incidence and mortality have also been carried out. Intake of flavonols and flavones were inversely related to incidence and risk of lung cancer but not to prostate, colorectal and stomach cancers in the ATBC (The Alpha Tocopherol Beta Carotene) study of 27,000 male heavy smokers in Finland aged 50-69 years (Hirvonen *et al.*, 2001). Data was collected from validated dietary questionnaires and diaries. On the other hand 3 large scale studies, ATBC, CARET (Beta-Carotene and Retinol Efficacy Trial) and the Health Professionals Study concluded that dietary supplementation with β-carotene provided no protection against lung cancer (Pryor *et al.*, 2000), with the ATBC study identifying a higher risk of lung cancer for those in the β-carotene intervention group as compared to those in the control group (The ATBC Study Group, 1994). Likewise, long-term supplementation with alpha-tocopherol and β-carotene provided no preventative effect on urinary track cancers (Virtamo *et al.*, 2000) but did lower the risk of occurrence of prostate cancer (Heinonen *et al.*, 1998) in the ATBS Study. The Linxian Cancer Study in China, supplementation with combinations of vitamins and micronutrients, especially vitamins C and E and selenium, reduced total mortality mainly due to significant a significant reduction in cancer rates, especially stomach cancer and oesophageal cancer (Blot *et al.*, 1993).

Alcohol containing drinks have now been identified as factors that increase the risk of several forms of cancer (IARC, 1988). At high intakes, alcoholic drinks are highly associated with increased cancer risk especially cancers of the larynx, liver, breast and oesophagus but this relationship is weak for red wine (Barra *et al.*, 1990). However other studies have highlighted a protective and beneficial effects of drinking wine, white and red, in reducing cancer risk. Klatsky *et al.*, (1989) found no relationship between moderate consumption of wine and colorectal cancer incidence, similarly Gronbaek *et al.*, (1998) found that moderate wine intake did not increase the risk of upper digestive tract cancer, whereas moderate intake of beer or spirits did increase the risk. In a similar study, Prescott *et al.*, (1999) reported the risk of lung

cancer was associated with a high consumption of beer and spirits whereas wine may be protective. Overall, population studies show that moderate consumption of wine, especially red wine is associated with cancer incidence less often than for consumption of similar quantities of other alcohol-containing beverages. The inconclusive nature of the epidemiological studies may reflect in part, potential biases in recall and the difficulty of separating the effects of red wine from those of alcohol.

The mechanisms by which red wine may protect against cancers are yet undetermined but some studies *in vitro*, now support a role for wine in protecting against cancer. The red wine polyphenol, *trans*-resveratrol has been indicated to have anti-carcinogenic effects in cancer cell lines being able to induce dose-dependent inhibition of proliferation and DNS synthesis. Red wine concentrate with the alcohol removed, has dose-dependent anti-proliferative effects on breast cancer cell lines (Damianaki *et al.*, 2000) and consumption of dehydrated dealcoholised red wine delays the onset on tumours in transgenic mice that subsequently develop visible tumours without carcinogen pre-treatment (Clifford *et al.*, 1996).

1.9 Absorption & bioavailability of red wine polyphenols

Absorption from the diet is a prerequisite for a possible causal relationship between the intake of polyphenols from red wine and prevention from CHD. However information on the extent of absorption, bioavailability in the body and the metabolic fate of ingested polyphenols is limited and often controversial.

Very few studies have provided an estimate of the extent of polyphenol absorption from wine in humans. The ingestion of a glass of red wine results in rapid and transient increase in total phenol content of the plasma (Serafini *et al.*, 1998). Ingestion of white wine did not alter plasma polyphenol level. As

of yet it is unclear which of the many polyphenols present in red wine are the most bioavailable. Although there is an increasing body of evidence supporting the view that flavonol conjugates are preferentially absorbed and that the nature of conjugation is important (Hollman *et al.,* 1995, Aziz *et al.,* 1998). Following the consumption of red wine the presence of catechin metabolites, predominately as sulphate conjugates and conjugates containing both glucuronide and sulphate residues were identified in human plasma (Donovan *et al.,* 1999), however the overall levels identified were comparatively low compared to the catechin levels of the wine itself. Cyanidin-3-*O*-glucoside, an anthocyanin present in red wine has also been identified in the plasma of orally dosed rats (Tsuda *et al.,* 1999) and was found with its methylated metabolites to accumulate in the kidney.

1.10 Introduction to tea

1.10.1 The History of Tea

According to Chinese mythology in 2737 BC, the Chinese emperor Shen Nung, the scholar and herbalist was sitting beneath a tree while his servant boiled drinking water. A leaf from the wild tree dropped into the water and Shen Nung decided to try the brew. The resulting drink was said to be both healthy and refreshing, Shen Nung noted in his Pen ts'ao, or medical book, "It is good for tumours and abscesses that come about the head, or for aliments of the bladder. It gladdens and cheers the heart" (Liao *et al.,* 2001).

From the earliest times tea was renowned for its properties as a healthy refreshing drink. By the time of the Song Dynasty (960 – 1271 AD) the major tea plantations in China extended southeast, and people were offering tea as tribute in place of grain and money. After the Ming Dynasty (1368 – 1644 AD), tea became the national drink of China (Liao *et al.,* 2001). Tea became a necessity of life equal to fuel, rice, oil and salt. People were said to go without

rice for 3 days but would not go without tea for a single day (Yamanishi, 1995).

1.10.1.1 The history of tea in Britain

The early beginnings of tea in Britain are obscure. The East India Company under the charter granted by Elizabeth I to the directors had the monopoly of importing goods from outside Europe and recorded ships reaching Britain in 1637, but no recording of tea dealings with Chinese merchants appears until 1644. Sailors bringing packets of tea as presents and stories its health benefits led to its introduction into London coffee houses. The merchant, Thomas Garraway, was among the first to trade tea in Britain. People flocked to his coffee shop in London's Exchange Alley to try a drink that was said to among other things to "help the headache, clean the kidneys and clear the sight". He offered it in dry and liquid form, holding his first public sale in 1657. Tea rapidly gained in popularity and by 1700 was on sale in more than 500 coffee houses in London. By the middle of the 18th century, tea had become the drink of the masses and had become Britain's most popular beverage (www. tea.co.uk).

1.11 From plantation to cup

At one time two species of tea plants, *Camellia sinensis* (northern China form) and *Camellia assamica* (southern China form) were recognised by botanists. However these plants are now considered different strains of *Camellia sinensis*, members of the Theaceae family (Liao *et al.*, 2001). As a member of the Camellia family, tea is a perennial evergreen tropical plant which yields crops through out the year in tropical regions and for 6 to 8 months in subtropical regions. Free growing tea plants have shiny green pointed leaves similar to that of privet leaves, white flowers with yellow anther resembling the wild rose and the tea fruit containing 3 seeds (Yamanishi, 1995). In its wild state, tea grows best in regions which enjoy a warm, humid climate with a rainfall measuring at least 100 cm a year. Ideally

it likes a deep, light, acidic and well-drained soil. Given these ideal conditions, tea will grow in areas from sea-level up to altitudes as high as 2,100 meters above sea-level. Tea is also grown across a wide range of latitudes, from 45°N (Russia) to 30°S (South Africa), and from 150°E (New Guinea) to 60°W (Argentina). Tea varies in flavour and characteristics according to the type of soil, altitude, latitude and climate conditions of the area in which it's grown (Yamanishi, 1995).

1.11.1 Manufacturing of tea

Tea manufacturing includes different technological processes (Table 6). Tea production technology is aimed at modifying the chemical composition of the raw material, the fresh tea leaf, in order to produce new tastes and aroma compounds responsible for the taste, colour and aroma of the manufactured tea that are so highly valued by the consumer (Bokuchava & Skobeleva, 1980). Classification of processed tea is well established and is based on quality and processing. There are 6 main types of tea produced: green, yellow, dark, white, oolong and black, with green, black and oolong teas being the main tea types consumed today. This division is based on the degree of fermentation and oxidation of the polyphenols present in the tea leaves.

1.11.1.1 Tea Leaf Plucking

Once the correct tea leaf age and size has been achieved, the tea leaf is gathered in mainly by hand. Due to this, tea is regarded to be the most labour-consuming crop. The tea leaf must be gathered as fast as possible as the slightest delay may destroy tender flushes and decrease the harvest (Bokuchava & Skobeleva, 1980).

1.11.1.2 Withering

Withering is the first important step in tea manufacturing. The withering step is carried out to biochemically and physically prepare the freshly plucked

tea leaves for further processing procedures. The leaf is spread on trays and left to wither in air at 25-30°C. The freshly plucked tea leaf contains about 75-80% water and the withered leaf contains 62 to 64% (Liao *et al.*, 2001). Withering also induces changes in the overall catechin content and the protein and amino acid composition (Bokuchava & Skobeleva, 1980). The withering process involves both physical and chemical changes which exert a significant effect on the subsequent technological processes of rolling and fermentation and as a result on the quality of the manufactured tea.

1.11.1.3 Rolling & Crushing

The second important step in tea manufacture is the rolling and subsequent crushing of the tea leaf. The main purpose of the rolling step is complete maceration of tea leaf tissues to induce fermentation. The rolling step proceeds slowly, providing a gradual excretion of tea leaf juices.

1.11.1.4 Fermentation

The most important stage in the manufacture of black tea is fermentation. The biochemical changes developed in the withering stage proceed at a rapid rate during fermentation. These changes result in profound changes in tea leaf constituents contributing to the formation of the new taste and aroma responsible for the character of black tea. Following rolling and crushing, the tea leaves are laid out in a cool humid atmosphere for 3-4 hours to ferment or oxidise. Fermentation is mediated by oxidative enzymes present in the tea leaf, mainly polyphenol oxidases (Bokuchava & Skobeleva, 1980). During fermentation the rolled leaves lose their green colour acquiring a coppery-red colour, due to the strong oxidative reactions occurring. During fermentation the catechins present in the fresh tea leaf are rapidly converted to the characteristic theaflavins and thearubigens of black tea, the ratio of which are responsible for the strength of the tea brew (Liao *et al.*, 2001).

1.11.1.5 Firing

Following fermentation the tea leaf immediately undergoes firing at high temperature of 90 to 95°C reducing tea moisture content to 3-4% (Bokuchava & Skobeleva, 1980). The main purpose of firing is to stop fermentation at the very moment when the amount of valuable substances accumulated in the tea leaf is highest. During firing the tea undergoes physical and chemical changes, which depend on firing temperature and rate of air flow.

1.11.1.6 Cleaning, sorting & packing

The dried tea is sifted to prepare the final product. The tea is sorted into grades or leaf particle size by being passed through a series of wire mesh sifts of varying size into containers before being weighed and packed into chests or tea sacks. Tea storage is followed by chemical changes in the content of essential oils, tannins and other soluble constituents which all gradually decrease which deteriorates the taste and aroma of the finish tea product (Bokuchava & Skobeleva, 1980).

1.11.2 World tea production

Tea like red wine is a rich source of polyphenols. Although native to south east Asia, tea is now cultivated in more than 25 countries worldwide (Table 7) with the major commercial producers being India, Japan, Africa, Sri Lanka, China and Indonesia (Tea Council, 2000). It is generally accepted that next to water, that tea is the most consumed beverage worldwide, with a per capita consumption of 120 mL per day (Ahmad & Mukhtar, 1999). World tea production and consumption levels are presently the highest levels achieved in the last decade and rising. To date about 3 billion Kg (Graph 1) are produced per year (Tea Council, 2000) with 78% of tea produced accounting for black tea, 20% as green tea and 2% as oolong tea (Yang & Landau, 2000). Approximately 90% of the tea consumed in Britain is known as the popular brand leading blends.

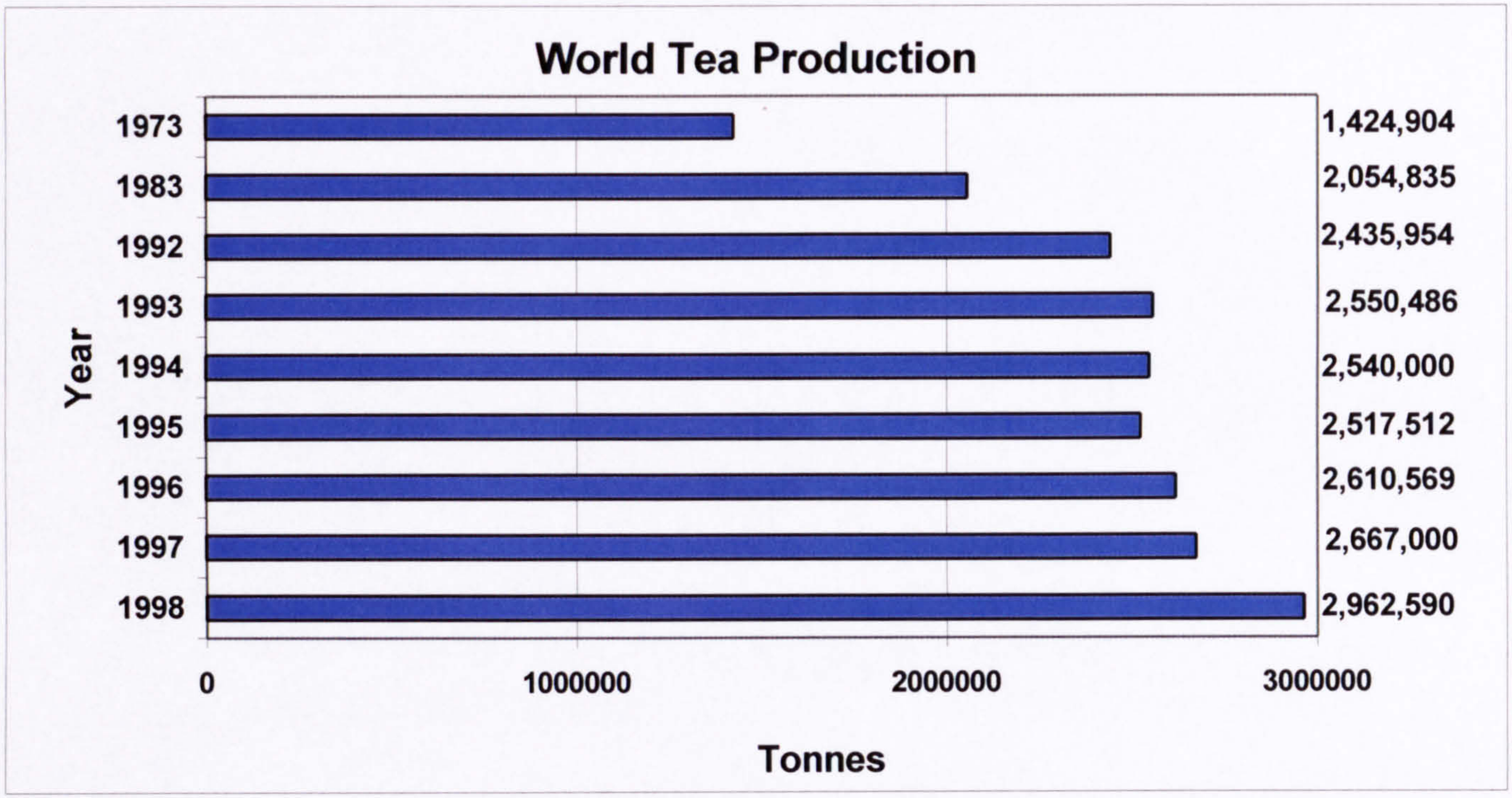
Table 6 Summary of the processes involved in the manufacture of tea

Process	Method
Plucking	the new growth is picked, usually by hand
Withering	the leaf is allowed to dry slowly and the leaf becomes more pliable
Rolling	the softened leaf is rolled, resulting in a large and wiry end product
Crushing	the leaf is crushed, shredded and rolled, resulting in a granular end product
Fermentation	the green leaf oxidises, changing in both colour and flavour
Firing	the tea is dried at high temperature to stop fermentation and reduce moisture (<3%)
Cleaning	the coarse stalk and large leaf is removed from the dried tea
Sorting	the leaf is passed through a series of meshes to produce standard grades
Packing	the graded tea in bulked in silos and packed into paper sacks

Table 7 Tea production around the world

Country	% production of world exports
India	14%
Sri Lanka	21%
Africa	25%
Kenya	
Malawi	
Tanzania	
Zimbabwe	
China	18%
Indonesia	8%
Non-Tea Council Members	34%
Australia	
Turkey	
Japan	
South America	

Adapted from the Tea Council, 2000



Graph 1 The World Market - Tea Productionn (Tea Council, 2000)

1.11.3 Tea types

There are three main categories of tea consumed in the world today (Table 8) which are manufactured due to differences in the aforementioned tea processing procedures and which give rise to the characteristic colour, flavour and aroma of each specific tea type. They are:

1.11.3.1 Green Tea

Green tea is a very valuable food product which is widely consumed throughout the world but predominately in China and Japan. With respect to the world tea market, green tea is inferior only to black tea (Liao *et al.*, 2001). The manufacture of green tea based is on the steaming and withering of the fresh tea leaf. In green tea manufacture the development of oxidative processes is regarded as an adverse factor, the fresher the tea leaf the better the tea produced (Yamanishi, 1995). Therefore, enzymic activity is arrested by high temperatures, at an early stage in production and replaced with thermal treatment of the raw tea, applied as sweating or roasting to make the tea tender for rolling.

The raw tea in the production of green tea is initially steamed to induce leaf withering at temperatures up to 95-100°C. The leaves are then dried to remove excess moisture, at temperatures from 100 to 110°C, before rolling to crush the leaf blades and stalks (Bokuchava & Skobeleva, 1980). The tea is then sifted and cooled before firing to reduce the overall residual moisture content to 3 to 5% and to produce a finished dry, olive-green in colour, stable product of high quality (Bokuchava & Skobeleva, 1980) (Figure 7).

1.11.3.2 Black tea

Black tea is the most commonly consumed type of tea in the market today accounting for up to 80% of all tea produced and consumed

predominately consumed in western countries, and is one of the most widely consumed beverages in the world (Liao *et al.*, 2001).

In the manufacture of black tea, the fresh tea leaves are allowed to initially wither to about 55% of their original leaf weight altering the chemical and physical composition of the raw tea leaf and to prepare them for rolling and crushing and the onset of fermentation (Bokuchava & Skobeleva, 1980). Inadequate withering at this initial stage results in an inferior quality of tea. The leaves are then rolled and crushed to induce the gradual excretion of tea leaf cell juices and to initiate fermentation. In black tea manufacture, rolling is in essence the first stage of fermentation usually requiring about 2-3 hours, with the second stage of extensive fermentation and leaf oxidation continuing thereafter via the release of polyphenol (*o*-diphenol) oxidase from the leaf endoplasmic reticulum (Bokuchava & Skobeleva, 1980). Fermentation is the major technological process in the manufacture of black tea and is based on these oxidative transformations which are responsible for formation of black tea aroma, flavour and colour. Following fermentation, the leaf is fired, moisture content is evaporated and the leaf turns a dark brown to black (Figure 7).

1.11.3.3 Oolong tea

Oolong tea (or red tea) is mainly produced in China and Taiwan. The first stage in oolong tea manufacture is withering followed by mild rolling and brief partial fermentation. The latter is halted when the leaf tips and edges become reddish brown and a specific aroma appears. The tea leaves are then fired to reduce the moisture content to 4 to 5% (Bokuchava & Skobeleva, 1980, Yamanishi, 1995) (Figure 7). Oolong tea is a semi-green or semi-fermented tea with a chemical composition between that of green and black teas.

Oolong tea has a pleasant, mild but at the same time has an astringent taste and strong stable aroma which is best drunk without milk (Liao *et al.*, 2001).

Table 8 Main varities of tea avaliable and country of origin

Green	Oolong	Black
Genmaicha (Japan)	Ti Kuan Yin (China)	Assam (India)
Spider Leg (Japan)	Formosa Oolong (Taiwan)	Ceylon (Sri Lanka)
Gyokuro (Japan)	Pu-erh (Cpaulhina)	Darjeeling (India)
Sencha (Japan)		Yunnan (China)
Gunpowder (China)		Keemun (China)
Longjing (China)		Sikkim (India)
Baozhong (China)		

Adapted from Duthie & Crozier, 2002

1.11.4 Herbal & Fruit teas

Fruit and herbal teas are not made of tea leaves but are an infusion of fruit and herbs. Fruit teas are a blend of ingredients with one or more predominant fruit flavours. Such teas may contain up to eight or nine different ingredients each with their own role to play in the flavour of the infusion. Fruit teas are noted for their refreshing and delicious taste and are a modern alternative to tea and coffee. Herb teas are the leaves, seeds and flowers of a particular herb, harvested and dried into a tea with a fresh clean taste. These teas are traditionally believed to have properties to treat specific medical conditions especially those which are related to stress or the digestive system (www. tea.co.uk). Examples of such teas include Camomile, renowned for its soothing and calming properties, Echinacea to maintain a healthy immune system, Ginseng for its stimulating and energising benefits and Peppermint renowned for its distinctive flavour and stimulating effect on the digestive system (Yamanishi, 1995).

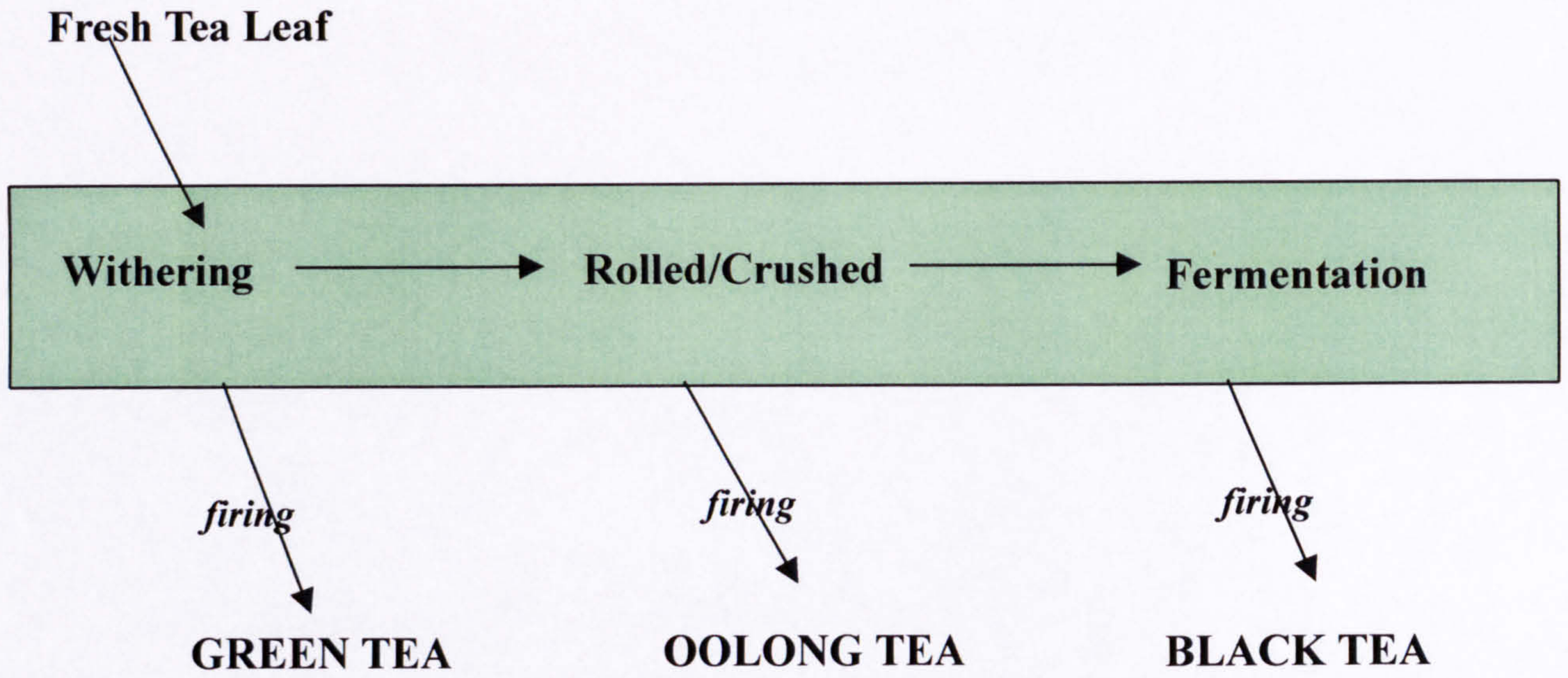


Figure 7 Tea manufacturing process (Adapted from Balentine *et al.,* 1997)

Fruit and herb teas are generally caffeine free, usually contain less than 5 calories per cup and have no added sugar and make a refreshing alternative to other hot beverages.

In addition there are a number of scented teas also available today such as, Earl Grey (black tea with oil of bergamot), Lady Grey (black tea with orange, lemon peel and oil of bergamot), Lapsang Souchong (black tea scented with smoke) and Jasmine (green tea scented with Jasmine flowers) (Duthie & Crozier, 2002).

1.11.5 Tea composition

On average British people drink about 3.5 cups of tea a day (National Drinks Survey, 1996), with it being stated that "increasing this average consumption by just one cup a day may yield significant health benefits on a population basis, not at least due because of its contribution to fluid intake".

Tea taken on its own has no calories however as most people take it with milk, tea is a valuable provider of the nutritional content of milk, which can account for up to 16% of the recommended daily amount of calcium and significant amounts of folic acid, riboflavin (B2) and vitamin B6 (pyridoxine) (Liao *et al.*, 2001) (Table 9).

Tea is also a rich source of manganese, essential for bone growth and the body's development, potassium which is vital for maintaining fluid levels in the body, zinc which is essential for growth and development and fluoride, 0.5 – 0.8 mg per day (Liao *et al.*, 2001) for protection against tooth decay and gum disease. The tea drunk today may also be less rich in micro-nutrients than in previous decades as tea infusion times have decreased dramatically from 5 – 6 mins to about 40 –60 seconds decreasing the overall nutrient content of the brew (Duthie & Crozier, 2002).

Table 9 Nutrients value of tea

Amount provided by 650mL of tea* with semi-skimmed milk

Parameter	Contribution
Energy	200 kJ (48 Kcal)
Protein	3.41 g
Carbohydrate	4.77 g
Fat	1.36 g
Minerals:	
Calcium	109 mg
Potassium	300 mg
Zinc	0.68 mg
Vitamins:	
Thiamine (B1)	70 mg
Riboflavin (B2)	270 mg
Vitamin B6	70 mg
Folate	20 mg

* Average daily consumption of tea is 3.43 cups/day (650 mL) (National Drinks Survey, 1996).

1.11.5.1 Caffeine content

Tea contains caffeine, a naturally occurring purine alkaloid (Duthie & Crozier, 2002). Apart from tea, caffeine can also be found in chocolate, soft drinks, coffee, cold remedies and pain relievers. Caffeine is a powerful stimulant which can increase concentration, accuracy, sensitivity to taste and smell and which overall benefits performance. Extensive studies have failed to show that caffeine, in moderation has any deleterious effects on blood cholesterol levels, the cardiovascular circulation or that it may give rise to the formation of cancers and heart disease (Liao *et al.*, 2001).

Table 10 displays the difference in caffeine content of a wide range of regularly consumed drinks. The amount of caffeine in a cup of tea depends on the plant variety and how long you let the tea brew infuse. On average a 200 mL cup of tea contains about 40 mg of caffeine, about half the amount

contained in an average cup of coffee (www.tea.co.uk) and which accounts for an average daily intake of 0.15 g of caffeine (Duthie & Crozier, 2002).

Table 10 Typical caffeine content of selected beverages

Beverage	**Caffeine content (per 200 mL cup)**
1 cup ground roasted coffee	115 mg
1 cup instant coffee	65 mg
1 can regular cola	50 mg
1 cup tea-leaf or bag	40 mg
1 cup instant tea	30 mg

(Adapted from www.tea.co.uk)

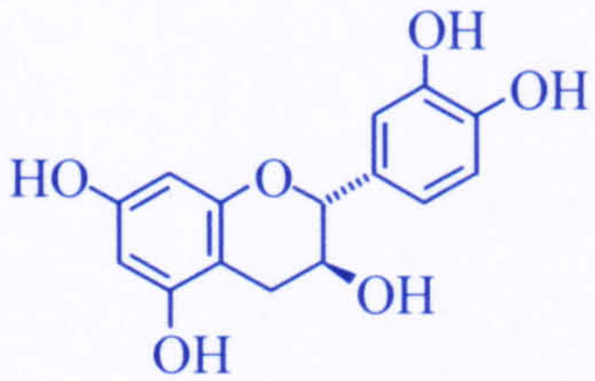
1.12 Chemical composition of tea

Green tea leaves are very unique in that they are very rich in catechins, caffeine and the amino acid, theanine. These constituents are soluble in hot water and impart flavour and taste to green tea beverages (Balentine *et al.*, 1997). As discussed before, the amounts of these compounds in tea beverages varies considerably depending on the manufacturing process, the strain of tea plant, climatic and geographical factors (Balentine *et al.*, 1997).

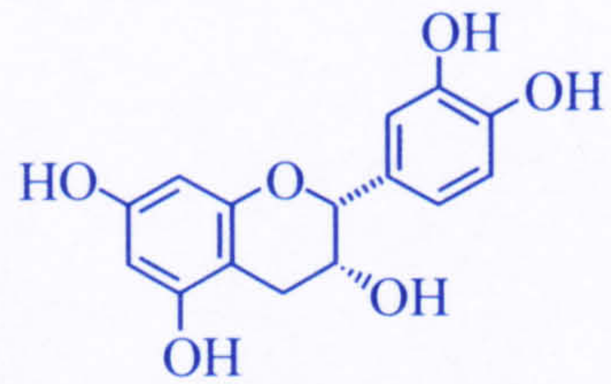
1.12.1 Catechins

Tea leaves contain in amounts equivalent to 30 – 40% of the overall dry weight of the tea (Balentine *et al.*, 1997), a range of polyphenols particularly the flavan-3-ols (+)-catechin and (-)-epicatechin (EC) and their gallate and galloyl derivatives; epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and catechin gallate (CG) (Figure 8). The type and proportion of catechins present in the tea leaf varies with the season,

Catechin monomers

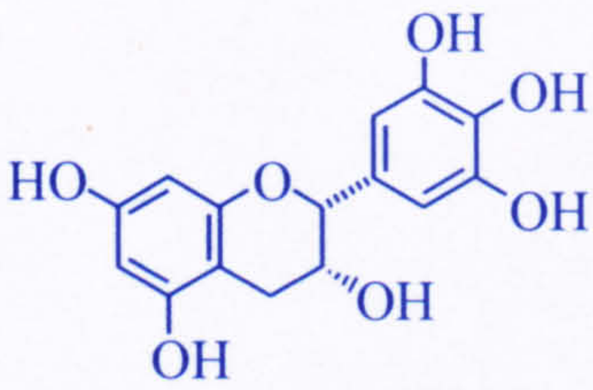


(+)-catechin

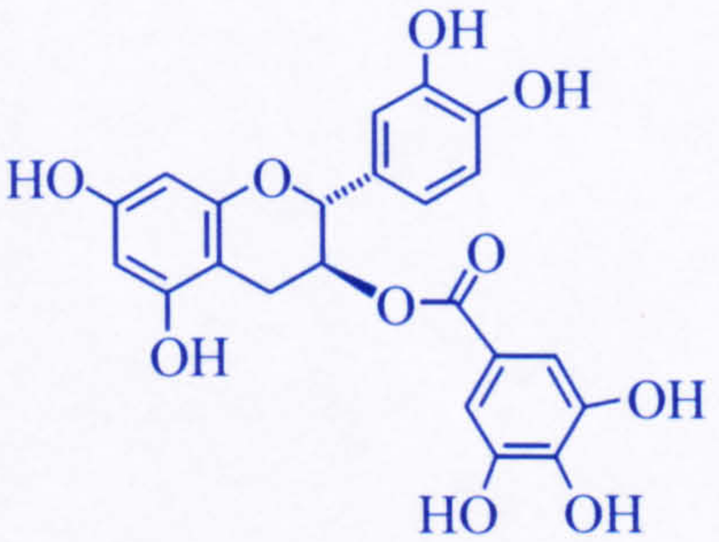


(-)-epicatechin (EC)

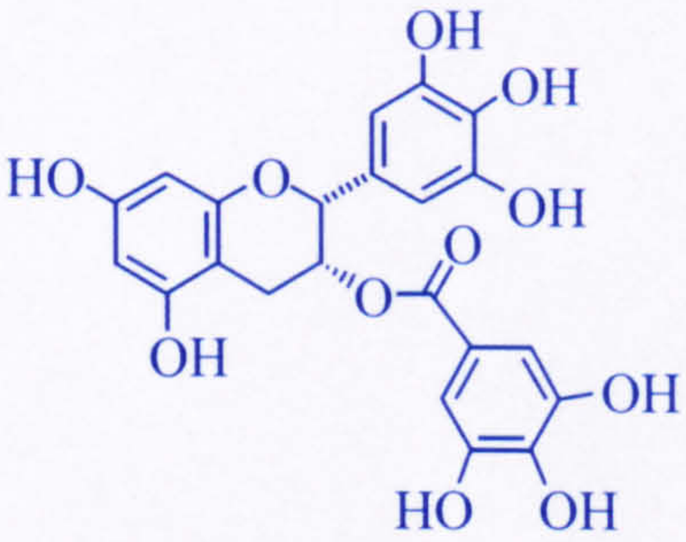
Gallocatechins and gallate esters



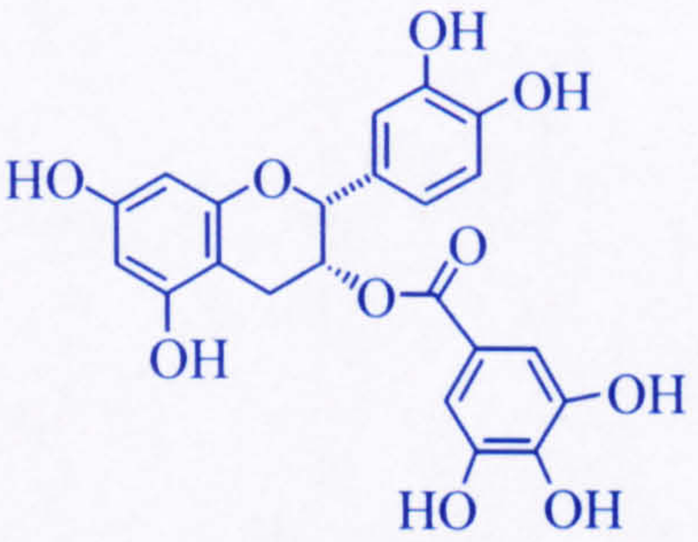
epigallocatechin (EGC)



catechin gallate (CG)



epigallocatechin gallate (EGCG)



epicatechin gallate (ECG)

Figure 8 Structure of the main catechins and epicatechins present in tea

climate and horticultural practices (Bokuchava & Skobeleva, 1980).

More than 80 to 90% of green tea polyphenols are catechins (Liao *et al.*, 2001). Catechins (3,3′,4′,5,7-pentahydroxyflavan) contain a hydroxypyranone ring system attached to a hydroxylated phenyl ring, the latter of which is referred to as the "B" ring (Balentine *et al.*, 1997). Two chemical configurations within the catechin family are possible, but the majority of the catechin mass is in the so-called "epi-catechin" form. When there is the presence of 3 tri-hydroxyl (OH) groups present on the B ring, compounds are known as gallo-catechins. The term "gallo" comes from its analogy to gallic acid,1,2,3 trihydroxy benzoic acid (Robb & Brown, 2001). The addition of a gallic acid group on the pyran "C" ring leads to compounds being known as catechin gallates. It is thought that gallocatechin gallate (GCG) and catechin gallate (CG) are not indigenous to the tea plant, but are the product of a epimerisation reaction with EGCG caused by the high temperatures of the firing process during manufacturing (Robb & Brown, 2001). EGCG is the most abundant catechin found in green tea and accounts for 25 to 40% of the total catechin content. In tea leaves, EC is synthesized from flavanone and acetyl-Co A, while gallic acid is synthesized from shikimic acid (Figure 1) (Liao *et al.*, 2001).

The biological function of catechins in tea plants is not clear and why the tea leaves accumulate high concentrations of catechins in their leaves is yet to be determined (Liao *et al.*, 2001). The occurrence of lower catechin levels in shaded leaves as compared to apical leaves may indicate that they may be used to protect the plant from UV damage and excessive sunlight. The bitter taste that catechins confer on the tea beverage may function as an anti-microbial protection and the antioxidant properties of catechins may also act as a deterrent and protect the tea bush against insects, infection and environmental damage (Cook & Samman, 1996).

1.12.2 Theaflavins

Catechins undergo many chemical reactions both in the processing of different tea types and in the preparation of the tea beverage itself.

During the fermentation process of the manufacturing of black tea, ungallated catechins undergo condensation reactions with gallated catechins (Balentine *at al.*, 1997). This reaction is initiated by polyphenol oxidase. The products of such reactions are called theaflavins (Table 11).

Table 11 Theaflavin reactants and products

Catechin reactant	Gallocatechin reactant	Theaflavin product
(-)-epicatechin	(-)-epigallocatechin	theaflavin
(-)-epicatechin	(-)-epigallocatechin gallate	theaflavin-3-gallate
(-)-epicatechin gallate	(-)-epigallocatechin	theaflavin-3′-gallate
(-)-epicatechin gallate	(-)-epigallocatechin gallate	theaflavin-3,3′-digallate

Adapted from Robb & Brown, 2001

Theaflavins, which are characterised by a benzotropolone ring system (Figure 9) contribute to the overall astringency and brightness of black tea (Robb & Brown, 2001). Common to black tea are 4 main theaflavins (Table 11, Figure 10) that vary in degree of gallation and several minor theaflavins, including isotheaflavin and neotheaflavin. The total theaflavin content of black tea leaves does not usually exceed 2% and can be as low as 0.3% (Balentine *et al.*, 1997). Theaflavins undergo further condensation to form undefined water-soluble thearubigens (Figure 9). During fermentation the fresh tea leaf catechins can be reduced by as much as 85%, only 10% of which are accounted for as theaflavins with the balance of catechins forming the indistinct thearubigens (Balentine *et al.*, 1997).

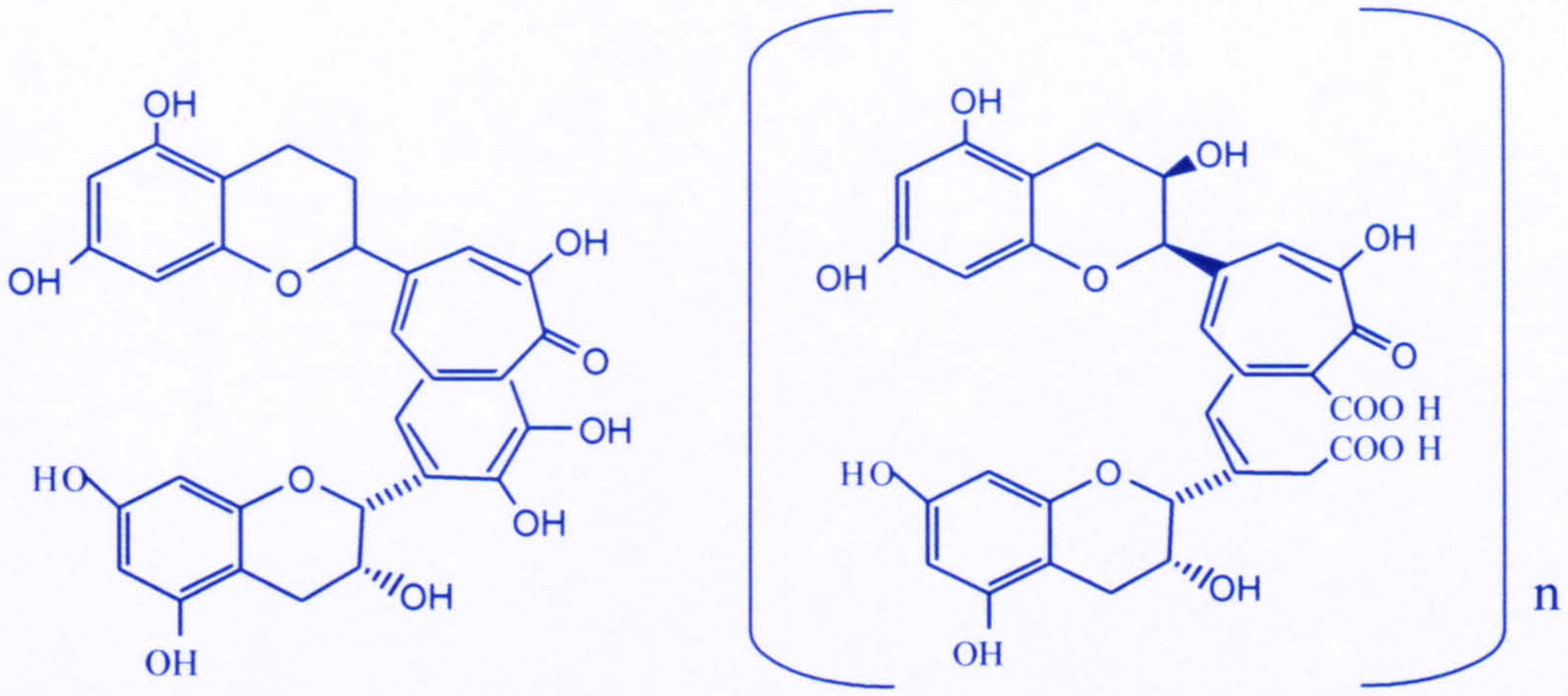
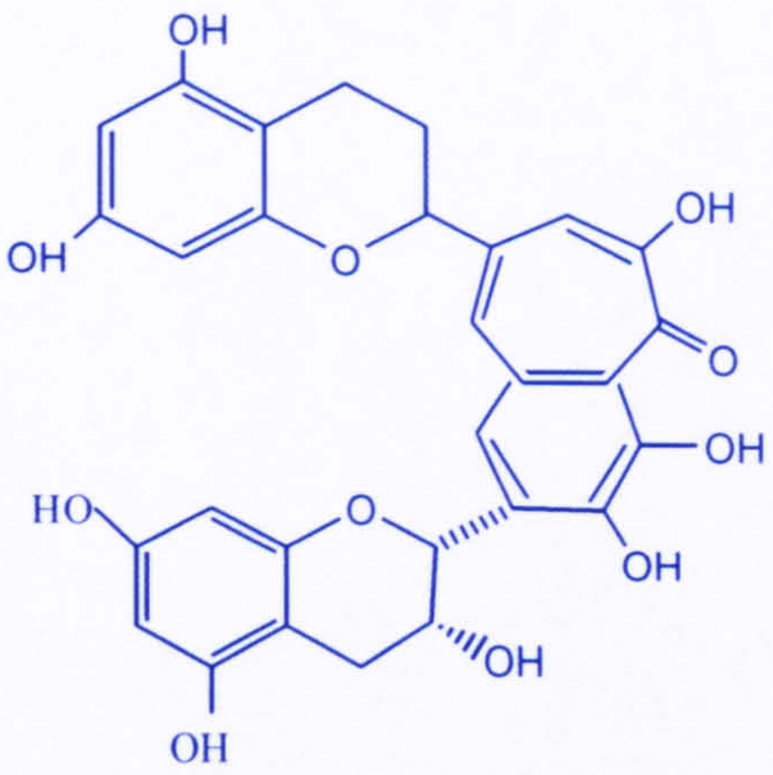


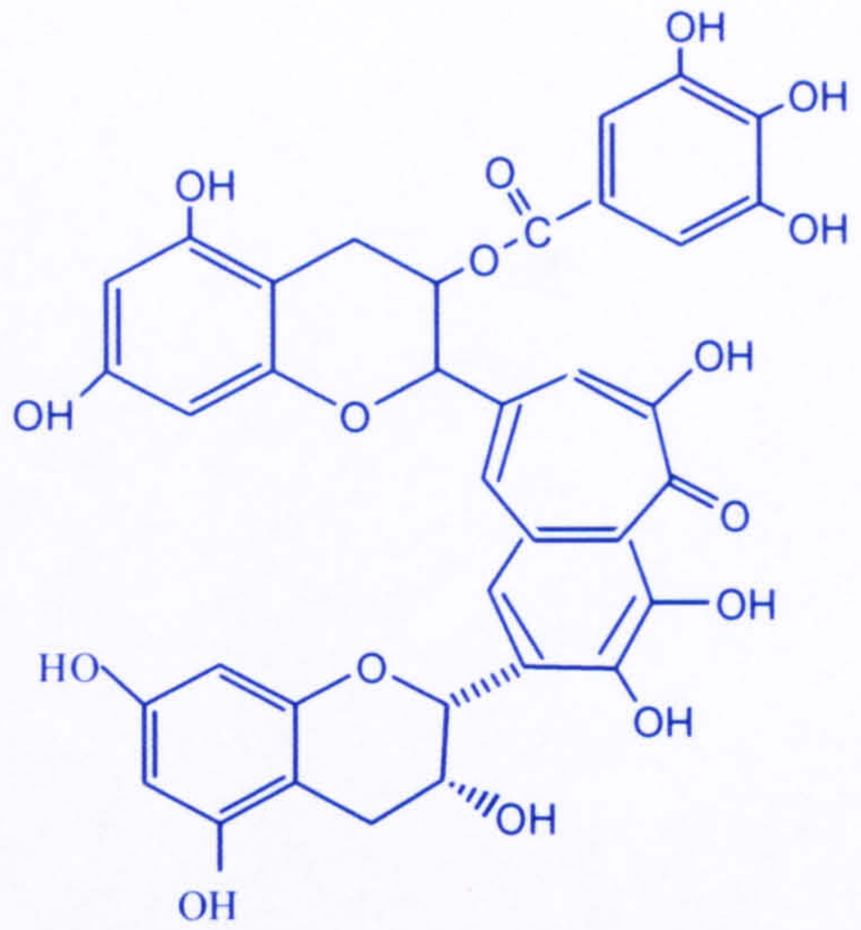
Figure 9 Structure of the theaflavins & thearubigens of black tea

1.12.3 Other components

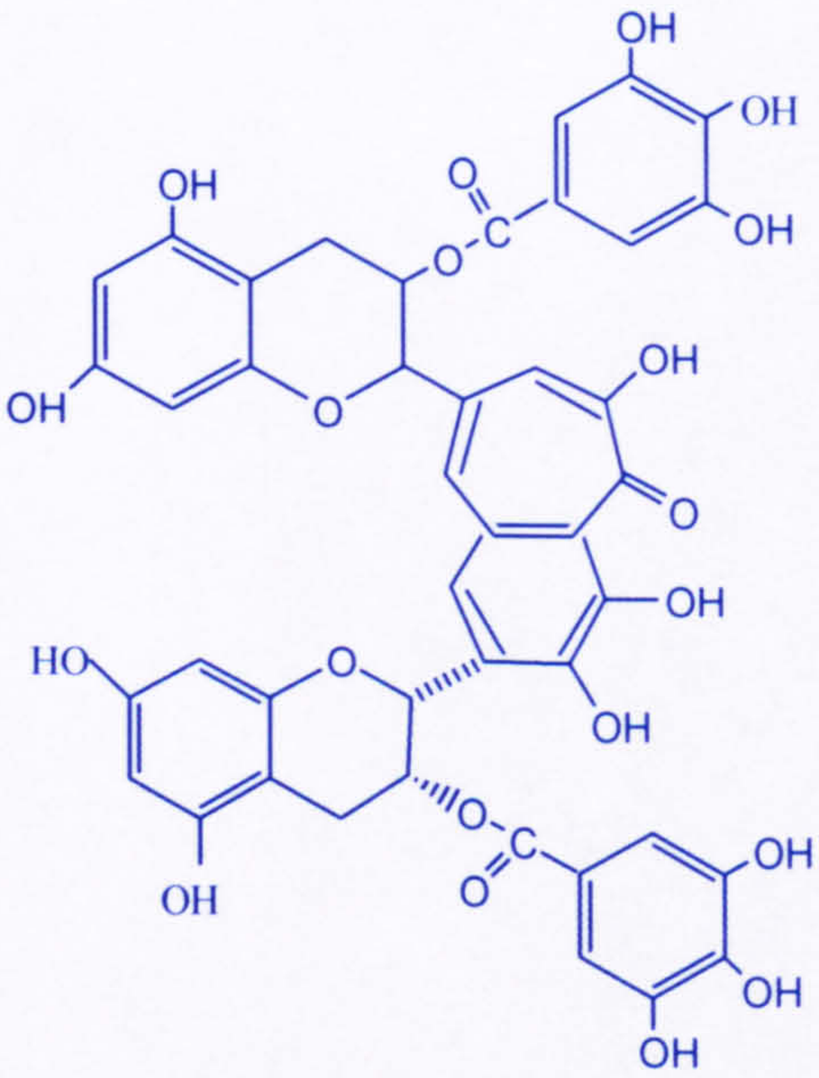
Tea also contains the flavonols, quercetin, kaempferol and rutin and the phenolic acids, caffeic, quinic and gallic acids (Dufresne & Fransworth, 2001). Both green and black tea infusions have been shown to contain conjugated quercetin, kaempferol and myricetin with the total flavonol content ranging from 21.0 to 32.8 mg/L (Table 2) (Crozier *et al.,* 2000). Tea is also a good source of methylxanthines primarily in the form of caffeine and the amino acid theanine, accounting for about 50% of the total amino acid content and which imparts a pleasantly sweet taste. Theanine is degraded to glutamic acid and has been shown to be able to reduce blood pressure and to have a relaxation effect in humans (Yokozawa *et al.,* 1998). The aromatic flavour of tea has been attributed to a number of alcohols (amyl alcohol, pentenols), theaspirane and hexenoate derivatives and sulphur compounds. Some of these compounds are present in green tea as glycosides, are known aroma precursors which are enzymatically hydrolysed during fermentation to alter tea aroma and flavour (Guo *et al.,* 1995). Green tea also has about 600 mg of vitamin C and 80 mg of vitamin E per 100 g of freshly dried leaves (Liao *et al.,* 2001).



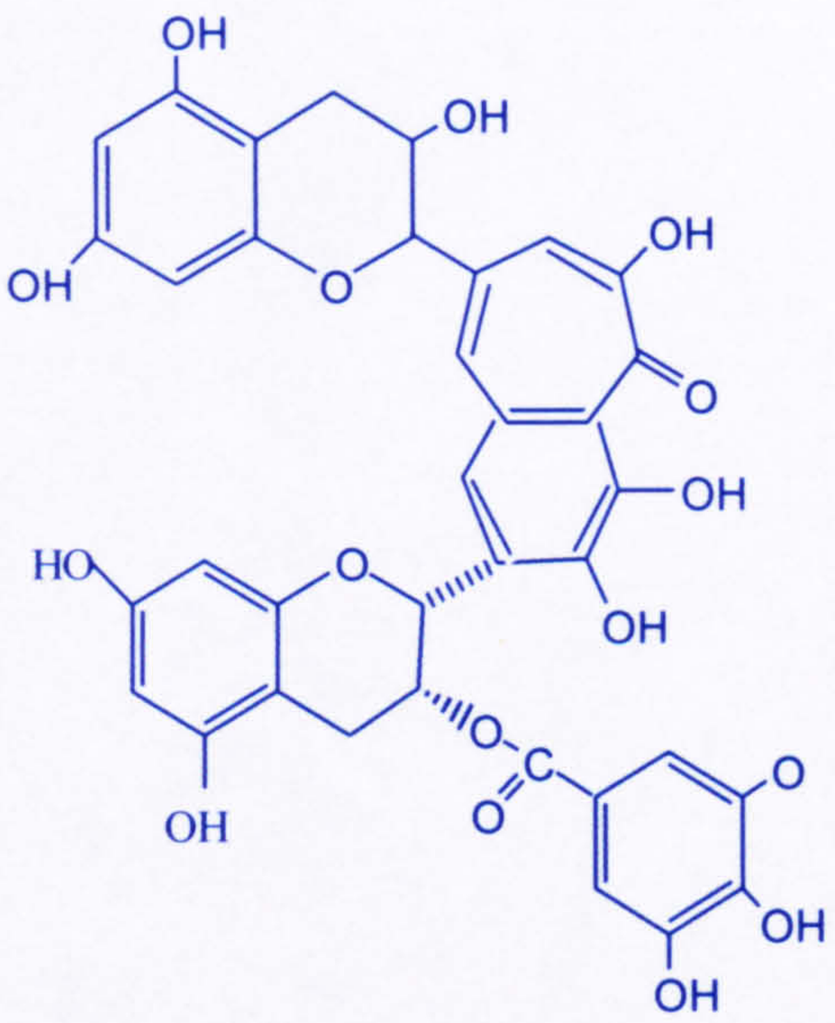
theaflavin



theaflavin-3-gallate



theaflavin-3,3'-digallate



theaflavin-3'-gallate

Figure 10 Theaflavins present in black tea

1.13 Physiological activity of tea catechins

1.13.1 Tea as an antioxidant

Oxidative damage by free oxygen radicals is known to be one of the prevalent mechanisms in the development of chronic diseases such as CHD and cancers. The mechanisms of action of antioxidants are discussed in detail in section 1.7.1.

Evaluation of the antioxidant potential of tea and tea catechins based on chemical and biological *in vitro* systems and redox potentials indicate that tea catechins are effective antioxidants and free radical scavengers and in some cases display up to 5 times the antioxidant activity of vitamin E and vitamin C (Wiseman *et al.*, 1997). Similar levels of antioxidant activity are reported for the theaflavins and the fact that the overall antioxidant capacity of infusions of black tea are similar to green tea suggest that thearubigens are equally as effective as antioxidants as catechins (Balentine *et al.*, 1997). A recent study has shown that the theaflavins present in black tea possess at least the same antioxidant potency as catechins present in green tea (Kwok *et al.*, 2001). The conversion of catechins to theaflavins during fermentation in the manufacturing of black tea does not appear to significantly alter their free radical scavenging ability (Kwok *et al.*, 2001).

The scavenging activity of different tea and tea catechin molecules is related to the number of *o*-dihydroxy and *o*-hydroxyketo groups, C_2 –C_3 double bonds, solubility, concentration, the accessibility of the active group to the oxidant and the stability of the reaction product (Wiseman *et al.*, 1997). The addition of glycosidic groups to the flavonoid molecule reduces the antioxidant activity. Structurally catechins therefore have the potential to donate electrons and subsequently delocalise and stabilise unpaired electrons. Gardner *et al* (1998) identified EGCG to be clearly the most effective antioxidant in several chemical systems followed by ECG. The gallic acid ester moiety at the 3-position on the C-ring of the catechin is an important

determinant of antioxidant potential as substitution of this group by hydroxyl in EGCG or ECG to form EGC or EC respectively, markedly decreases the overall antioxidant activity (Gardner *et al.*, 1998).

Studies dealing investigating the *in vivo* increase of antioxidant effects are limited at present and results conflicting. Green and black tea both represent an excellent source of antioxidant polyphenols, green tea being about five times more potent than black tea (Serafini *et al.*, 1996) with the antioxidant activity of tea being directly proportional to its total polyphenolic content. Green tea was found to be 20 – 25% more effective at radical quenching than black tea in both water and lipid soluble media (Gardner *et al.*, 1998). Total antioxidant capacity of plasma was seen to be significantly increased (7%) after 60 mins and 6.2% after 120 mins at baseline values following consumption of 300 mL of green tea and up to 12% following consumption of 450 mL of tea after 60 mins and 12.7% after 120 mins (Sung *et al.*, 2000). Total increase in plasma antioxidant concentration was tea dosage dependent. However Serafini *et al* (1996) reported that after consuming 300 mL of green tea total radical antioxidant capacity, using the TRAP assay, showed the highest increment of 40% at 30 mins after ingestion and decreased thereafter reaching baseline values at 80 mins following ingestion, different results to the previous study. Using the FRAP assay, Benzie *et al* (1999) reported a 4% increase in antioxidant power 40 mins after consuming 400 mL of green tea, returning to basal levels after 2 hours. Ingestion of 300 mL of red wine was seen to show a similar antioxidant effect to 450 mL of green tea (Whitehead *et al.*, 1995). The green tea epicatechins, EGCG, EGC and EC, the main polyphenols with an antioxidant effect, reported a maximum plasma concentration time of 1.5 to 2.5 hours after consumption (Yang *et al.*, 1998).

On the other hand, while catechins are effective antioxidants that act as radical scavengers and maybe able to protect cell components from free radical damage, these antioxidants can also be pro-oxidants under certain conditions and can generate hydroxyl radicals especially in the presence of

iron (Fe^{II} or Fe^{III}), silver (Ag^{I}) or copper (Cu^{II}) (Liao *et al.*, 2001). In the presence of these metal ions and under aerobic conditions, tea catechins (EC, EGC and EGCG) can generate radicals that can cleave DNA, chromatin and can accelerate the peroxidation of unsaturated fatty acids.

1.13.2 Tea & vasorelaxation

In addition to the proposed effects of moderate tea consumption on general health through its role as a potent antioxidant, a small number of animal studies from one group have shown that tea and tea catechins may also affect blood vessel function (Huang *et al.*, 1998, 1999). The role of vasorelaxation in the protection of the cardiovascular system is discussed in detail in section 1.7.4. The vasodilator effect and pathways of action of black tea have not been investigated to date.

Green tea epicatechins derivatives isolated and purified from jasmine green tea were seen to induced vasorelaxation in isolated rat mesenteric artery rings with intact endothelium following preconstricted with various pharmacological vasoconstrictors (Huang *et al.*, 1998) including phenylephrine (PE), glibenclamide, the ATP-sensitive K^+ channel blocker and charybdotoxin, the Ca^{2+}-activated K^+ channel blocker. EGCG was identified to be the most potent vasodilator with all epicatechins inducing vasorelaxation with similar potencies, in a concentration-dependent manner following preconstriction with PE. All epicatechins reduced the maximal contraction induced by PE. Relaxation induced by green tea epicatechins was unaffected by glibenclamide and charybdotoxin ruling out the involvement of ATP-sensitive and Ca^{2+}-activated K^+ channels in vasorelaxation induced by these compounds (Huang *et al.*, 1998). The vasodilator activity of green tea epicatechins occurred via endothelium-dependent and –independent mechanisms and has been attributed to the release of NO, the inhibition of calcium influx and activation on NO (Huang *et al.*, 1999).

Although evidence at present is scarce, these studies provide novel evidence for the possible mechanisms involved in the relaxation of vascular tissue by green tea epicatechin components.

1.13.3 Tea as an anti-carcinogen

Many mechanisms have been proposed concerning the inhibitory action of tea and tea catechins against carcinogenesis. The anti-carcinogenic properties of green tea polyphenols are likely a result of the inhibition of biochemical markers of tumour initiation and promotion, induction of apoptosis and inhibition of cell replication rates retarding the growth and development of tumours. Other main mechanisms include the antioxidative effect of tea catechins, the direct interaction between the reactive mutagens and the nucleophilic tea catechin and the inhibition of the bioactivation of promutagens (Liao *et al.*, 2001). Catechins can act at the level of cancer initiation by preventing chemically induced mutagenesis which is achieved by the inhibition of the mutagen bioactivation process or enhancement of the deactivation of reactive mutagens by enzymes like glutathione-*S*-transferase, glutathione peroxidase and catalase (Yang & Wang, 1993). Catechins can also influence the post-initiation process by suppressing cell proliferation (Liao *et al.*, 2001).

Many studies have demonstrated the inhibition of cancer activation by tea and tea polyphenols *in vitro*, but such activities have only been observed in a very small number of studies *in vivo*. The anti-proliferative effects of tea polyphenols have been demonstrated in the NNK-induced lung tumourgenesis model in mice. When green or black tea polyphenols were given to mice 24 hours after a dose of NNK, bronchiolar hyperproliferation was significantly inhibited (Fujiki *et al.*, 1992). Similarly, treatment with black tea also inhibited cell proliferation in NNK-induced tumours in mice (Yang *et al.*, 2000). Inhibition of cell transformations and cell growth by purified catechins and theaflavins have also been reported. These have been attributed to the inhibition of MAP-kinase activity and phosphorylation. In addition tea

polyphenols have been shown to inhibit the binding of epidermal growth factor to its receptor (Liao *et al.,* 2001). Purified tea polyphenols, especially EGCG can also inhibit apoptosis in cancer cells (Mukhtar & Ahmad, 2000) is able to arrest the cell cycle in human epidermal cancer cells and may be able inhibit the process of tumour formation by blocking cellular signal transduction pathways. Additional studies are needed to clarify the mechanisms of tea and tea polyphenols in cancer prevention.

1.14 Tea & coronary heart disease

When the complex nature of CHD is considered, tea and tea catechins may play a protective role via a number of different mechanisms, previously discussed working in synergy. The aetiology of cardiovascular disease is discussed in detail in section 1.8.1.

Although a small number of epidemiological studies have not provided clear-cut evidence for a link between tea consumption and cardiovascular disease (van het Hof *et al.,* 1997, 1999; Hollman *et al.,* 1999), the majority of studies have shown that tea consumption is associated with a lower risk of cardiovascular disease (Klatsky *et al.,* 1990; Stensvold *et al.,* 1992; Hertog *et al.,* 1993; Knekt *et al.,* 1996). However from such a wide range of studies it is not possible to establish whether the relationship between tea intake and disease risk is causal or chance.

Tea consumption is difficult to measure or estimate from questionnaires. Such methods employed to estimate total dietary intake and tea consumption are open to substantial potential error and bias and the effect of confounding factors. Methods employed in estimating tea consumption include various dietary assessment models including 24 hour dietary recall, completion of food diaries and self or investigator administered diet history frequency questionaires. Various factors may affect the recall of dietary information including subjects honesty, competence and memories. In terms of tea consumption and CHD various factors may affect exposure. These include

frequency of consumption, duration of tea leaf extraction on brewing, the addition of milk, type of tea and size of cup. Many questionnaires used are general dietary intake surveys and are not designed to specifically quantify tea consumption and do not investigate potential confounding factors such as social class or ethnic group.

A large-scale epidemiological study carried out in Norway, compared the cholesterol levels and blood pressures of 9,856 men and 10,233 women, without a history of cardiovascular disease or diabetes (Stensvold *et al.,* 1992). This study found that both factors were lower and inversely related to increasing tea consumption, when individuals were consuming less than one cup per day as compared to individuals consuming more than one cup or more than five cups per day (Stensvold *et al.,* 1992). Likewise in the recent prospective Rotterdam study of over 3,000 men and women aged over 55 years, a significant inverse relationship between tea intake and radiographically quantified atherosclerosis was identified (Geleinjnse *et al.,* 1999). The Boston Area Health Study of 340 subjects and age, sex and community matched controls also found that subjects who drank one or more cups of black tea a day had approximately half the risk of a heart attack as those who did not drink tea at all (Sesso *et al.,* 1999).

Welsh men, however in the Caerphilly Study had a positive association between black tea consumption and CHD (Hertog *et al.,* 1997) determined using a semi-quantitative food frequency questionaire. It was thought that the addition of milk to the tea may have however abolished the antioxidant potential of the tea, but subsequent human studies on this topic, have shown that the addition of milk to the tea does not affect plasma antioxidant level or urinary excretion of catechins (Serafini *et al.,* 1996, van het Hof *et al.,* 1999).

The effects on tea on the cardiovascular system have also been reported in experimental studies with animals. Green tea significantly reduces serum and liver cholesterol levels and liver weight by lowering lipid deposition in hypocholesterolemic diet-induced rats (Yokozawa *et al.,* 1998). Likewise rats

fed with 2.5% green tea leaves in the diet over a long period of time were found to have a reduction in blood triglycerides and total cholesterol content. Green and black tea fed at lower levels also improved plasma lipid profiles and reduced LDL and vLDL (very low-density lipoprotein) oxidation in hamsters fed a normal of high cholesterol diet (Vinson *et al.*, 1998).

Oxidation of LDL is recognised as an important step leading to atherosclerosis and there are several reports indicating that tea and tea catechins inhibit the oxidation of LDL *in vitro* (Wiseman *et al.*, 1997). Studies testing the antioxidant effect of tea polyphenols on LDL and vLDL oxidation all indicate that EGCG is the most potent inhibitor of lipid peroxidation and has a lipoprotein bound antioxidant activity greater than that of vitamin C, tocopherol and β-carotene (Vinson *et al.*, 1995). Black tea extract has also been shown to increase the resistance of LDL to oxidation in a concentration dependent manner. Green tea catechins, have also been shown to suppress of inhibit the proliferation of smooth muscle cells in bovine aortic vessels which produce connective tissue leading to luminal narrowing and atherosclerosis (Yokozawa *et al.*, 1995). Similarly in human aortic vessels, tea extracts, catechin and epicatechin all exhibit a dose-dependent inhibition of the formation of early lipid peroxidation products (Pearson *et al.*, 1998). Such effects may be due to the direct scavenging of the oxidising species by the polyphenols or may results from the polyphenol-mediated regeneration or protection of vitamin E in LDL.

Consumption of tea and tea catechins may also have other possible beneficial effects in the prevention of CHD by other mechanisms aside from its antioxidant capabilities. The vasodilatory effects as discussed previously, of tea extracts and epicatechins, may enhance optimal blood flow by affecting the enhancement of NO release from endothelial cells, the accumulation of cyclic 3′,5′-guanosine monophosphate (cGMP) and inhibition of calcium influx into the smooth muscle cell (Huang *et al.*, 1999). At present the suggested vasodilatory effects of tea catechins in protecting vascular function is not well documented. Tea extracts have also been shown to prevent platelet

adhesion and aggregation by inhibiting the cyclo-oxygenase pathway and reducing cyclic 3′,5′-adenosine monophosphate (cAMP) response of platelets to prostaglandin I_2 (Gryglewski *et al.*, 1987, Tijburg *et al.*, 1997). Caffeine in tea may also reduce blood coagulation by inhibiting thrombin-stimulated thromboxane formation (Duthie & Crozier, 2002).

1.14.1 Tea & cancer

Initiation, promotion and progression in cancer development are modulated by many factors relating to the diet, metabolism and environmental factors (Dufresne & Farnworth, 2001). Accumulation of reactive oxygen species (ROS) in cells and resultant changes and modifications in DNA structure, enzymatic activity and cell defence mechanisms all influence the development of cancer pathogenesis (Yang *et al.*, 2000).

The experimental and epidemiological data from a wide range of studies have provided evidence that polyphenolic antioxidants present in green tea may be able to confer protection against cancer initiation and its subsequent progression and development (reviews by Kohlmeier *et al.*, 1997; Bushman, 1998). The majority of studies demonstrating cancer chemo-preventative effects have been conducted using green tea, where as only a limited number have assessed the effects of black tea.

In a study in China, green tea drinkers who consumed more than two cups of tea per day were found to have the risk of esophageal cancer reduced by 50% (Gao *et al.*, 1994) especially in non-smokers and non-alcohol drinkers as assessed by food frequency and lifestyle questionaires. Another large study, also from China, which controlled for confounding factors found that green tea drinkers had a 40-50% reduction in stomach cancer incidence (Yu *et al.*, 1995). Similarly studies from northern Italy have suggested a protective effect of tea against oral, pharyngeal and laryngeal cancers. Japanese women consuming more than 10 cups of tea daily have been shown to have a lower risk for cancer, at all sites, with increased tea consumption being associated

with a lower risk of breast cancer development and recurrence (Nakachi *et al.*, 1998). Likewise, a prospective cohort study of postmenopausal women in Iowa, USA, consumption of black tea has been shown to be associated with a lower risk of digestive and urinary tract cancers (Zheng *et al.*, 1996). Data was collected from validated food questionnaires and diaries. In contrast, in the Netherlands Cohort study on diet and cancer, consumption of black tea was not found to affect the risk of developing stomach, colorectal, lung and breast cancers (Goldbohm *et al.*, 1996). Similarly, a recent study carried out in middle-aged Finnish men indicated a positive association between increased tea consumption and colon cancer risk (Hartman *et al.*, 1998).

However in contrast to the epidemiological studies, animal studies have demonstrated the inhibition of carcinogenesis by tea and tea catechins. Studies have shown that oral consumption or topical application of green tea and/or its polyphenolic components provide protection against chemically-induced or ultraviolet radiation-induced skin carcinogenesis in the mouse model (Mukhtar *et al.*, 1994). In other animal model systems, the polyphenols isolated from green tea have been shown to provide protection against chemically-induced carcinogenesis in lung, fore-stomach, pancreas, breast, colon and oesophagus (Kohlmeier *et al.*, 1997). Recent studies have also suggested that much of the chemo-preventive effects of green tea are mediated by its major polyphenolic constituent, EGCG. Although it is important to note that the other polyphenolics present in green tea also contribute to this effect (Liao *et al.*, 2001).

There are currently a number of different mechanisms suggested by which tea and tea catechins may influence the three stages of cancer development. These include the direct and indirect antioxidant protection of DNA by neutralising the pro-carcinogen by their strong oxygen scavenging activity before cell injury occurs (Vinson *et al.*, 1995) by the modulation of enzyme systems, such as cytochrome P450 complexes which metabolise carcinogens or pro-carcinogens to genotoxins and by the modulation of malignant transformations, apoptosis and gene expression (Duthie *et al.*,

2000). Phenolics from green tea can also prevent the *in vitro* formation of nitrosamines, a group of carcinogens also found in tobacco (Gordon, 1996). Tea polyphenols can also inhibit the formation of heterocyclic amines from cooked meat and fish which are genotoxic carcinogens and are associated with cancer of the breast, colon and pancreas (Liao *et al.*, 2001).

1.15 Absorption & metabolism of tea catechins

Knowledge of the absorption and metabolism of catechins are crucial to the understanding of whether these compounds or their metabolites have the potential to exert the biological activity *in vivo*.

The bioavailability of polyphenolic compounds and their distribution throughout the body following administration, either orally or locally, have been investigated using sensitive methods to analyse the metabolites in biological fluids and tissues. Consumption of a single dose of green tea results in a small but significant increase in plasma concentrations of total catechins (van het Hof *et al.*, 1999) peaking at about 2 hours following consumption. The effect is less for black tea. This may be due to the presence of the high molecular weigh theaflavins and thearubigens in black tea (van het Hof *et al.*, 1999). As stated before, the addition of milk, at the typical UK level of 10-15% volume does not appear to affect the absorption of catechins from tea. Much larger amounts of milk may impair absorption but evidence is limited (Serafini *et al.*, 1996). Likewise, almost 50% of orally administered radio-labelled catechin was excreted in the form of urinary metabolites, indicating that catechins and their gut flora metabolites are well-absorbed (Das *et al.*, 1971) with plasma concentrations of catechins peaking between 1.5 to 2.6 hours on all subjects and declining to undetectable levels after 24 hours. Although the concentrations of catechins in the plasma are low, the total amount of catechins present in the total blood volume at any time is only a minute proportion of the total amount consumed (van het Hof *et al.*, 1998). Tea catechins appear in the urine soon after consumption, but the total amount

excreted in the urine again only represents a very small proportion of the total present in a cup of tea (He & Kies, 1994).

The tea epicatechins, EGCG, EGC, ECG and EC have all been identified in the portal vein of rats following the oral administration of a single dose of isolated green tea catechins indicating that these compounds can cross intestinal membranes (Hollman *et al.*, 1997). The limited number of pharmacokinetics studies on tea catechins suggest that they are rapidly absorbed with the bioavailability of EGCG being lower than that of EGC and EC (Hollman *et al.*, 1997).

Absorption of tea catechins, based on the current evidence appears to be limited and inconclusive. Catechins maybe highly absorbed but rapidly metabolised to other forms which cannot yet been detected or possibly sequestred in specific organs of the body (He & Kies, 1994). Absorbed catechins have been shown to be extensively metabolised and converted to glucuronides and sulphates predominately in the liver, with some being methylated by catechol-*O*-methyl transferase (Hollman *et al.*, 1997). Unabsorbed catechins and catechins conjugates are secreted with bile into the small intestine and are degraded by bacteria in the colon, as previously mentioned. At present there is no published data concerning the absorption of theaflavins and thearubigens from black tea.

In order to fully understand the bioavailability of tea catechins, more human studies are needed that focus on the identification and quantification of metabolites in body fluids.

Chapter 2 Materials and Methods

To investigate the activity and various properties of polyphenolic compounds *in vitro*, several complementary methods were employed in the course of this study.

2.1 Vasorelaxation of vascular tissue by polyphenolic compounds

2.1.1 Rabbit aortic preparation

Male New Zealand rabbits (3.5 Kg) were anaesthetised by i.v. administration of pentobarbitone (100 mg kg^{-1}) with 1000 IU heparin into the marginal ear vein. All rabbits used in the course of this study were obtained from Harlan UK, Ltd, a credited commercial supplier of laboratory animals. Once the animal was dead the descending thoracic aorta was then carefully removed and cleaned of adhering fat and connective tissue and cut into transverse ring segments of about 3-5 mm in length. The rings were suspended from force displacement transducers in 10 mL organ baths (Figure 11) filled with Kreb's buffer solution (pH 7.4) [composition in mmol: NaCl 118.4; $NaHCO_3$ 25; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 2.5; D-glucose 11], continuously oxygenated with 16% O_2, 5% CO_2 and 79% N_2, and maintained at 37°C, to mimic the internal environment as closely as possible. Aortic ring segments were set-up in the organ baths within 20 mins of the sacrifice of the animal. Rings were placed under the optimal resting tension of 2 g (oxygen tension of 120 mm Hg). This tension was kept constant throughout the course of all experiments. Vessel contraction/relaxation was measured via an isometric force transducer, linked in turn to a data handling system (Chart V3.5, MacLab Data Acquisition System, Version 8E, AD Instruments Pty Ltd, Australia) to record all changes in vascular tension.

2.1.2 Experimental Protocol

After an equilibration period of 45 min, vessels were maximally contracted twice with KCl (50 mM). After each contraction, vessels were washed out with Kreb's solution. In some experiments, the endothelium was mechanically removed by gently rubbing the luminal surface with ridged forceps. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (1 μM) to induce more than 50% relaxation in rings pre-contracted with phenylephrine (1 μM). Vessels were considered to be denuded of functional endothelium when there was no relaxation response to acetylcholine.

2.2 Determination of the grape and tea extract vasodilator effects in vitro

After washing and returning to initial baseline tension, aortic rings with and without functional endothelium were precontracted submaximally with phenylephrine (PE, 10^{-7} M). In some experiments where indicated, the thromboxane-mimetic U46619 (10^{-9} M) [G, 11-dideoxy-11A-GA-epoxy-methano-PGF_{2A}] was used to elevate vascular tone. After precontraction of the aortic rings the effect of the grape skin and tea extracts on vascular tone were studied. Once a stable plateau had been reached, cumulative concentration response curves (CCRC) for the diluted grape skin, powder and seed extracts, green and black tea extracts and theaflavin extract (2×10^{-4} – 14 mg mL^{-1}; 0.0002 - 14 mg mL^{-1}) were obtained. Dilutions of increasing concentration were added directly to the organ bath at 15 mins intervals for each individual extract. Time matched controls for each extract were run to demonstrate the effect of each extract on vascular tension as opposed to the natural relaxation response of each vessel with time.

The mechanisms and pathways of vasodilation in the grape and tea based extracts *in vitro* was also investigated. In some vessels, the NO synthase

inhibitor, L-NAME (100 μM) or the prostaglandin cyclo-oxygenase inhibitor, indomethacin (1, 100 μM) was added 40 min prior to precontraction with PE and the addition of the extracts. In some experiments, where indicated, the thromboxane antagonists ICI 192,605 (10 μM), 1 μM GR 32191 or the soluble guanylate cyclase inhibitor ODQ (1H-[1,2,4] Oxadiazolo [4,3-a]quinoxalin-1-one, 10 μM) were added 15 mins prior to precontraction with PE. To rule out the presence of prostanoid receptors in the rabbit aortic smooth muscle, CCRC's were obtained to prostacyclin (PGI_2, 10^{-10} – $3x10^{-7}$ M) and the selective prostacyclin receptor agonist, cicaprost (10^{-12} – $3x10^{-8}$ M). The combined effects of endothelium removal, and incubation with L-NAME, indomethacin and ICI 192,605 were also investigated.

Following preconstriction with PE, the absolute preconstriction of the vessel was measured. With each addition/dose of each individual extract to the organ bath the ensuing vasorelaxation was measured and expressed as a % of the degree of preconstriction with PE. This was repeated in at least 6 vessels and the % relaxation for each dose calculated and expressed as the % vasorelaxation induced by each grape and tea based extract following precontraction with PE. Data shown as mean % relaxation ± standard error of mean (n = 6-7) for the grape and tea based extracts and as mean % relaxation ± standard error of mean (n=3) for the theaflavin extract.

2.3 Determination of green tea catechins & epicatechins vasodilator effects in vitro

After washing and returning to starting baseline tension, aortic rings with and without functional endothelium were submaximally precontracted with PE (10^{-7} M). After precontraction of the aortic rings the effects of the catechin derivatives: epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), catechin and catechin gallate (CG) on vascular tension *in vitro* were studied. Once a stable plateau had been reached and was maintained, CCRC to the diluted catechin

derivatives (10^{-8} – $3x10^{-4}$ M) were obtained. Catechin and epicatechin dilutions of increasing concentration were added directly to the organ bath at 15 mins intervals. Time matched controls for each derivative were also run to demonstrate the effect of each derivative on vascular tension as opposed to the natural relaxation response of each vessel with time.

To investigate the role of nitric oxide in the vasodilator effect induced by the catechin derivatives, L-NAME (100 μM), was added to selected vessels, 40 mins prior to precontraction with PE and the addition of the diluted catechin and epicatechin derivatives.

Following preconstriction with PE, the absolute preconstriction of the vessel was measured. With each addition/dose of each catechin and epicatechin derivative to the organ bath the ensuing vasorelaxation was measured and expressed as a % of the degree of preconstriction with PE. This was repeated in at least 5 vessels and the % relaxation for each dose calculated and expressed as the % vasorelaxation induced by each green tea catechin and epicatechin derivative following precontraction with PE. Data shown as mean % relaxation ± standard error of mean (n = 5-6).

2.4 Determination of green tea fractions vasodilator effects in vitro

After washing and returning to starting baseline tension, aortic rings with functional endothelium were submaximally precontracted with PE (10^{-7} M). Following precontraction of the aortic rings the effects of the green tea fractions on vascular tension *in vitro* were studied. Once a stable plateau had been reached and was maintained, 20 μg mL^{-1} (twice 10 μg mL^{-1} aliquots) of each green tea fraction concentrate was added to the organ bath at 15 minute intervals to induce vasodilation in the aortic ring segments. Time matched controls for each fraction were also run to demonstrate the effect of each

fraction on vascular tension as opposed to the natural relaxation response of each vessel with time

Following preconstriction with PE, the absolute preconstriction of the vessel was measured. With each addition/dose of each green tea fraction to the organ bath the ensuing vasorelaxation was measured and expressed as a % of the degree of preconstriction with PE. This was repeated in 2 vessels and the % relaxation for each fraction addition calculated and expressed as the % vasorelaxation induced by each green tea fraction following precontraction with PE. Data shown as mean % relaxation for 2 vessels ($n = 2$).

2.5 Drugs and Solutions: vasodilation method

Acetylcholine (ACh), phenylephrine (PE), L-NAME, indomethacin and U46619 were purchased from Sigma-Aldrich Chemical Co. (Dorset, England). ICI 192, 605, and ODQ was purchased from Tocris Cookson Ltd (Bristol, England). All drugs were dissolved in distilled water, with the exception of indomethacin, which was dissolved in 0.5 M $NaHCO_3$, U46619 which was dissolved in absolute ethanol and ICI 192,605 which was dissolved in DMSO (dimethyl sulfoxide). The French grape skin and French grape seed extracts were obtained from Prof. P. L. Teissedre, University of Montpellier, France (acquired from E. Fesquet & N. Urban, La Gardonnenqye, 30 360 Cruviers-Lascours, France), and the grape powder, grape skin, green tea and black tea and theaflavin extracts were obtained from Dr G.G. Duthie and Dr P.T. Gardner, Rowett Research Institute, Aberdeen, Scotland (acquired from Unilever, Wageningen, Netherlands). All grape and tea based and theaflavin extract used in this study were received in the dry powdered form. 1:10 dilutions of an initial 30 mg of extract was fully dissolved in Kreb's solution and dilutions freshly prepared for each experiment. All catechins and epicatechin compounds used in this study (EC, ECG, EGC, EGCG, Catechin and CG) were obtained from Sigma-Aldrich Chemical Co. (Dorset, England), and were prepared in distilled water.

The chemical composition of selected grape and tea based extracts (g 100 g^{-1}) received from Dr G.G. Duthie and Dr P.T. Gardner, Rowett Research Institute, Aberdeen, Scotland (acquired from Unilever, Wageningen, Netherlands) was determined by HPLC and is shown in Tables 12 to 14. Chemical composition analysis of the extracts by HPLC was carried out by Thomas J Lipton Inc. (London, UK).

2.6 Fractionation of green tea & black tea

Samples of green and black tea were fractionated with the aim of isolating and identifying the zone of antioxidant and vasodilatory activity and to determine the phenolics, specifically catechins, present.

2.6.1 Pre-fractionation tea preparation

An initial amount of 1g of Tetley's (Dr. J. Leah, The Tetley Group, 325/347, Oldfield Lane North, Greenford, Middesex, UB6 0AZ) coarse grain green tea (code B-50048) and dry medium ground black tea (code U-1704) was brewed in 100 mL of boiling water for 10 mins to allow the full infusion of the tea leaves into solution. The brewed tea was then allowed to cool at room temperature for 30 mins before being filtered and centrifuged to separate any particles from the tea solution and stored at -80°C for further analysis.

2.6.2 Fractionation of green tea

A preparative HPLC system was used for a large-scale fractionation based on differences in polarity of the various green tea catechins. The system comprised of a Rheodyne injector model 3725 with a 10 mL injector loop (HPLC technology, Herts, UK), Capital 25 cm x 30 mm i.d. 10 μm ODS-H-optimal HPLC column (Capital HPLC, Broxburn, UK), two LC-10A pumps, a CTO-6A column oven set at 40°C, a Shimadzu SPD 10Avp absorbance

Component	Grape skin extract ($g/100g^{-1}$)	Grape powder extract ($g/100g^{-1}$)
Gallic acid	1.540	1.929
Caffeic acid	1.842	2.727
Ferulic acid	0.192	1.428
Catechin	6.701	15.080
Epicatechin	4.089	2.583
Quercetin	1.421	1.939
Rutin	0.179	2.546
Myricetin	0.779	2.991
Cyanidin chloride	0.765	1.048
Malvidin chloride	0.128	1.504
Resveratrol	0.232	18.450
TOTAL	**17.868**	**52.125**

Table 12 Chemical composition of grape skin and grape powder extracts as determined by HPLC.

Data from Thomas J Lipton Ltd (London, UK)

Component	Green tea extract (g/100g^{-1})	Black tea extract (g/100g^{-1})
(+)-Catechin	1.85	1.70
(+)-Gallocatechin	0.80	2.40
(+)-Gallocatechin gallate	1.09	-
(-)-Epicatechin	5.57	9.80
(-)-Epigallocatechin	1.58	8.60
(-)-Epicatechin gallate	10.61	13.20
(-)-Epigallocatechin gallate	17.52	43.70
Theaflavins	12.93	**See Table 14 below**
Flavonols	1.64	n.s.
Gallic acid	4.53	n.s.
N-containing compounds	0.90	0.80
TOTAL	**59.02**	**80.20**

Table 13 Chemical composition of green and black tea extracts as determined by HPLC.

n.s = not specified. Data from Thomas J Lipton Ltd (London, UK)

Theaflavin component (%)		
Theaflavin	-	21.4
Theaflavin 3-gallate	-	29.9
Theaflavin 3'-gallate	-	15.2
Theaflavin 3,3'-digallate	-	27.5
Unknown	-	5.9

Table 14 Chemical composition of black tea theaflavin extract as determined by HPLC (g/100^{-1}g).

Data from Thomas J Lipton Ltd (London, UK)

(Glasgow, UK) 2700 data handling system and a Gilson FC 203 fraction collector (Gilson Medical Electronics Inc., USA) (Figure 12). Samples were eluted by a 7 – 30% gradient of acetonitrile in 2% formic acid and held at 30% for 5 mins. Operating at 20 mL min^{-1}, fifty successive fractions were collected every 30 seconds from an injection of 10 mL of green tea, with each fraction containing 10 mL of eluent. The first 6 mins of eluent was discarded accounting for the dead volume of the column. Four identical 10 mL injection system runs were carried out, with a total pool of 40 mL of each fraction being collected overall. All fractions were stored at -80°C.

2.6.3 Fractionation of black tea

A preparative HPLC system was used for a large-scale fractionation based on differences in polarity of the various tea catechins present in black tea (Figure 12). The system comprised of a Rheodyne injector model 3725 with a 10 mL injector loop (HPLC technology, Herts, UK), two LC-10A pumps, a CTO-6A column oven set at 40°C, a Shimadzu SPD 10Avp absorbance detector set at 280 – 365 nm (Shimadzu, Kyoto, Japan), linked to a Reeve analytical (Glasgow, UK) 2700 data handling system and a Gilson FC 203 fraction collector (Gilson Medical Electronics Inc., USA). Samples were run using a Capital 15 cm x 20 mm i.d. ODS-H optimal column (Capital HPLC, Broxburn, UK), over a 5 – 15 % gradient of acetonitrile in 2% formic acid over the initial 20 minutes, then 15 – 35 % over the remaining 20 – 30 minutes. The first 6 min of eluent was discarded accounting for the dead volume of the column. Operating at 10 mL/min, sixty successive fractions were collected every 30 seconds from an injection of 10 mL of black tea, with each fraction containing 5 mL of eluent. All fractions were stored at -80°C.

2.6.4 Mass Spectroscopy analysis of green & black tea fractions

The phenolic profile of selected green and black tea fractions exhibiting high antioxidant activity was analysed by high performance liquid chromatography – mass spectroscopy – mass spectroscopy (HPLC-MS-MS., Thermoquest, San Jose, California). A LCQ Duo Instrument MS (Thermoquest, UK) comprising a TSP AS 3000 series auto-sampler and a TSP P4000 quaternary pump. Reversed phase separations were carried out at 40°C using a 250 x 4.6 mm i.d. MAX-RP 80A HPLC column (Phenomemex, Macclesfield, UK). The mobile phase was a 5 – 30% linear gradient over 60 mins of acetonitrile in 1% formic acid eluted at a flow rate of 1 mL min^{-1}. Short isocratic runs (75% B) were used to analyse the preparative HPLC tea fractions. In all cases, 100 μL of each selected fraction was analysed in both positive and negative ion mode (scanning from 100.0 – 2000.0 amu), using data dependent MS/MS. Column eluent was directed to a UV-vis 6000 photodiode array detector operating at wavelengths 250 - 700 nm.

Spectral and colorimetric methods

Throughout the course of this study, the use of colorimetric and spectral assays have played an important role in determining the antioxidant capacity and presence of total phenolics and catechins in various samples. These assays have been used in several different sections throughout the course of this study, enabling us to highlight individual features of individual samples, or allow comparison between samples in each study, in relation to their activity, chemical composition and content.

2.7 Spectral Assays

2.7.1 Electron Spin Resonance: a measurement of antioxidant capacity

The ability of phenolic compounds to reduce radicals in the aqueous phase was estimated by their ability to reduce the Fremy's salt (potassium nitrosodisulphonate) radical, determined by adding equal volumes of the Fremy's free radical and the compound of interest. The ESR spectra of the low-field resonance of the Fremy's radical was obtained 6 mins after the addition of the mixture by which time the reaction was complete. Signal intensity was obtained by double integration and the concentration calculated by comparison with a control reaction with distilled water (Gardner *et al.*, 1998).

Spectra were obtained at 21°C on a Bruker ECS 106 spectrometer operating at *ca* 9.5 GHz (X-band frequency) and equipped with a cylindrical (TM110 mode) cavity. A microwave power of 2 mW and a modulation amplitude of 0.01 mT were used.

2.7.1.1 ESR Analysis: grape and tea extracts

The ability of the grape and tea extracts and to reduce radicals in the aqueous phase were estimated by adding 3 mL (0.0025% solution) of the extract to an equal volume of a 1 mM solution of Fremy's free radical salt (potassium nitrosodisulphonate) in an ethanol/water mix (12:88 v/v), operating on the system described above (Section 2.7.1). All samples were run in duplicate, standardised using Trolox. Results expressed as the mean number of radicals reduced/L.

2.7.1.2 ESR Analysis: green tea fractions

The ability of the green tea fractions to reduce radicals in the aqueous phase was estimated by adding 3mL (0.0025% solution) of a fraction to an equal volume of a 50 μM solution of Fremy's free radical salt (potassium nitrosodisulphonate) in an ethanol/water mix (12:88 v/v), operating on the system described above (Section 2.7.1). All samples were run in duplicate, standardised using Trolox, and are expressed as the mean number of radicals reduced/L.

2.7.2 Ferric Reducing Ability of Plasma: an index of antioxidant activity

Plasma antioxidant capacity was estimated from its ferric reducing ability (FRAP) where development of an intense blue colour resulting from the conversion of a Fe (III)-2,4,6-Tri (2-pyr-idyl)-s-triazine (iron chloride-TPTZ) complex to Fe (II)-TPTZ (iron sulphate-TPTZ), with an concomitant increase in absorbance at 593 nm, being directly related to the amount of the reductant present in each sample. Grape and tea extracts, epicatechin and catechin derivatives (1 mM) and green and black tea fractions were run on the spectrometer (Cecil 3000 series UV/visible spectrophotometer, Cecil Instruments Ltd, Cambridge, UK) 4 mins after the addition of the FRAP reagent (acetate buffer, pH 3.6; $FeCl_3$, TPTZ in 40 mM HCl) to the reaction mixture (0.025% solution). The absorbance change of the aliquot diluted in distilled water is due to the combined reductive activity of all the reacting antioxidants present in the sample. Optical density was compared to a standard curve prepared with 0 - 1.0 mM ferrous sulphate ($FeSO_4$). Grape and tea extract, epicatechin derivates and green and black tea fractions were all run in duplicate. Results are expressed as the mean concentration of Fe (II) produced/mM of 2 samples (Benzie & Strain, 1996).

2.8 Colorimetric Assays

2.8.1 Determination of Total Phenol Content

The total phenol content is determined using the Folin-Ciocalteu method of Singleton and Rossi (1965) In brief, 0.1 mL of grape or tea based extracts, green tea epicatechin and catechin derivatives and green and black tea fractions were added to 5 mL of a 1:10 dilution of Folin-Ciocalteu reagent and 0.9 mL of distilled water. After five minutes, 3.5 mL of 115g Na_2CO_3/L was added and left at room temperature for 2 hours. Absorbance was read at 765 nm against a water blank using a Cecil 3000 series UV/visible spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). Optical density was compared to a standard curve prepared with 0 to 500 μL gallic acid mL^{-1}, with results being expressed as mean μg/mL gallic acid equivalents (GAE). Grape and tea extracts, epicatechin derivates and green and black tea fractions were all run in duplicate.

2.8.2 Spectral Assay for Determination of Total Catechins

The total catechin content of the epicatechin and catechin derivatives and the green and black tea fractions was determined using a method adapted by Kivitis *et al.*, (1997). Total catechin content of the green tea derivatives and tea fractions was determined by adding 3 mL of 6 mM DMACA (dimethylaminocinnamaldehyde) in a methanol/percholoric acid /water mix (v/v 8:1:1) to 1 mL of the tea fractions or epicatechin derivative. The absorption spectrum of the DMACA/fraction mix was measured from 500 – 750 nm, 4 min after the addition of the DMACA reagent. All samples were run in duplicate. The concentration of catechin present was calibrated, from 0 to 100 μg mL^{-1}, and related to the peak area of absorption. Sample blanks were also measured. To quantify the total amount of catechin present in a given fraction, the absorbance was measured between 2 wavelengths specific

for catechins, 604 nm and 684 nm. With the addition of the DMACA, solutions are seen to develop a highly specific colour complex, turning from pale yellow to a dark blue with increasing catechin content. Results are expressed as mean total catechins (mg/L).

2.9 Chemicals: HPLC, spectral & colorimetric analysis

Methanol (HPLC grade), ethanol and acetonitrile (HPLC Grade) were obtained from Rathburn Chemicals (Walkerburn, UK). Formic acid, percholoric acid, hydrochloric acid, acetic acid, Folin and Ciocalteu Reagent (2.0 normal), DMACA, TPTZ, potassium nitrodisulphonate, sodium acetate, Trolox (α tocopherol analogue), gallic acid, catechin, iron sulphate ($FeSO_4$) and iron chloride ($FeCl_3$) were supplied by Sigma-Aldrich Chemical Co. (Poole, Dorset, UK). Disodium carbonate (Na_2CO_3) were obtained from BDH Laboratory Supplies (Poole, UK).

2.10 Statistical analysis of vasodilatory, spectral & colorimetric assays

All vasodilatory experimental results are expressed as mean values ± standard error of mean of *n* experiments, where *n* = the number of animals for each set of experiments. Analysis was carried out using a one-way analysis of variance (ANOVA), followed by a Dunnet's Multiple comparisons test, where ns = not significant, *P<0.05, **P<0.01, ***P<0.001 as compared to experimental control.

Multivariate correlation analysis of experimental data was performed according to Pearson. Correlation calculations quantified the relationship between two sets of experimental variables. The correlation coefficient *r*, quantified the direction and magnitude of the correlation and ranged from –1 to +1. Values where P<0.05 were considered statistically significant.

Due to the complex nature of the biphasic extract response curves, pIC50 (inhibitory concentration which induces 50% of maximum vasorelaxation) values were not calculated. pIC50 values have been calculated for ACh CCRC. Threshold concentrations for the induction of the second phase vasodilator response, were calculated from each individual experimental trace to reflect differences in potency for each individual grape and tea based extract and green tea catechin and epicatechin derivates used in the course of this study. The threshold concentration is defined as the lowest concentration of extract that caused a significant vessel relaxation.

All spectral and colorimetric assay results with rape and tea extracts and epicatechin derivatives are expressed as mean values, where all samples have been run in duplicate and calibrated against relevant standards. Analysis was carried out using a one-way analysis of variance (ANOVA), followed by a Dunnet's Multiple comparisons test, where ns = not significant, *$P<0.05$, **$P<0.01$, ***$P<0.001$, as compared to relevant control for each individual assay.

The Dunnet's Multiple comparisons test, like that of the Bonferroni, Tukey and Newman-Kuels, are post test which are modifications of standard t-tests. They account for multiple comparisons as well as the fact that the comparisons maybe interrelated. Dunnet's Multiple comparisons test allows the control data to be compared to all other variables but not to one another.

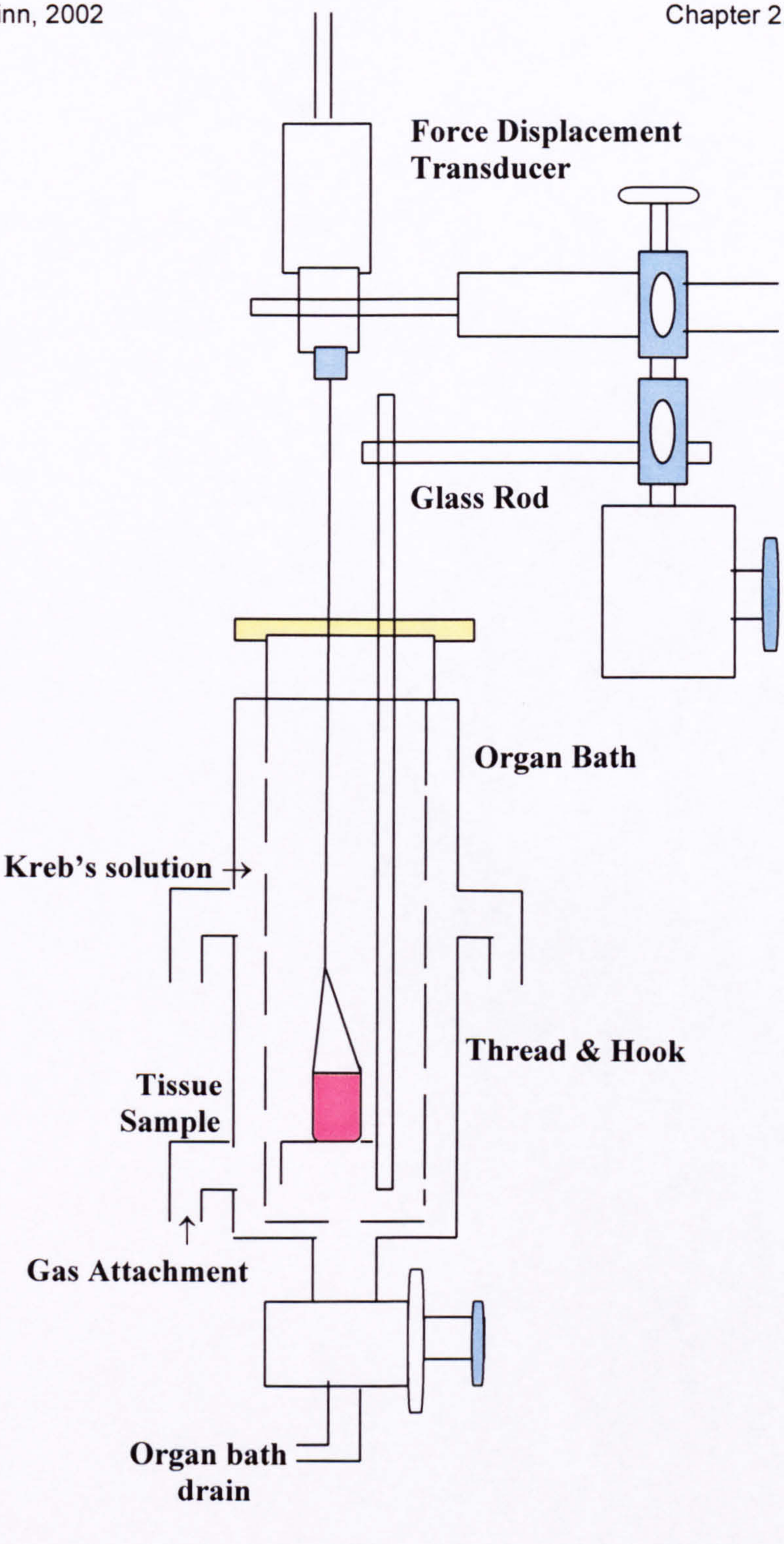


Figure 11 Organ bath set-up

Diagram of a simple organ-bath set-up, consisting of a standard 10 mL bath, filled with Krebs solutions and continuously oxygenated with a standard gas mixture. Tissue sample is attached via a hook, thread and glass rod to an isometric force transducer, which in turn is linked to a data handling system recording changes in vessel contraction/relaxation.

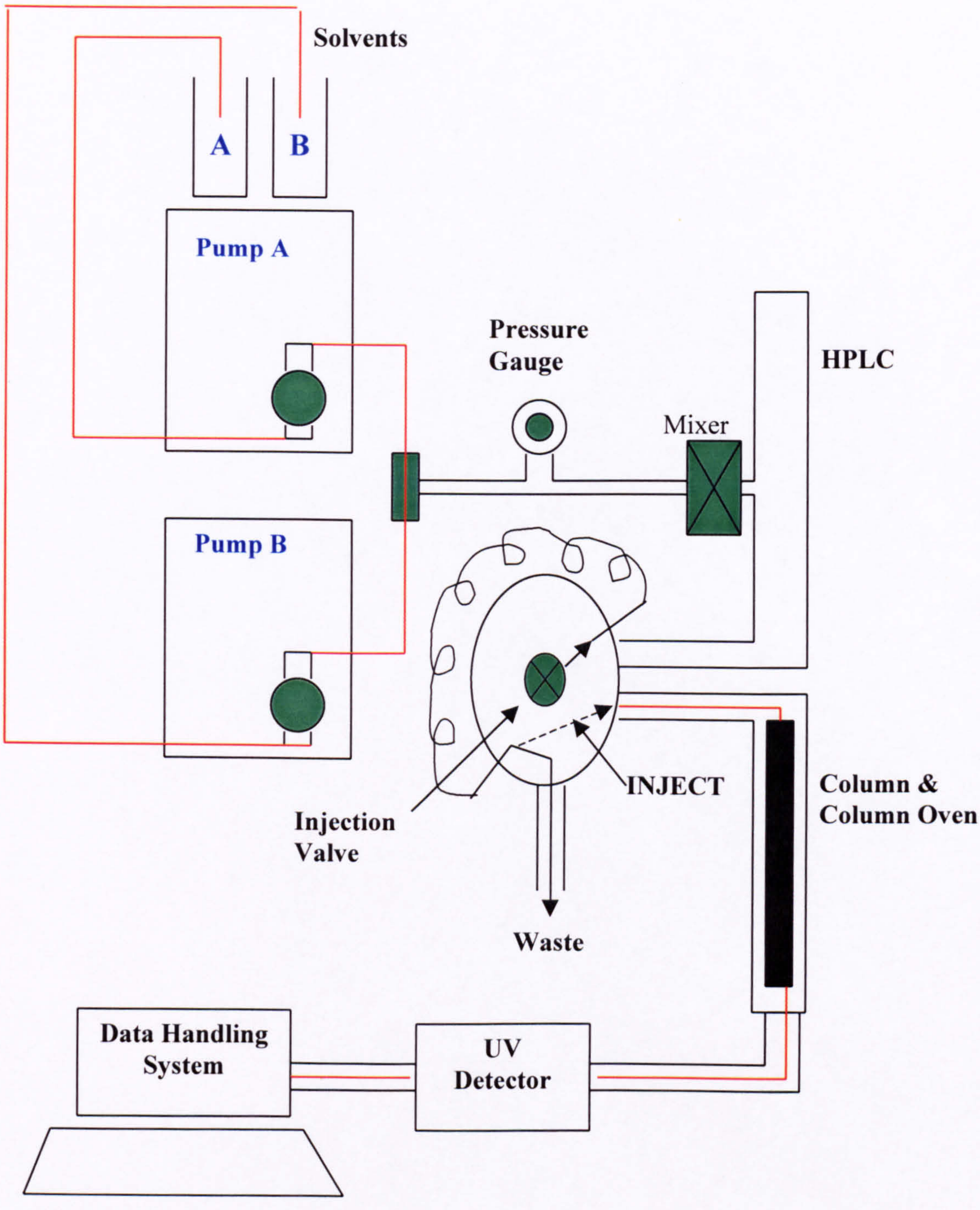


Figure 12 Preparative HPLC system

Chapter 3 Vasodilation and antioxidant activities of grape and tea based extracts

3.1 Introduction

The incidence of CHD mortality in France has been shown to be significantly lower than that of other western industrialised countries with similar high dietary fat intakes and smoking tendencies. This phenomenon discussed in more detail in Chapter 1, has been referred to as the "French Paradox" and has been attributed to the high consumption of red wine by the French (Renaud & de Logeril, 1992).

There is evidence that the phenolic components of red wine may be cardio-protective and decrease the risk of CHD mortality. Ingestion of red wine significantly increases plasma phenolic acid concentration (Duthie *et al.*, 1998; Cacetta *et al.*, 2000). Polyphenols inhibit human platelet aggregation and eicosanoid synthesis (Demrow *et al.*, 1995; Pace-Asiak *et al.*, 1996) and reduce the susceptibility of human plasma low-density lipoprotein to lipid peroxidation (Frankel *et al.*, 1993; Furhman *et al.*, 1995). Their alleged beneficial effects have been attributed to their antioxidant activity.

Natural dietary phenolics have been shown to induce endothelium-dependent vasodilation in both human and rodent arteries (Andriambeloson *et al.*, 1998; Flesch *et al.*, 1998). The mechanism of this endothelium-dependent vasodilation is considered to be via nitric oxide (NO) and subsequent increases in cGMP (Fitzpatrick *et al.*, 1993; Andriambeloson *et al.*, 1997; Flesch *et al.*, 1998). However, the phenolic component(s) within the grape which are responsible for inducing this event and the exact mechanisms by which these compounds produce this effect remain unknown.

Tea, like red wine, is a rich source of polyphenols. Tea leaves have been found to contain more than 35% of their dry leaf weight as polyphenols

(Serafini *et al.*, 1996). Both green and black tea posses strong antioxidant activity and can enhance plasma antioxidant capacity (Xie *et al.*, 1993; Serafini *et al.*, 1996; Yokozawa & Dong, 1997). Black tea has also been shown to have anti-atherosclerotic activity (Yokozawa *et al.*, 1998). The vasodilator activity of green tea has been attributed to its (-)-epicatechin content, to the release of NO from vascular endothelial cells, and to the inhibition of calcium influx (Huang *et al.*, 1998; 1999). The vasodilator effect of black tea has not been investigated to date.

Recently a striking correlation has been identified between the ability of red wines to induce vasodilation in the isolated rabbit aorta and the antioxidant activity of the same wines (Burns *et al.*, 2000). This study will determine the vasodilator effects of grape and tea based extracts *in vitro*, investigate their mechanisms of vasodilation and to relate this to the antioxidant activity and phenolic content of each extract.

3.2 Materials & Methods

3.2.1 Vasodilation activity

In brief, isolated rabbit aortic vessels were suspended in standard 10 mL organ baths (Figure 11) filled with Krebs buffer solution, continuously oxygenated with 16% O_2, 5% CO_2 and 79% N_2 and maintained at 37°C, to mimic the internal environment. Vessels were placed under an optimal resting tension of 2 g. Vessel contraction/relaxation was measured via an isometric force transducer.

After an equilibration period, vessels were twice maximally contracted with 50 mM KCl and washed out thoroughly with Krebs solution. In some experiments the luminal endothelium was removed prior to the start of the experiment. After washing and returning to initial baseline tension, vessels with and without functional endothelium were precontracted with

submaximally PE (10^{-7} M). In some experiments where indicated, the thromboxane-mimetic U44619 (10^{-9} M) was used to elevate vascular tone.

Once a stable plateau had been reached, CCRC for the diluted grape and tea based extracts (0.0002 – 14 mg mL^{-1}) were obtained. The mechanisms of vasodilation were investigated in the presence of several pharmacological inhibitors, namely, the NO synthase inhibitor, L-NAME (100 μM), the prostaglandin cyclo-oxygenase inhibitor, indomethacin (1, 100 μM), the thromboxane antagonists, ICI 192,605 (10 μM) and GR 32191 (1 μM) and ODQ (10 μM), the soluble guanylate cyclase inhibitor, individually and in combination.

3.2.2 Antioxidant activity

Antioxidant activity of the grape and tea based extracts in this study, were determined using specific spectral and colorimetric assays discussed in detail in Chapter 2.

Total antioxidant activity of each extract was determined using ESR spectroscopy, where the ability of the extracts to reduce radicals in the aqueous phase was estimated by adding the extract to an equal volume of a 1 mM Fremy's salt free radical. Results were standardised using Trolox.

Plasma antioxidant capacity was also estimated from the extracts ferric reducing ability (FRAP) at 593 nm. The development of an intense blue colour resulting from the conversion of a Fe(III)-2,4,6-Tri-2-pyr-idyl)-s-triazine (TPTZ) complex to Fe(II)-TPTZ being directly related to the amount of reductant present in the sample. Results are expressed as the concentration of Fe(II) produced/mM (Benzie & Strain, 1996).

Total phenol content of the grape and tea based extracts was determined and expressed as gallic acid equivalents (GAE) using the Folin-Ciocalteu method at 765 nm (Singleton & Rossi, 1965).

3.3 Results

3.3.1 Vasodilator effect of grape & tea based extracts

Vasorelaxation was induced, to varying degrees, by all grape and tea based extracts investigated in this study. The CCRCs to each extract, from 0.0002 to 14 mg mL^{-1}, in isolated rabbit aortic vessels precontracted with PE is shown in Graph 2. Time matched controls for each extract were also run to demonstrate the effect of each extract on vascular tension as opposed to the natural relaxation response of each vessel with time (Graph 3). Relaxation induced by acetylcholine (ACh) in vessels with functional endothelium, induced a maximum relaxation of 97% with a pIC50 of 7.5 ± 0.1, and 23% in vessels without intact functional endothelium with a pIC50 of 1.2 ± 0.2 of PE-induced tone, as compared to vasorelaxation induced by each extract shown in Graph 4.

Figure 13 displays a sample vasorelaxation trace of vasodilation induced by ACh with and without functional endothelium following preconstriction with PE. Similarly, Figure 14 displays a sample trace of vessel maximal contractile responses to 50 mM KCl. Table 15 displays the threshold concentrations, that is the lowest concentration that caused a significant relaxation, for the second phase and maximum relaxation values for all extracts tested in this study. Hence, the extracts were less potent than ACh, inducing a maximum vasodilation of only 68%. Figure 15 show a sample trace of the vasorelaxation induced by green tea, grape powder and grape skin extracts.

Table 15 Maximum % relaxation values and threshold concentrations for relaxation obtained with grape and tea extracts

Extract	Max. % Relaxation	Threshold conc. for relaxation	*n*
French grape skin (FGSk)	55.0 ± 6	$3.4 \pm 1 \times 10^{-2}$ mg.ml^{-1}*	6
French grape seed (FGSd)	62.6 ± 4	$0.4 \pm 0 \times 10^{-2}$ mg.ml^{-1}	6
Grape skin (GS)	63.8 ± 6	$0.4 \pm 0 \times 10^{-2}$ mg.ml^{-1}	6
Grape powder (GP)	58.6 ± 11	$0.4 \pm 0 \times 10^{-2}$ mg.ml^{-1}*	6
Green tea (GT)	46.0 ± 10	$40.3 \pm 4 \times 10^{-2}$ mg.ml^{-1}***	7
Black tea (BT)	44.3 ± 5*	$40.0 \pm 0 \times 10^{-2}$ mg.ml^{-1}***	6

n = number of animals. Statistical analysis was carried out using an ANOVA followed by a Dunnets Multiple comparisons test *P< 0.05, **P< 0.01, ***P<0.001 as compared to control. Values expressed as mean % relaxation ± standard error of mean.

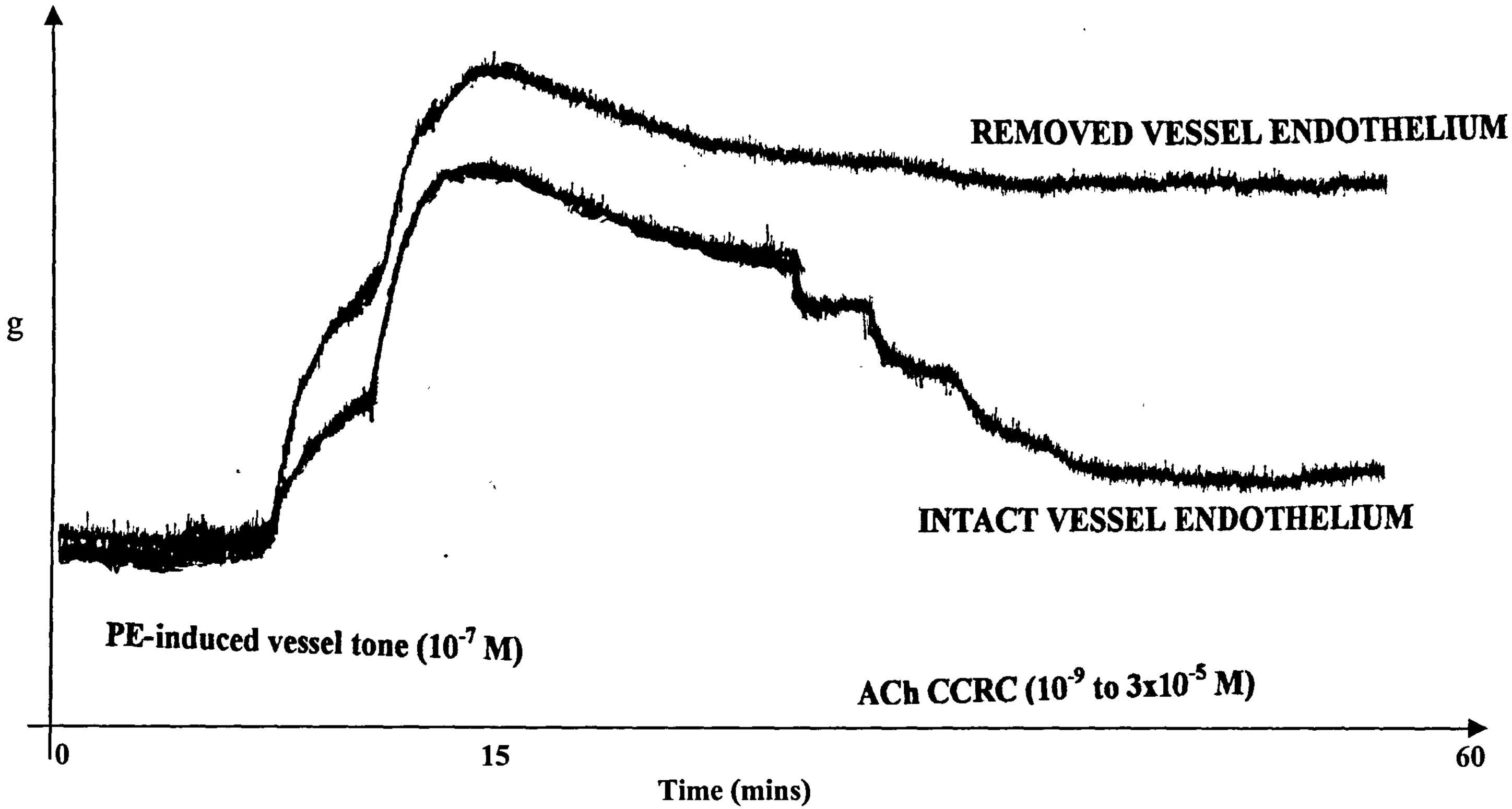


Figure 13 Vasodilation trace of ACh induced vasorelaxation with and without functional endothelium

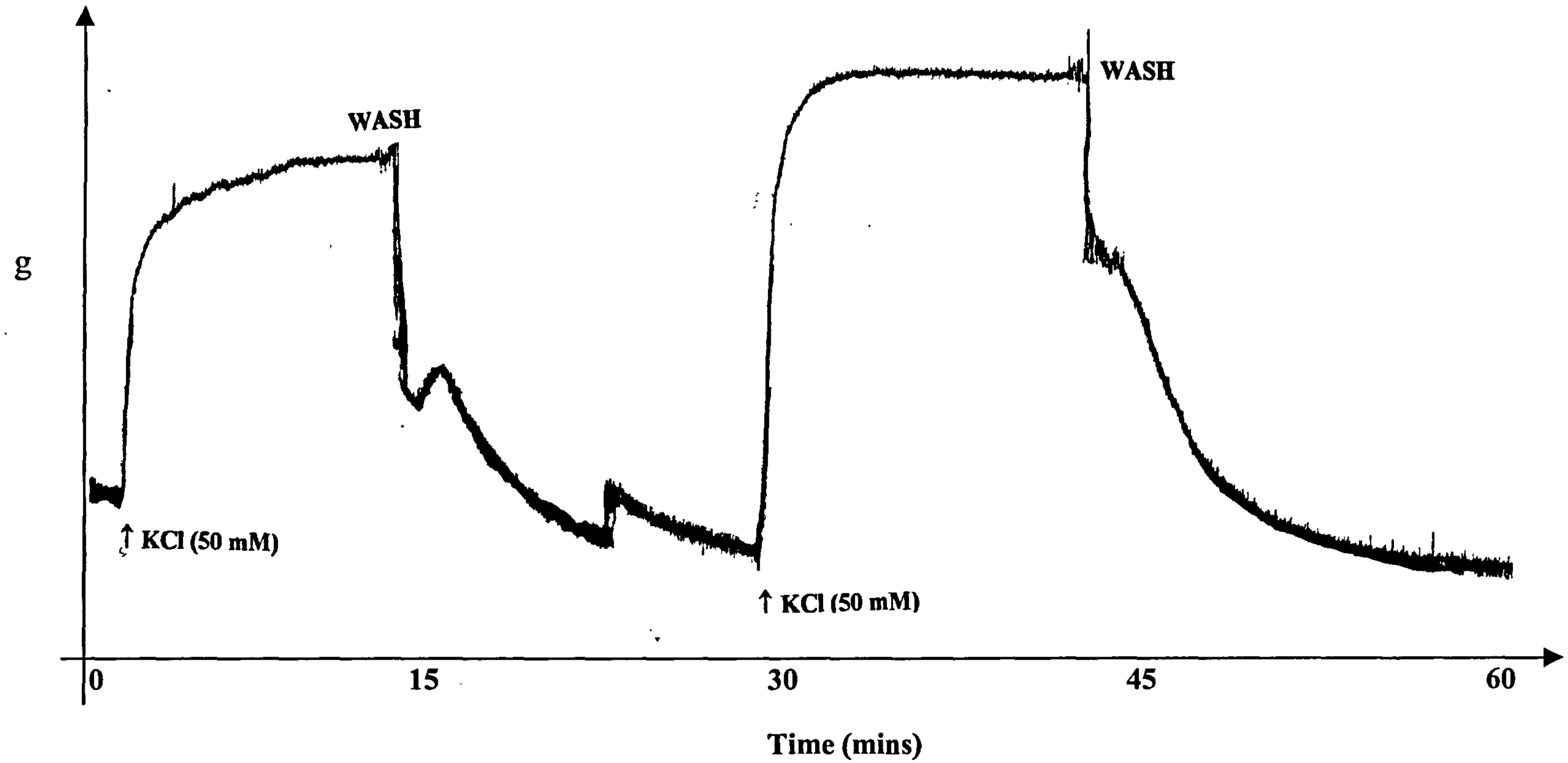


Figure 14 Trace of the maximum vessel contractile responses to 50 mM KCl

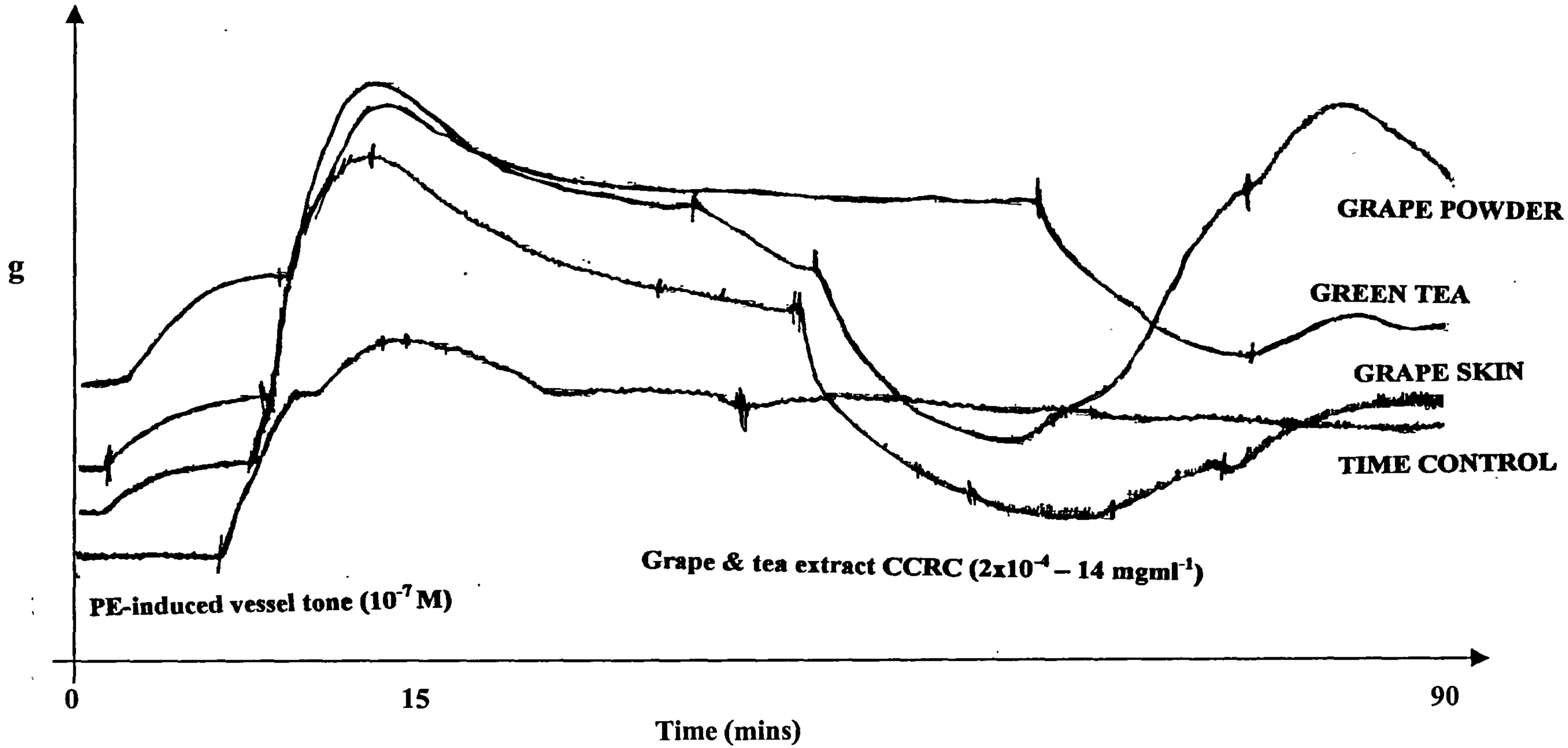


Figure 15 Trace of the vasodilation induced by grape and tea extracts *in vitro*

3.3.2 *Vasodilation induced by grape based extracts*

The CCRCs for grape skin, grape powder, French grape seed and French grape skin extracts were all biphasic. There is a small vasodilation (8 – 25% of maximum vasodilation) at low concentrations, which has been termed the first phase, and a more profound relaxation at higher increasing extract concentrations (~60% relaxation of PE-induced preconstriction), subsequently termed the second phase. In the case of all grape based extracts, this second phase was not maintained at higher extract concentrations, with vasodilation reversing in a concentration dependent manner (Graph 2). Graph 2 also demonstrates that the French grape seed extract, which although did not induced the highest maximum vasorelaxation value, was the most potent with a maximum relaxation being achieved by 0.04 mg mL^{-1} compared to other extracts which did not achieved vasodilation until concentrations greater than 0.14 mg mL^{-1}.

3.3.2.1 Grape skin extract

Grape skin extract induced the greatest degree of vasorelaxation (63.8% PE-induced preconstriction) of all extracts investigated in this study. The first phase of the vasodilator response was not abolished by the presence the of the nitric oxide synthase inhibitor L-NAME (100 μM) or removal of the endothelium (Graph 5) but was inhibited by the prostaglandin cyclo-oxygenase inhibitor indomethacin (100 μM, but not 1 μM) ($P<0.001$ at 0.014 mg mL^{-1}) and by 10 μM ICI 192,605 ($P<0.05$ at 0.014 mg mL^{-1}) (Graph 6). Neither indomethacin or ICI 192,605 inhibited the second phase, which was however, abolished by L-NAME and removal of the endothelium (Graph 5). This indicates that the grape skin extract induced endothelium-NO-independent prostaglandin related vasodilation in the first phase response and endothelium-NO-dependent vasorelaxation in the second phase.

The thromboxane A_2 (TXA_2) antagonist devoid of partial agonist activity, GR 32191 did not inhibit the first phase, but this was subsequently inhibited by 10 μM ODQ ($P<0.05$ for concentrations >0.1 mg mL^{-1}) (Graph

7). A combination of removal of the endothelium, presence of L-NAME and indomethacin (100 μM) were required to totally abolished the grape skin extract vasorelaxation at all concentrations of the extract (Graph 8).

The vasodilator response to the grape skin extract when the thromboxane mimetic U44619 (Table 16) was used to elevate vascular tone as compared to PE (10^{-9} M) demonstrated a concentration-dependent response. The biphasic nature of the response curve was retained, but the second phase was maintained at higher extract concentrations in U46619-precontracted vessels (Graph 9) as compared to PE-precontracted vessels where there was reversal of the relaxation response.

Table 16 Preconstriction to PE and U44619.

Degree of preconstriction to PE (10^{-7} M) and U46619 (10^{-9} M) in all experimental groups, in the presence of L-NAME (100 μM) and L-NAME and indomethacin (1 μM) as a % KCl contraction (50 mM) in intact aortic vessels.

Exp. Group	PE	*n*	U46619	*n*
Extract	67% ± 2	6	71% ± 1	6
Extract & L-NAME	68% ± 6	6	75% ± 4	4
Extract & L-NAME & indomethacin	79% ± 3	6	76% ± 2	4

All data expressed as mean % relaxation values ± standard error of mean. *n* = number of animals.

3.3.2.2 Grape powder extract

A grape powder extract was also able to induce vasorelaxation in preconstricted vessels. This extract constituted a powder extract of the whole grape, both skin, seeds and pulp. The grape powder extract induced biphasic vasorelaxation, with maximum relaxation value of up to 59% of PE-induced preconstriction (Table 15), then reversal of the vasodilator response with increasing extract concentrations. As with the grape skin extract, the presence

of L-NAME was not able to abolish the first phase response, but did abolish the second phase (Graph 10), indicating the grape powder extracts induces vasodilation via endothelium-dependent and independent mechanisms. Due to the limited supply of the grape powder extract, no further studies were carried out using this extract. Graph 11 shows a comparison of the vasodilator responses of the grape powder and skin extracts *in vitro*.

3.3.2.3 French grape seed & skin extracts

The vasodilation induced *in vitro* by a French grape seed extract and a French grape skin extract were also investigated. Both extracts induced significant vasorelaxation in vessels preconstricted with PE. As before, both extracts induced biphasic vasodilation, with an initial first phase of dilation at low extract concentrations (~ 10 % PE precontraction), then a further second phase with increasing concentrations that was not maintained with the highest extract concentrations inducing reversal of the relaxation response (Graph 12). French grape seed extract was found to be the most potent all extracts investigated in this study inducing vasorelaxation at a lower concentration than all other extracts.

In the case of both extracts, the concentration dependent response curve induced by both extracts was abolished, in the first and second phases, by the presence of L-NAME and removal of the endothelium (Graphs 13 & 14), indicating vasorelaxation by the 2 extracts to be via endothelium-NO-dependent mechanisms.

3.3.3 Vasodilation induced by tea based extracts

The CCRCs for green and black tea and black tea theaflavin derivatives, were also all biphasic (Graph 15) and remarkably similar to one another, with a first phase equivalent to that observed with the grape based extracts and slight reversal of the vasodilation response. The second phase of relaxation, of 25 – 40% PE-induced preconstriction, occurred at higher

threshold concentrations than those required to initiate the second phase contractions by the grape extracts (Graph 2).

Table 15 shows the threshold concentrations for the second phase and maximum vasorelaxation values induced in the isolated aortic vessels for the green and black tea extracts.

3.3.3.1 Green tea extract

The first phase of the response was not observed in the presence of 100 μM L-NAME, 100 μM indomethacin, 10 μM ICI 192, 605 or after the removal of the endothelium (Graphs 16 & 17). The presence of indomethacin (100 μM) and ICI (10 M) both induced vasoconstriction in the aorta (Graph 17). 10 μM ICI 192, 605, 100 μM L-NAME, 100 μM indomethacin and endothelial removal on their own did not inhibit the second phase vasodilation to higher concentrations of the extract (Graph 17). Only the combination of endothelial removal and indomethacin significantly reduced the second phase response ($P<0.01$ at 1.4 mg mL^{-1}; $P<0.001$ at 14 mg mL^{-1}).

3.3.3.2 Black tea extract

The first phase response was abolished in the presence of 100μ M L-NAME (Graph 18) and after removal of the endothelium, 100 μM indomethacin and 10 μM ICI 192, 605 (Graph 19). As with the green tea extract, the black tea extract also induced a vasoconstriction in the presence of indomethacin and ICI (Graph 19). 100 μM L-NAME and removal of the endothelium reduced the second phase vasodilator response to high extract concentrations ($P<0.05$ at 1.4 and 4 mg mL^{-1}). Combinations of L-NAME, indomethacin and endothelial removal were not able to abolish this second phase (Graph 19), although this combination did abolish these responses to the grape skin extract. Only a combination of endothelial removal and indomethacin appear to be able to abolish this response.

3.3.3.3 Black tea theaflavin extract

Theaflavins are formed by the enzymatic oxidation of catechins in green tea during fermentation in the manufacture of black tea. They are high molecular weight compounds which give black tea its characteristic astringent taste and black colour (Balentine *et al.*, 1997).

The vasodilation induced by the black tea theaflavin derivative was also investigated. This extract produce a similar biphasic response curve to that of the green and black tea extracts, with an initial first phase response at low extract concentrations (~12% of PE-induced preconstriction) and subsequent reversal of the response then a second profound phase of relaxation, to the extracts maximum vasodilatory response (28.3 % PE-induced preconstriction) at the highest extract concentrations (Graph 20).

3.3.4 Antioxidant activities of grape & tea based extracts

To investigate the antioxidant abilities of the grape and tea extracts, 3 specific spectral and colorimetric antioxidant assays were carried out. ESR, determines the radical quenching ability of the extracts, FRAP analysis, estimates their ferric reducing ability and the total phenol content. Table 17, displays the values obtained by each individual extract in each of the 3 assays.

3.3.4.1 Electron Spin Resonance (ESR)

The antioxidant activity by ESR for each of the grape and tea extracts, with the exception of the Theaflavin extract, is shown in Graph 21. All results are expressed as the number of radicals reduced by each individual extract/mg x 10^{18}. The green tea extract is found to have the highest antioxidant capacity followed closely by the black tea extract. All the grape based extracts have dramatically lower antioxidant capacity as compared to the tea extracts (Graph 21).

Table 17 Antioxidant capacity and total phenolic content of grape and tea based extracts

Extract	*n*	ESR[a]	FRAP[b]	Total Phenols[c]
French grape seed (FGSd)	2	1.11 ± 0.017**	2.23 ± 0.04**	231.90 ± 7.31**
French grape skin (FGSk)	2	0.58 ± 0.016*	0.86 ± 0.04**	88.29 ± 0.36*
Grape skin (GS)	2	0.37 ± 0.017	0.56 ± 0.04	45.38 ± 1.09
Grape powder (GP)	2	0.88 ± 0.013**	1.13 ± 0.03**	116.98 ± 1.46**
Green tea (GT)	2	3.32 ± 0.026**	2.88 ± 0.02**	230.87 ± 13.16**
Black tea (BT)	2	2.59 ± 0.153**	3.07 ± 0.009***	213.29 ± 4.38**

[a]Antioxidant capacity of the extracts, measured by ESR spectroscopy, presented as the number of Fremy's radicals reduced per mg of extract x 10^{18}. [b]FRAP antioxidant capacity of various extracts, as concentration of Fe^{II} produced per mg of extract. [c]Total phenol content of the extracts as determined by using the Folin-Ciocalteu method (mM gallic acid equivalents GAE mg/litre). All data expressed as mean values. All samples were run in duplicate ($n = 2$). Statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by a Dunnet's Multiple Comparisons test, ***P<0.001, **P<0.01, *P<0.05, as compared to control.

3.3.4.2 Ferric reducing ability (FRAP)

Antioxidant ability has also been determined by the ferric reducing ability of each extract, analysed by the FRAP assay. All results are expressed as the concentration of Fe(II) produced by each extract during its reduction from Fe(III). As with the previous spectral assay a similar pattern emerges, with the tea extracts being higher in activity than the grape based extracts (Graph 22). In addition, the French grape seed extract is also significantly higher in activity than the other related grape based extracts.

3.3.4.3 Total phenolic content

The total phenol colorimetric assay, is a non-specific assay which identifies the presence of gallic acid groups, with all results being expressed as gallic acid equivalents (GAE). As with the previous 2 spectral assays, the tea extracts and the French grape seed extract are higher in total phenols than the remaining grape extracts (Graph 23).

The statistical significance of the relationships between the antioxidant activity, total phenolic content and the maximum % vasodilation induced by each extract was analysed using Pearsons correlation (Graph 23). Using correlation analysis, maximum vasodilation was inversely correlated to antioxidant activity as determined by ESR (r = -0.87, P>0.05, P = 0.02) but not significantly correlated to antioxidant activity by FRAP analysis (r = -0.74, p = 0.09). The small P value indicates that this is a true relationship between the vasodilation and ESR and is not coincidental. Total phenol content was also determined to be significantly correlated with the maximum vasodilation induced by each extract (r = -0.95, P<0.01, P = 0.01). The negative r values indicate that as one variable increases the other decreases. It is therefore evident that higher the antioxidant activity of the extracts, the less active they are as vasodilators of isolated aortic vessels *in vitro*.

3.4 Discussion

These results demonstrate that all the extracts tested in this study, induced vasodilation in isolated aortic vessels, to varying degrees. The grape and tea extracts all produced easily distinguishable concentration response curves. All extracts induced a biphasic concentration-dependent vasorelaxation in aortic rings. The antioxidant capacity of the extracts consistently showed the tea extracts to be more effective antioxidants than the grape based extracts, with the exception of the French grape seed extract, the opposite pattern as highlighted by the vasodilation studies.

3.4.1 Grape skin extract

Vasorelaxation with the grape skin extract, in the first phase response, was unaffected by the removal of the endothelium, by the presence of the NO synthase inhibitor L-NAME or by indomethacin (100 μM), indicating that endothelium-derived NO is not responsible for this vasodilator effect. ODQ suppressed the first phase response of the grape skin extract induced suggesting that soluble guanylate cyclase activation may be involved in this response. ICI 192, 605, the TXA_2 inhibitor, also inhibited this first phase response, but this inhibitor only has partial agonist activity, so the effects of GR 32191, the TXA_2 antagonist devoid of partial activity was also investigated. GR 32192 did not affect the first phase vasodilator response indicating that the activation of TXA_2 receptors do not play a role in this response. Therefore, while the mechanism involved in this first phase vasodilator response induced by the grape skin extract is obviously multi-factorial, it occurs independent of the vascular endothelium and the release of NO.

The second phase of the grape skin extract vasorelaxation was inhibited by removal of the endothelium, L-NAME and ODQ indicating clearly that endothelium-derived NO is responsible for this vasodilation. This phase was resistant to indomethacin, ICI 192, 605 and GR 32191, ruling out a role for

prostaglandins and thromboxanes in this effect. The second phase results are consistent with previous studies that red wine and grape-derived products induce endothelium-NO-dependent vasorelaxation in isolated rat aortic vessels (Fitzpatrick *et al.,* 1993; Andriambeloson *et al.,* 1997; Flesch *et al.,* 1998). Work by these authors did not highlight a 2 phase response by red wine and grape products as shown in this study. Anthocyanins and oligomeric condensed tannins isolated from grape skin extracts have been previously shown to exhibit vasorelaxation to the same extent as the original red wine polyphenolic fraction (Andriambeloson *et al.,* 1997b).

As a combination of endothelium removal, L-NAME and indomethacin totally abolished the vasodilator responses, this supports the assumption that this extract induces vasodilation by complex mechanisms including endothelium-independent mechanisms, possibly via vasodilating prostaglandins, via prostacyclin and endothelial NO production.

3.4.2 Grape powder extract

The grape powder extract first phase vasodilator response was not inhibited by the presence of L-NAME, but abolished the second phase response, indicating that as with the grape skin extract vasodilation is occurring via endothelium-NO-independent and dependent mechanisms in the rabbit aortic vessels. On comparison of the grape skin and powder extracts, the grape powder extract is found to be more potent at the first phase vasodilator response than the grape skin extract. Indicating that the both the flesh and the seeds of the grape also have a potential for relaxation and not all vaso-active components are derived from primarily the grape skin.

3.4.3 French grape skin & seed extracts

Vasorelaxation with the French grape seed and skin extracts in the first phase vasodilator response was abolished by the presence of L-NAME and removal of the endothelium. The second phase response was also abolished by

the French grape seed and skin extracts indicating that vasorelaxation in the isolated rabbit aortic vessels was via NO derived endothelial-dependent mechanisms. The French grape skin extract produced a comparable CCRC to the original grape skin extract investigated but induced a lower maximum relaxation value.

The French grape seed extract was found to be the most potent of all tested in this study. Grape seeds contain flavan-3-ols or catechins as compared to the anthocyanins found in grape skin extracts accounting for their deep red colour (Fitzpatrick *et al.*, 1997). Previous studies have shown epicatchin-3-*O*-gallate isolated from grape seed extracts have potent vasodilatory activity *in vitro*. As shown with the antioxidant work in this chapter, the French grape seed extract had comparable antioxidant activity to the green and black tea and was consistently higher in all assays than other grape based extracts, which maybe due to differences in its chemical composition.

3.4.4 Green tea extract

Indomethacin, L-NAME, endothelial removal and ICI 192, 605 all inhibited the first phase vasodilator response. The presence of both ICI 192, 605 and indomethacin with increasing extract concentrations induced a vasoconstriction in the vessels, suggesting that a certain concentrations, green tea extracts may exert contractile effects which are normally masked by simultaneous increased activity of vasodilating prostaglandins. Caffeine, which is present in considerable amounts in green and black tea (Worth *et al.*, 2000), up to 40 mg per cup, may be involved in this vasoconstriction, as many of the flavonols present in green tea, quercetin, kaempferol, myricetin, apigigin and luteolin, with the exception of rutin, have all previously been shown to be potent vasodilators in isolated pulmonary arteries (MacLean *et al.*, 1997). Caffeine has recently been shown to induce vasoconstriction in many arteries including the rabbit aorta (Luo *et al.*, 2000) and has recently been identified during the large-scale preparatory fractionation of a sample of green tea (to be discussed in detail in Chapter 5). Therefore, the mechanisms

behind the first phase are more complex than those mediating the first phase vasodilator response of the grape skin extract. They may include endothelial prostacyclin and NO release.

The second phase vasodilator response induced by the green tea extract was reduced but not abolished by L-NAME and removal of the endothelium. The dilator response was also partially resistant to the effects of L-NAME, indomethacin and endothelial removal in combination, indicating that while endothelium-dependent NO/prostacyclin activity may be involved, other as of yet undetermined mechanisms play also play a role.

3.4.5 Black tea extract

As observed with the green tea extract, L-NAME, removal of the endothelium, indomethacin and ICI 192, 605 all inhibited the first phase response, with the black tea extract inducing a vasoconstriction in the aortic vessels in the presence of indomethacin.

The second phase of the vasodilator response to black tea extract was resistant to L-NAME, removal of the endothelium, indomethacin, ICI 192, 605 and all combinations of these. The second phase was only abolished in the presence of indomethacin and after removal of the endothelium. Therefore, the mechanism of vasodilation for the black tea extract is very different from that observed with the grape skin extract, in the both first and second phase responses, initiating vasodilation via similar mechanisms to the green tea extract. These results suggest that the vasorelaxation induced by this extract are due to the combined interactions between vasodilating, endothelium-dependent prostaglandins. No literature is available at present of the mechanisms and vasorelaxation induced by black tea.

3.4.6 Theaflavin extract

As observed with the green and black tea extracts, the theaflavin extract, a product formed from the oxidation of green tea catechins during fermentation, also induced a biphasic CCRC in the isolated rabbit aortic vessels. Due to the limited amount of extract available, only the basic cumulative concentration response curve for the extract was determined. The theaflavin extract induced a comparable CCRC to that induced by the black tea extract as shown. However, the theaflavin extract induced lower relaxation values at all concentrations, indicating that the black tea extract has other vaso-active components present. Catechins and other flavonoids which are present in black tea and have not yet been identified, may account for this role.

3.4.7 Antioxidant activity

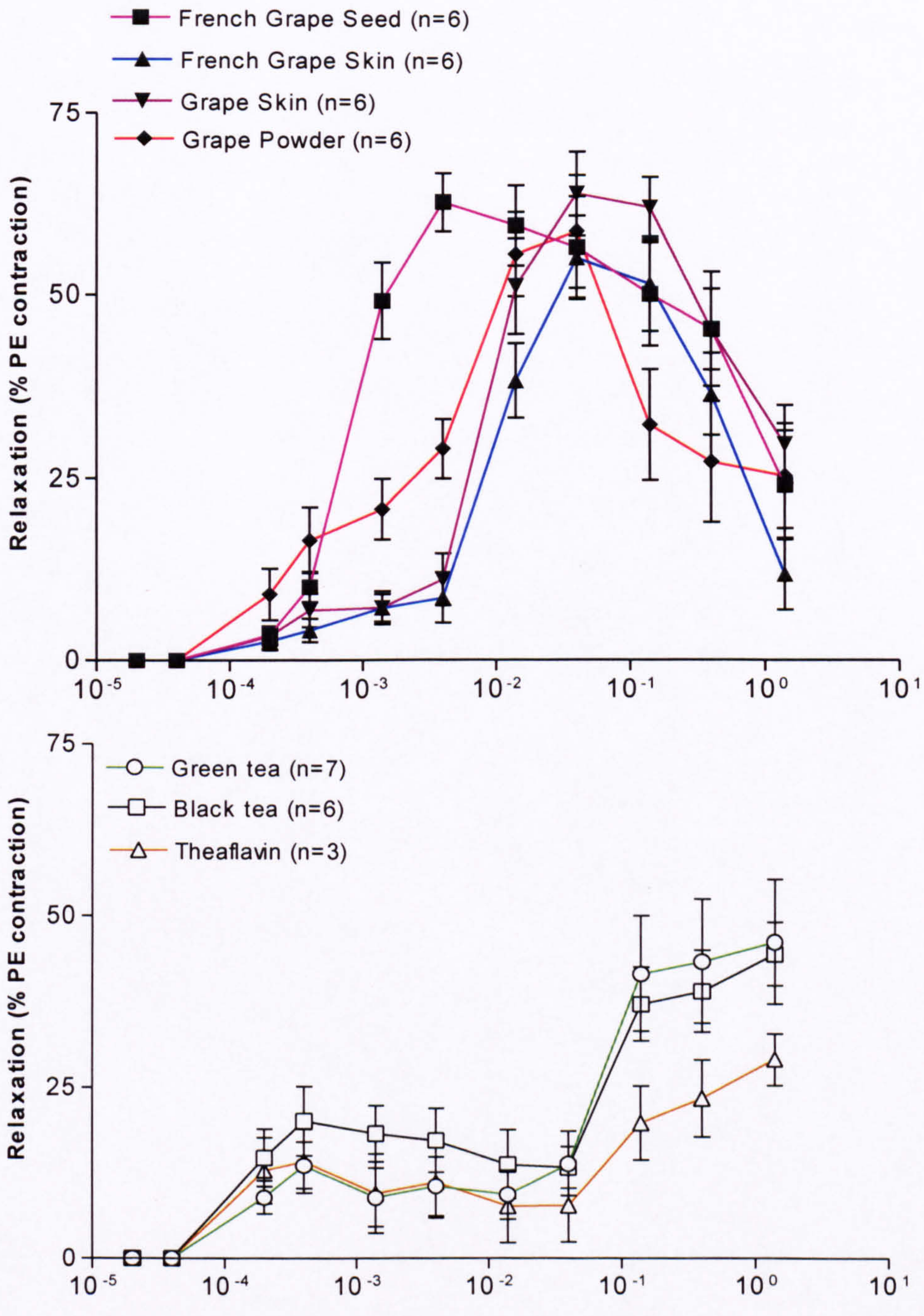
It has previously been demonstrated that red wine extracts induce vasorelaxation in rabbit aortic vessels and that the potency is directly correlated with their antioxidant activity and total phenolic content (Burns *et al.*, 2000). However, the results of this study demonstrate that the grape and tea extracts examined induce vasorelaxation that is inversely and significantly correlated to their antioxidant activity by ESR and their total phenolic content. The higher the antioxidant activity of the extracts, the less active they are as vasodilators and vice versa, as determined by multivariate correlation analysis. Study. This also indicates that the compounds responsible for their antioxidant activity are distinct from those responsible for inducing their vasodilator activity *in vitro*. The opposite nature of the results published by Burns *et al.*, (2000) as compared to the present study may be due to genuine differences in the initial starting products rather than differences in methodology. Alcohol free full red wine extracts were investigated for their antioxidant and vasodilatory activity as compared to various dried grape based extracts in this study. The red wine extracts may have contained a wider range of active compounds than the grape based extracts which may have enhanced their vasodilatory and antioxidant capabilities. Red wine will also have a different

composition to that of grape extracts because of the chemical changes that occur during extraction, vinification and maturation of the finished wine.

3.4.8 Conclusion

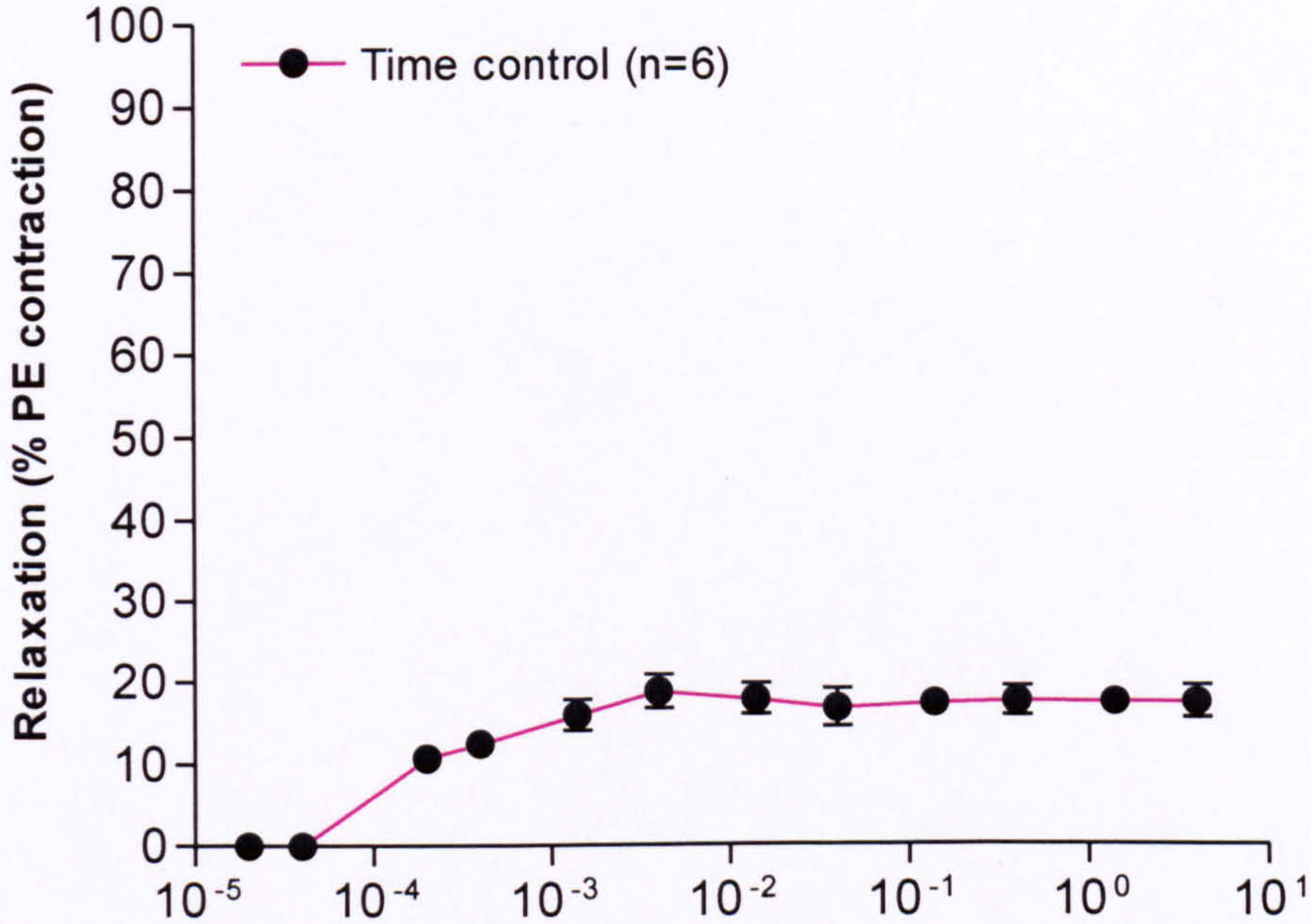
In conclusion, this study has demonstrated that a variety of grape and tea based extract induce vasorelaxation in isolated rabbit aortic vessels and has determined that they are also effective antioxidants *in vitro,* within a concentration range that may be reached by moderate wine and tea consumption. This study also indicates that consumption of green tea may have greater possible cardiovascular benefits compared to black tea in terms of their vasodilatory activity and antioxidant capacity. The data presented here however do not however take in account absorption and metabolism of grape and tea phenolics *in vivo*. The grape and tea extracts both produced easily distinguishable concentration response curves. The isolation and identification of the vaso-active substances present in these extracts and their possible effects *in vivo* continues to be fully determined. It seems likely that the alleged beneficial effects of red wine and tea are due to the cumulative effects of several polyphenols working in synergy rather than one individual compound present.

3.5 Graphs

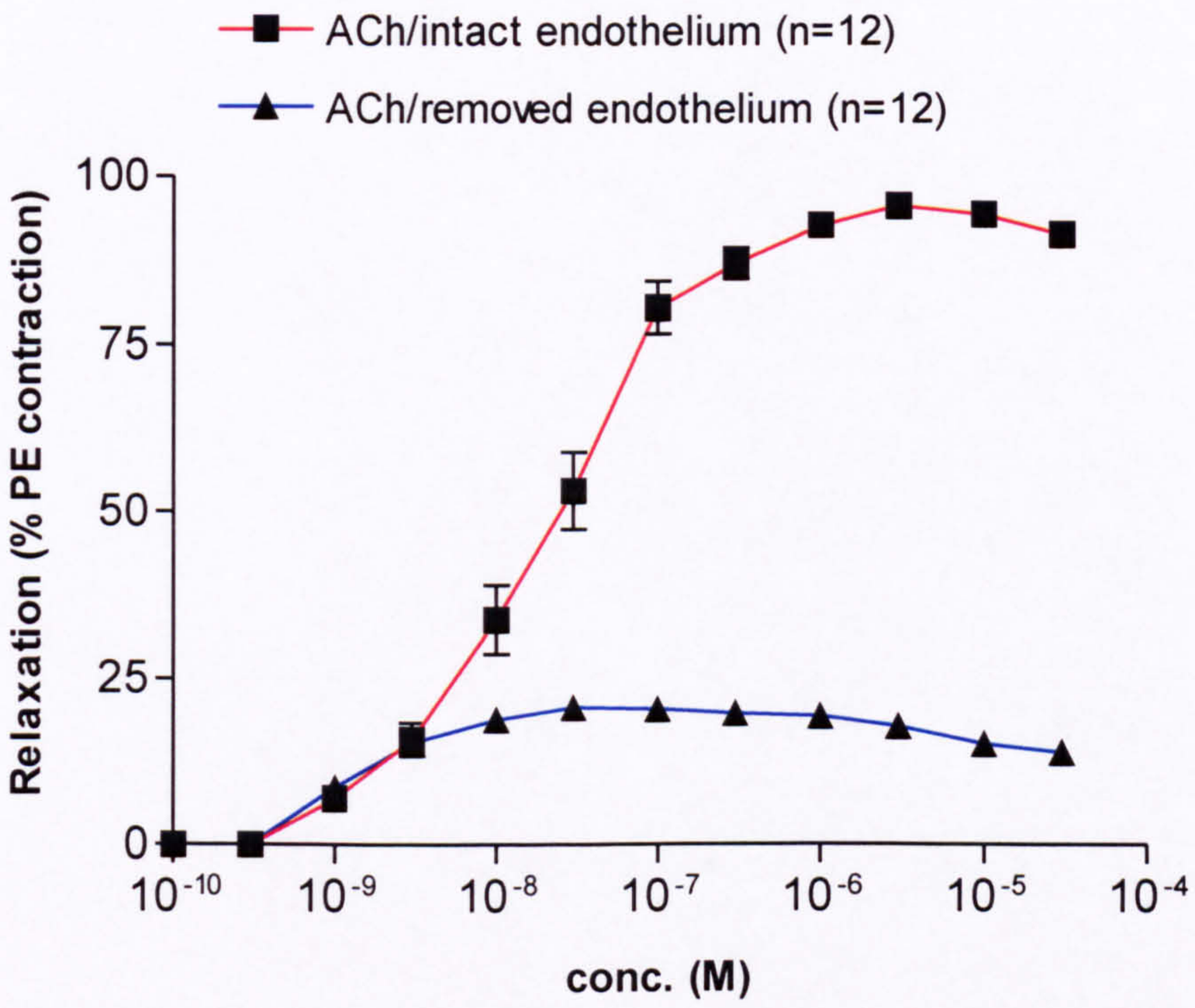


Graph 2 Vasorelaxation induced by grape extracts and tea extracts in aortic vessels

Values expressed as mean % relaxation ± standard error of mean. For individual maximum relaxation values see Table 15.

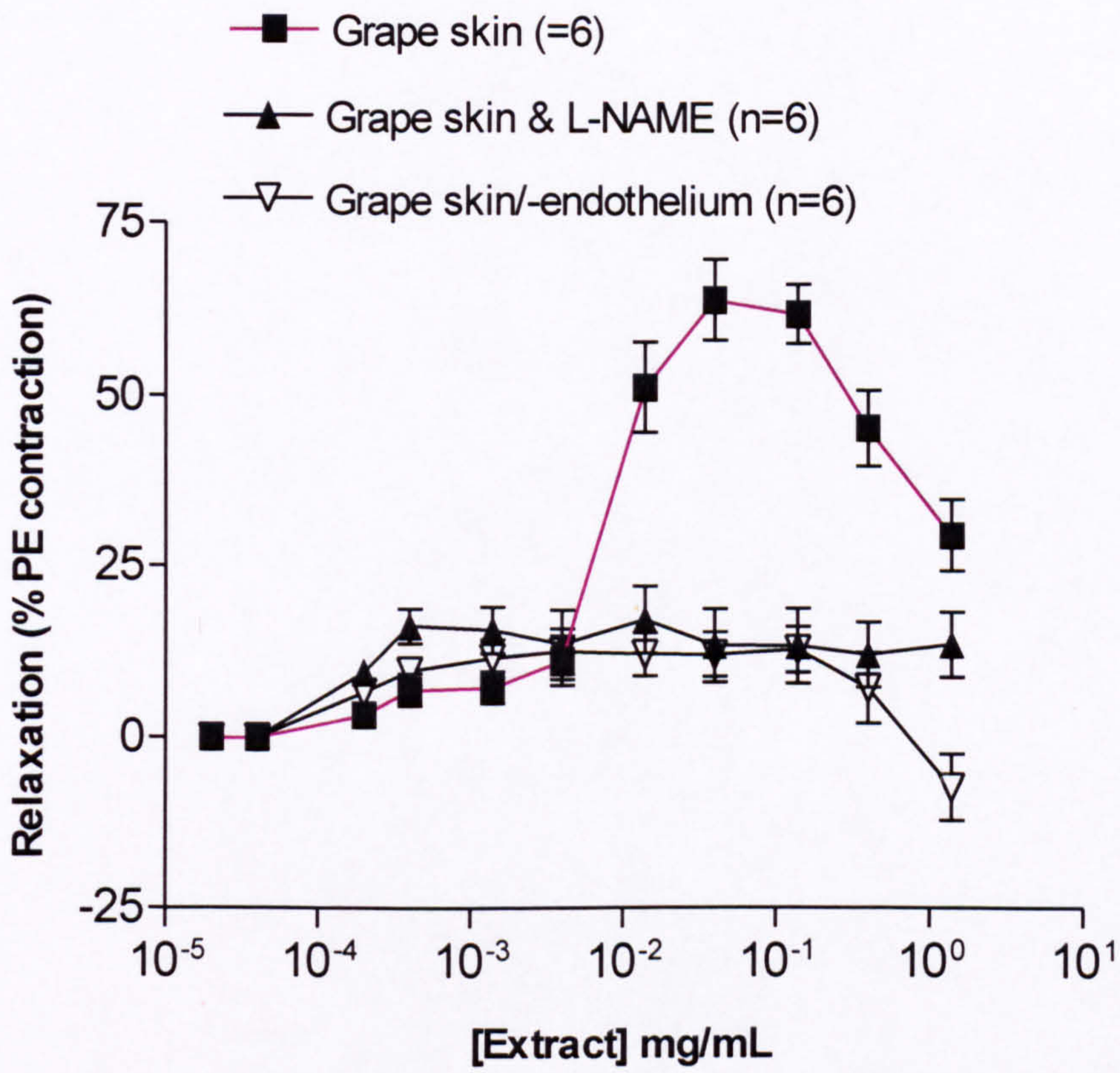


Graph 3 Vessel matched time controls for all experimental groups
Values expressed as mean % relaxation ± standard error of mean.



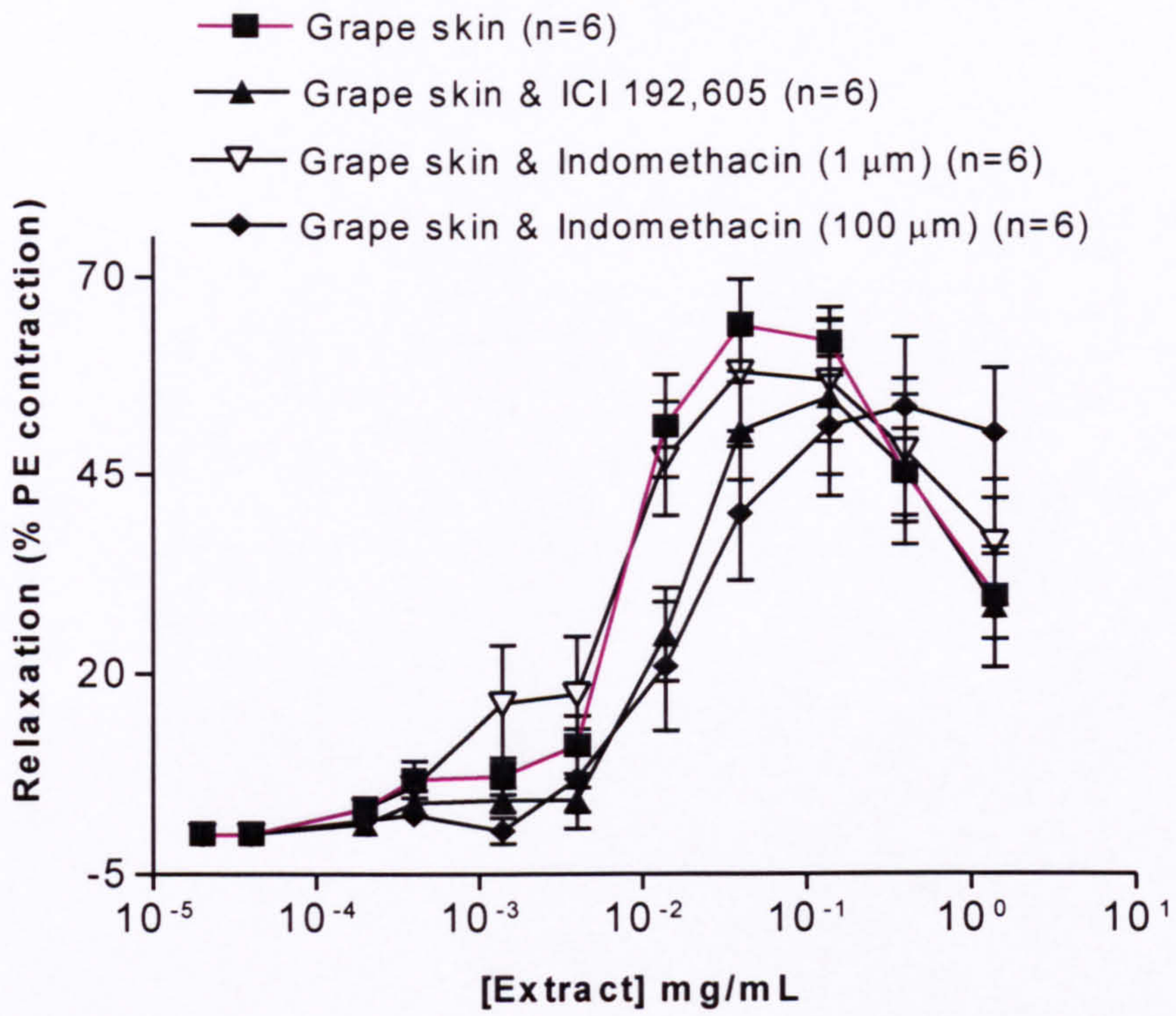
Graph 4 CCRC to ACh in vessels with and without functional endothelium following preconstriction with PE

Values expressed as mean % relaxation ± standard error of mean.



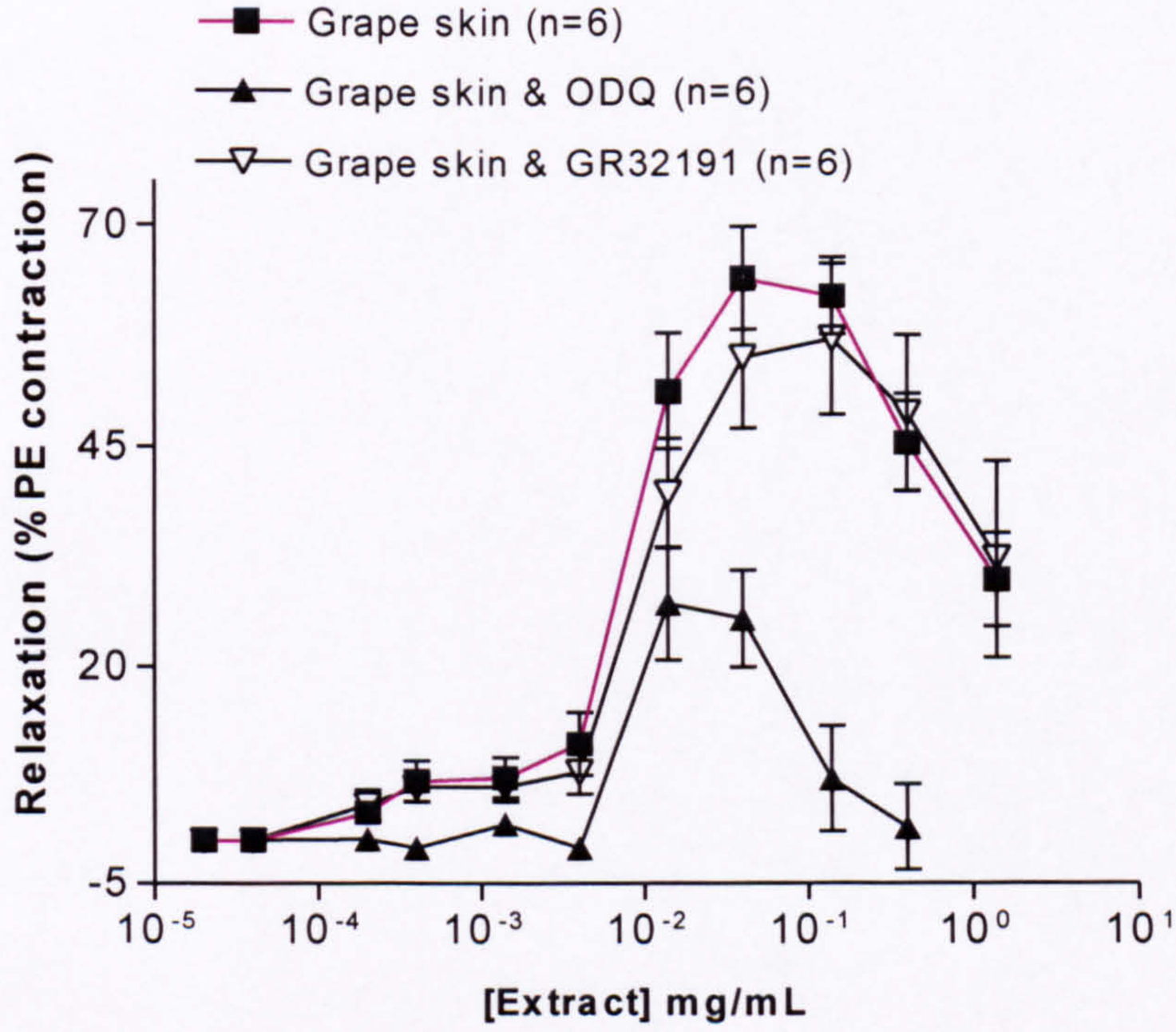
Graph 5 Grape skin extract induced vasorelaxation in the presence of L-NAME and after removal of the endothelium

Values expressed as mean % relaxation ± standard error of mean.



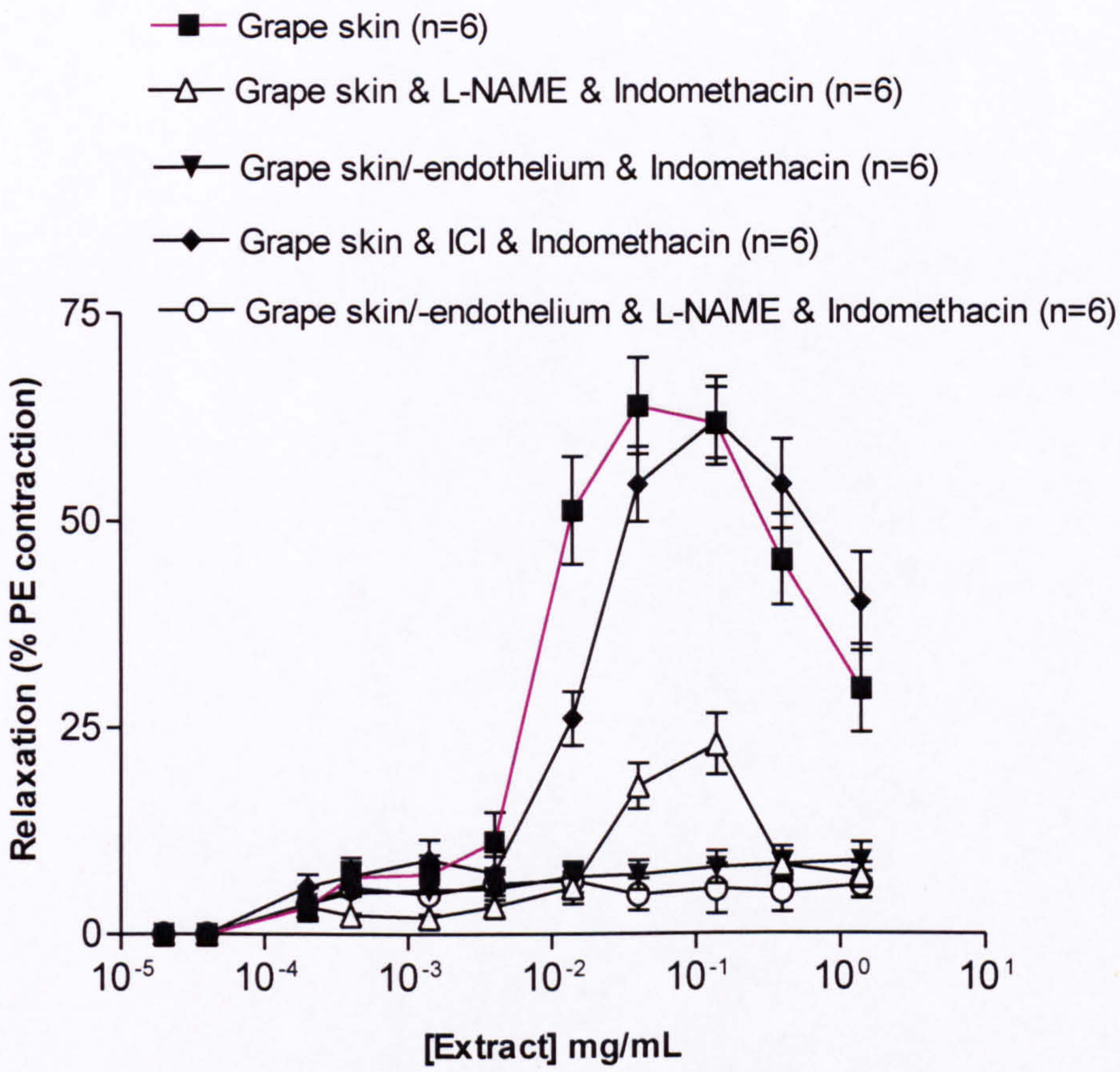
Graph 6 Grape skin extract induced vasorelaxation in the presence of indomethacin & ICI 192,605

Values expressed as mean % relaxation ± standard error of mean.



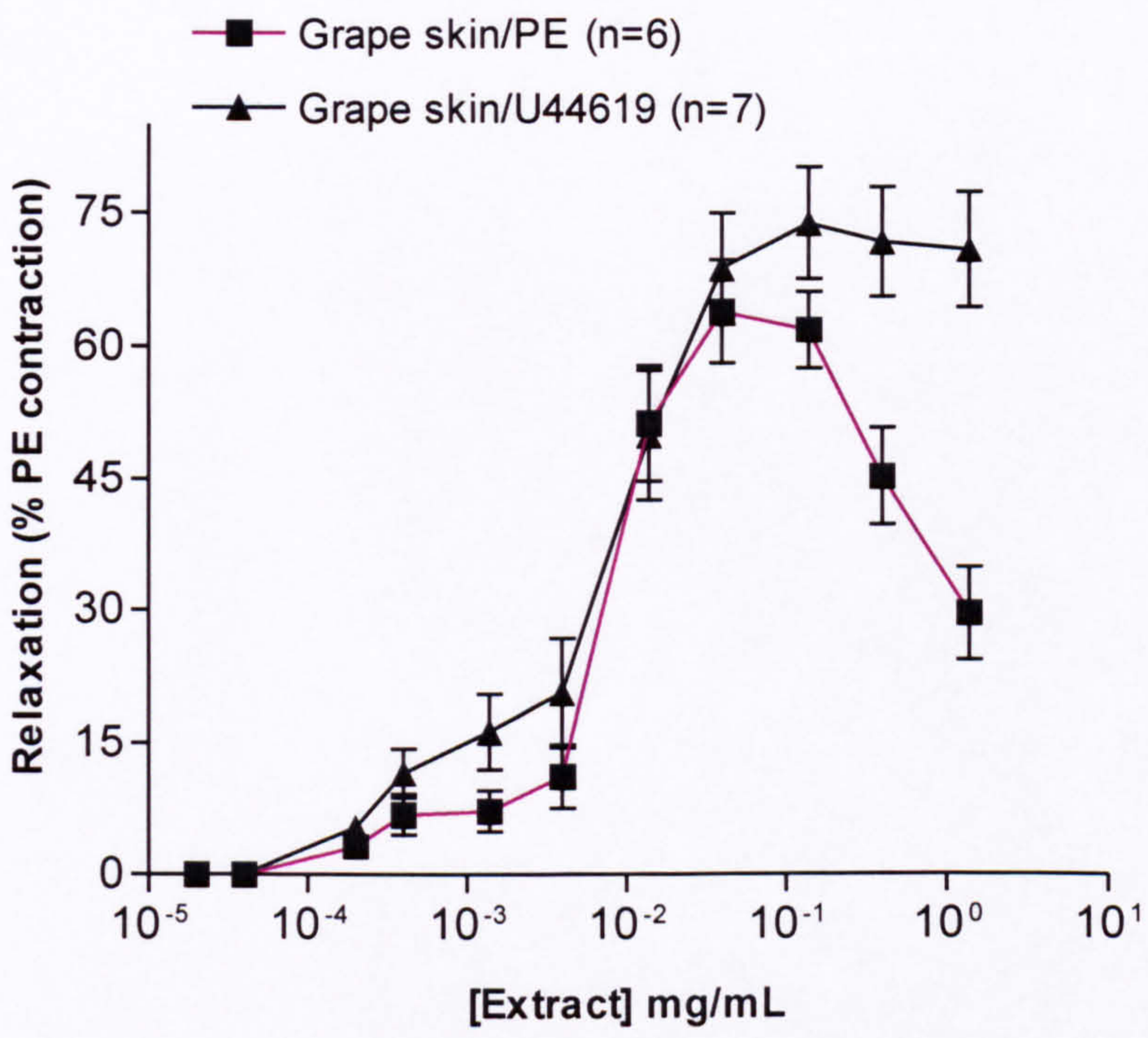
Graph 7 Grape skin induced vasorelaxation in the presence of ODQ and GR32191

Values expressed as mean % relaxation ± standard error of mean.



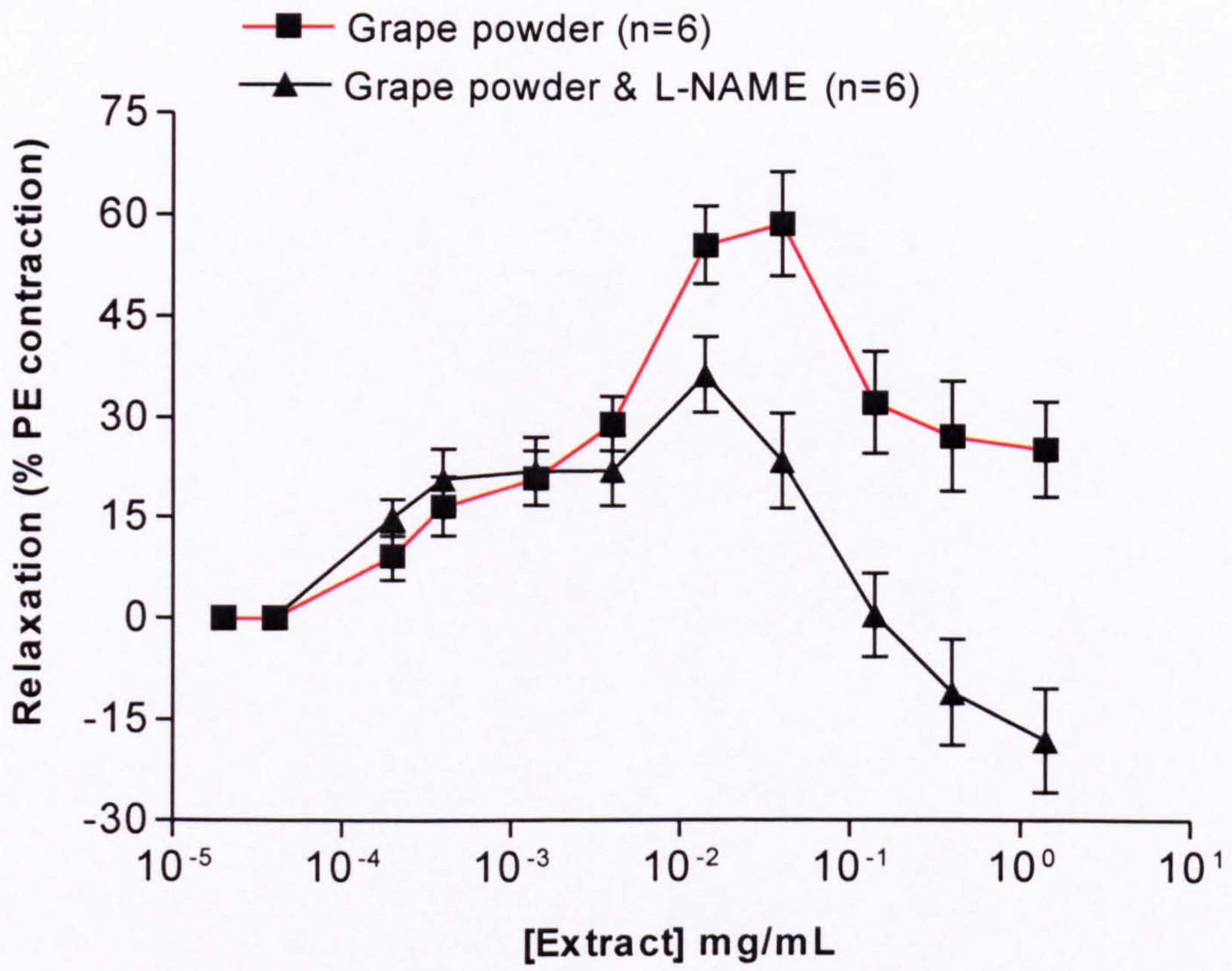
Graph 8 Grape skin extract induced vasorelaxation in the presence of L-NAME, indomethacin, ICI 192,605 and endothelial removal

Values expressed as mean % relaxation ± standard error of mean.



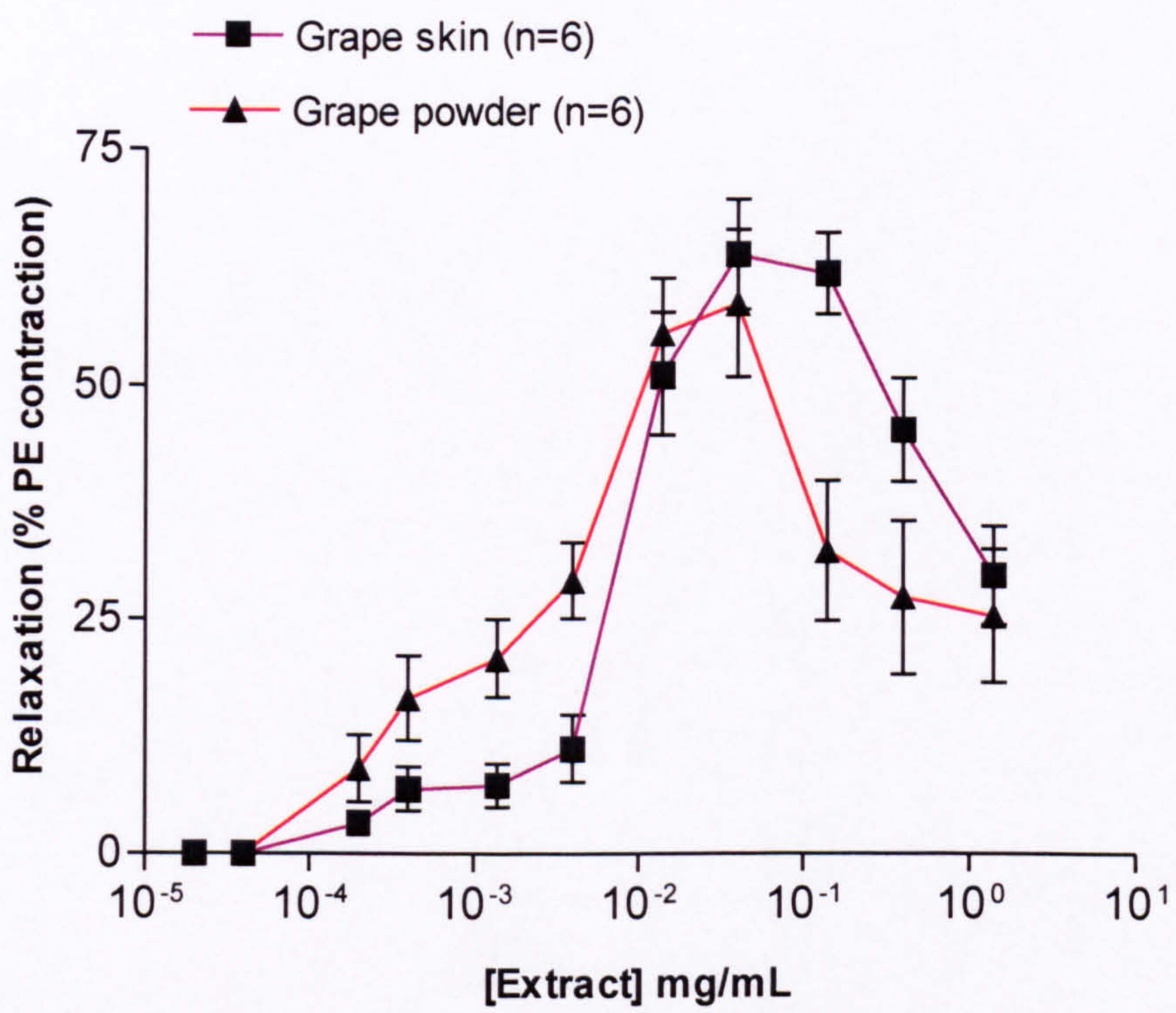
Graph 9 Grape skin extract induced vasorelaxation following preconstriction with PE & U46619

Values expressed as mean % relaxation ± standard error of mean.



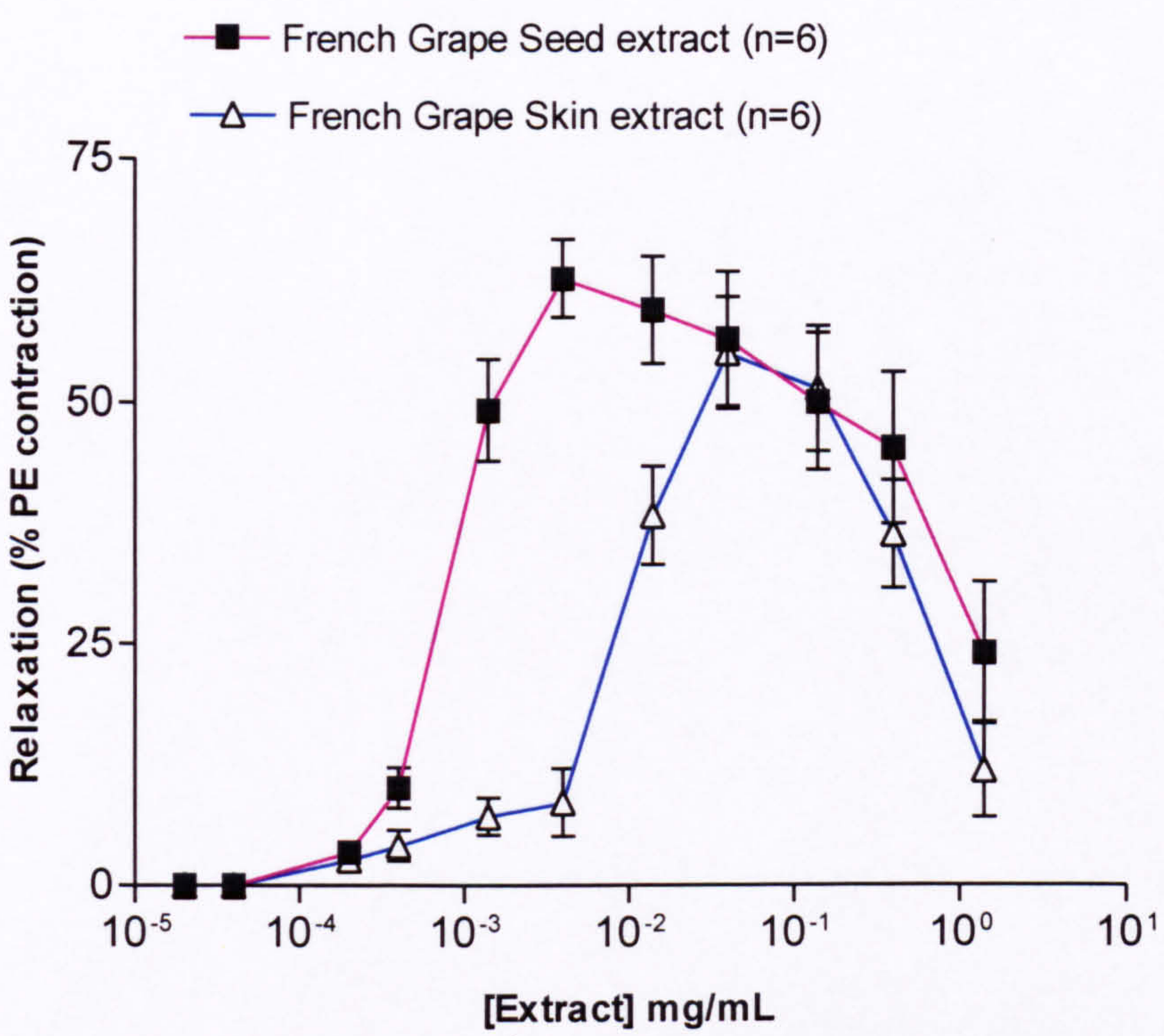
Graph 10 Grape powder extract induced vasorelaxation, and in the presence of L-NAME

Values expressed as mean % relaxation ± standard error of mean.



Graph 11 Comparison of the vasorelaxation induced by grape skin and grape powder extracts

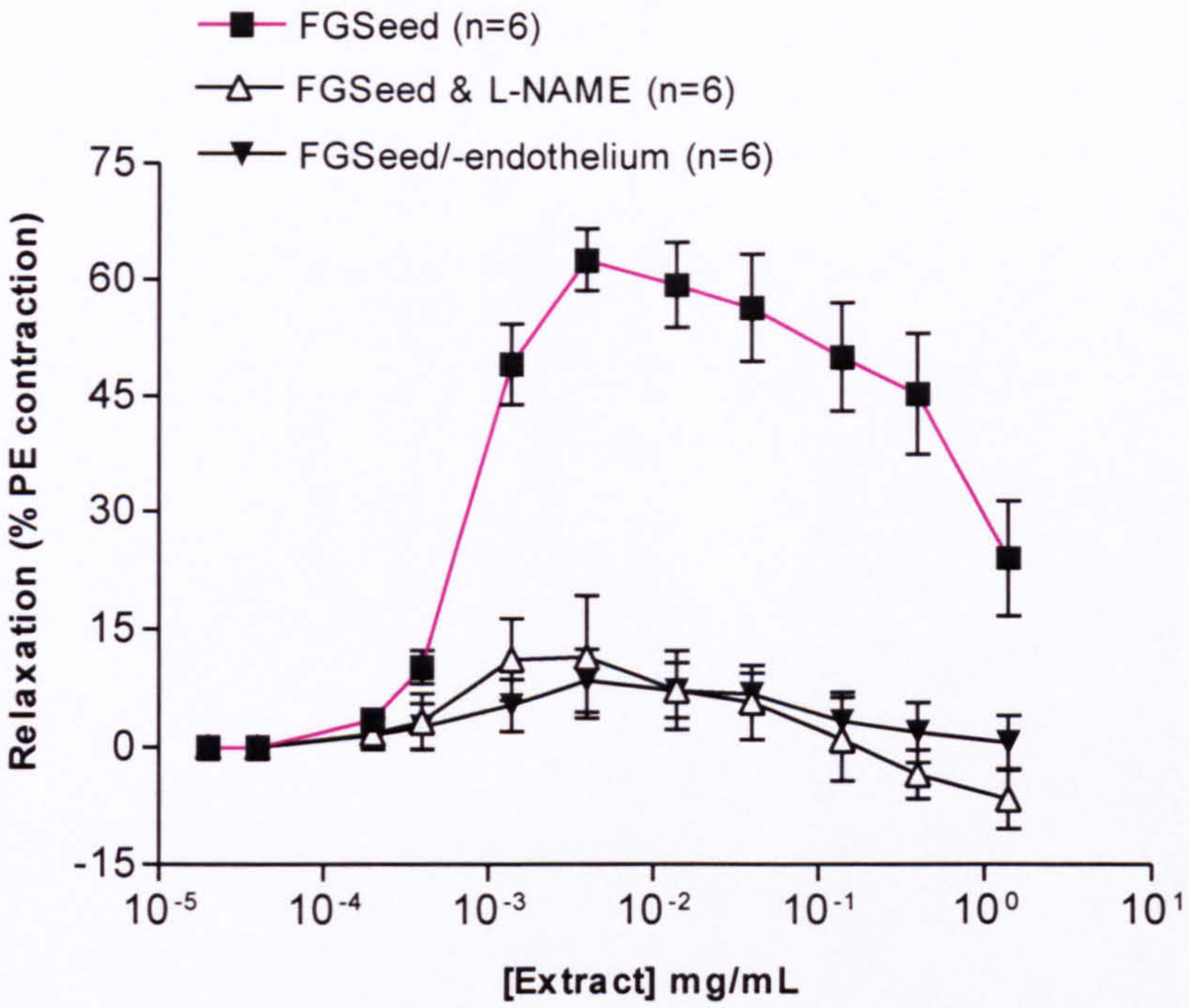
Values expressed as mean % relaxation ± standard error of mean.
For individual maximum relaxation values see Table 15.



Graph 12 Comparison of the vasorelaxation induced by French grape seed and skin extracts

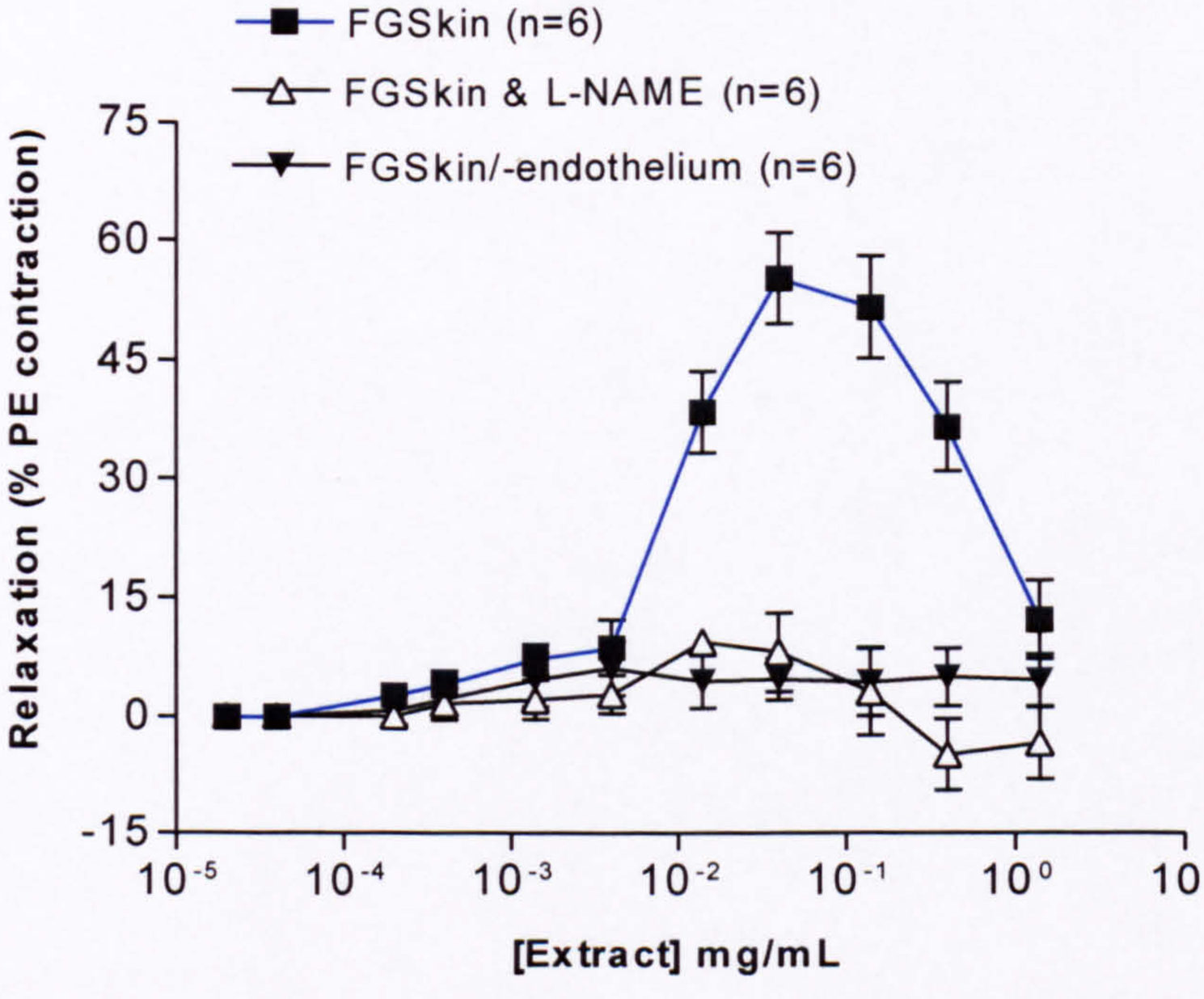
Values expressed as mean % relaxation ± standard error of mean.

For individual maximum relaxation values see Table 15.



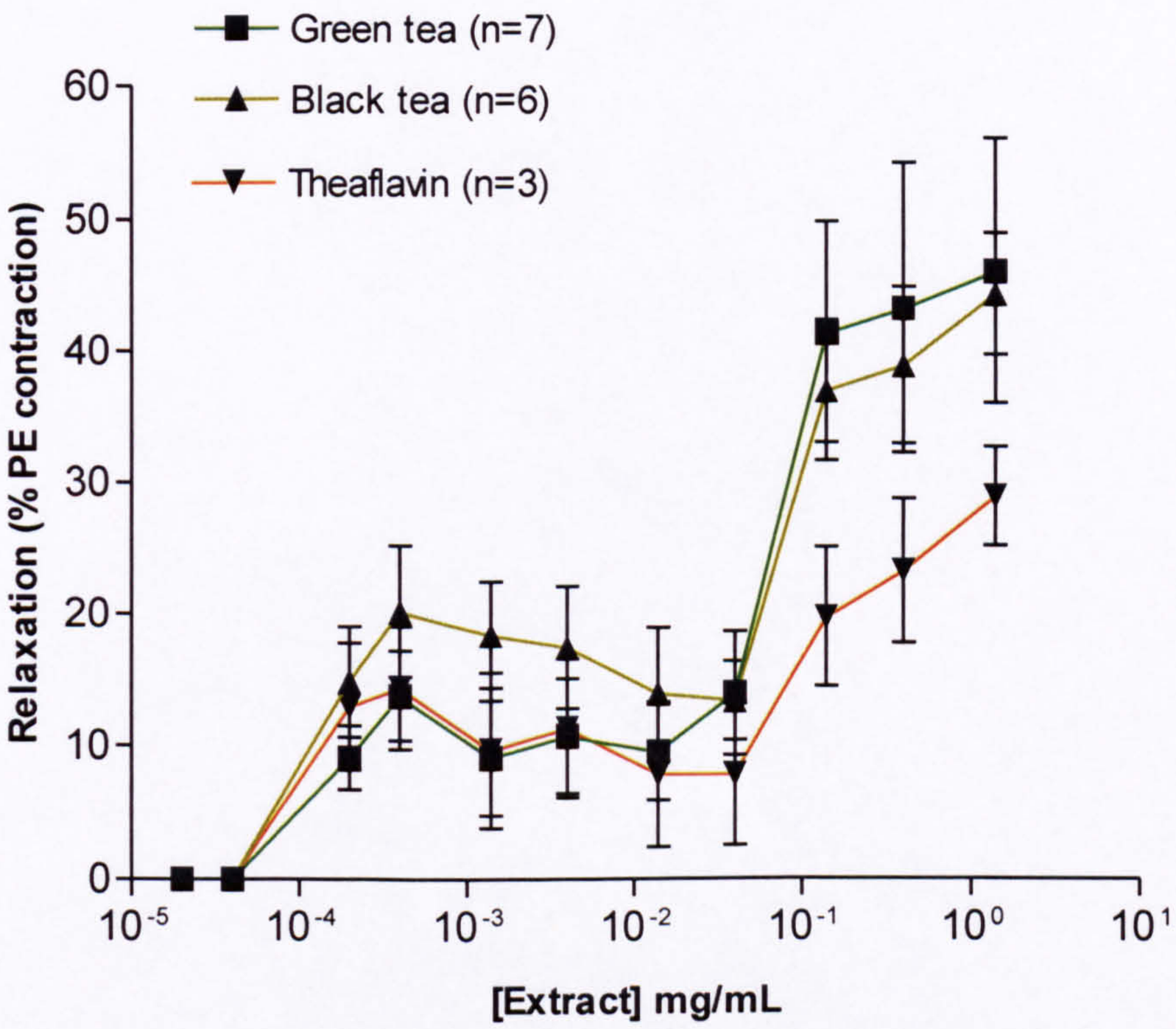
Graph 13 French grape seed (FGSeed) extract induced vasorelaxation in the presence of L-NAME and after removal of the endothelium

Values expressed as mean % relaxation ± standard error of mean.



Graph 14 Vasorelaxation induced by French grape skin (FGSkin) extract in the presence of L-NAME and after removal of the endothelium

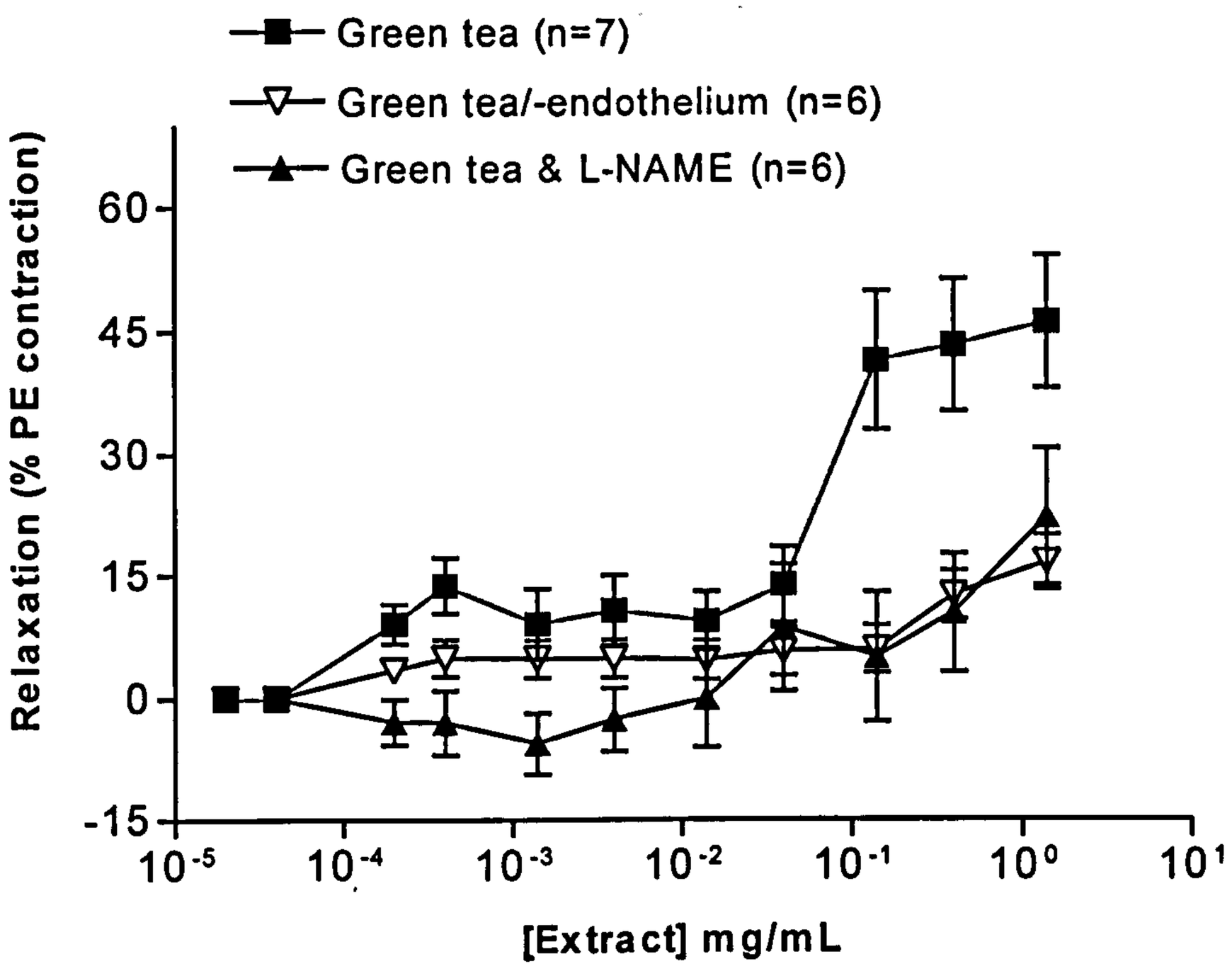
Values expressed as mean % relaxation ± standard error of mean.



Graph 15 Comparison of the vasorelaxation induced by green, black and theaflavin extracts

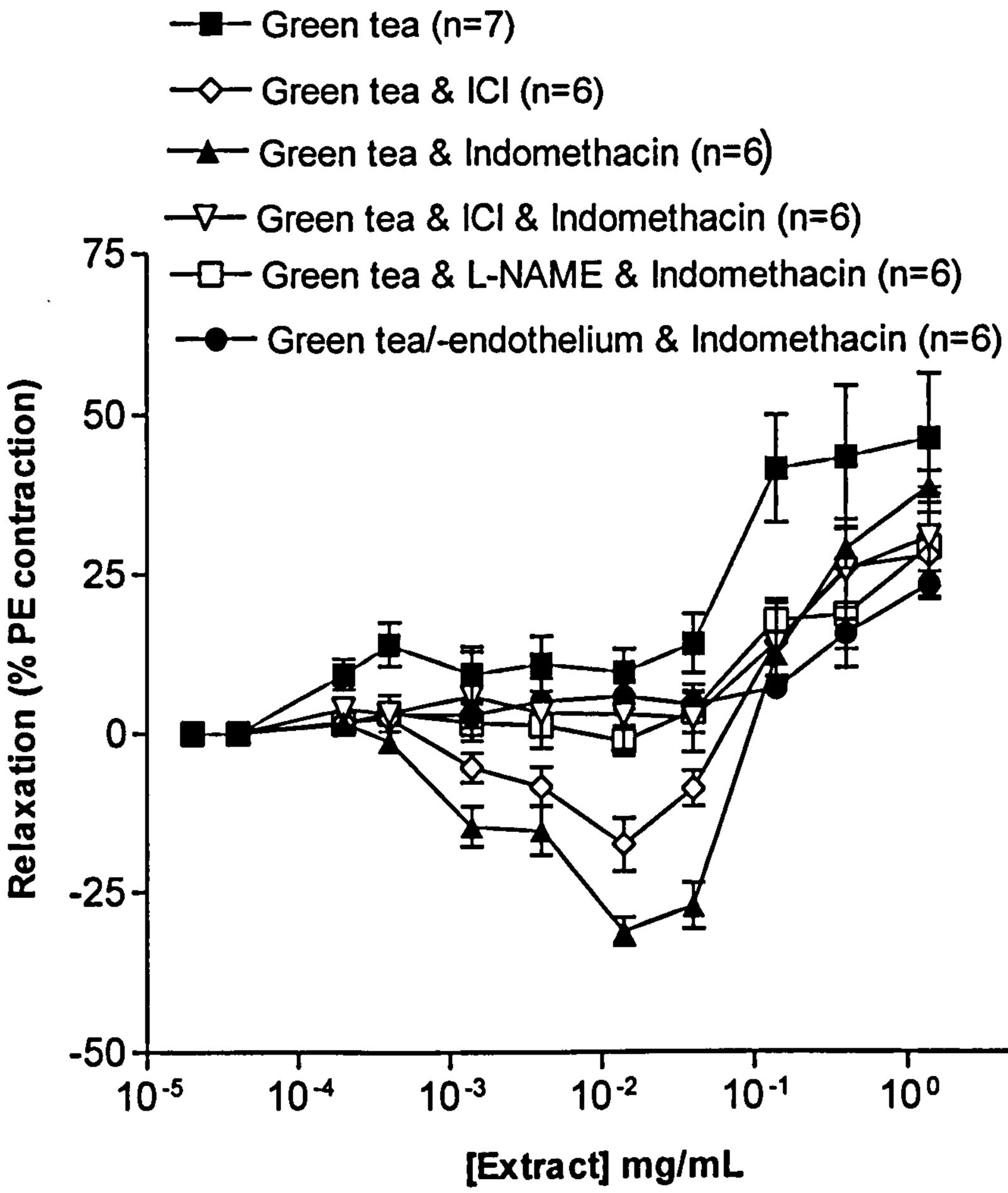
Values expressed as mean % relaxation ± standard error of mean.

For individual maximum relaxation values see Table 15.



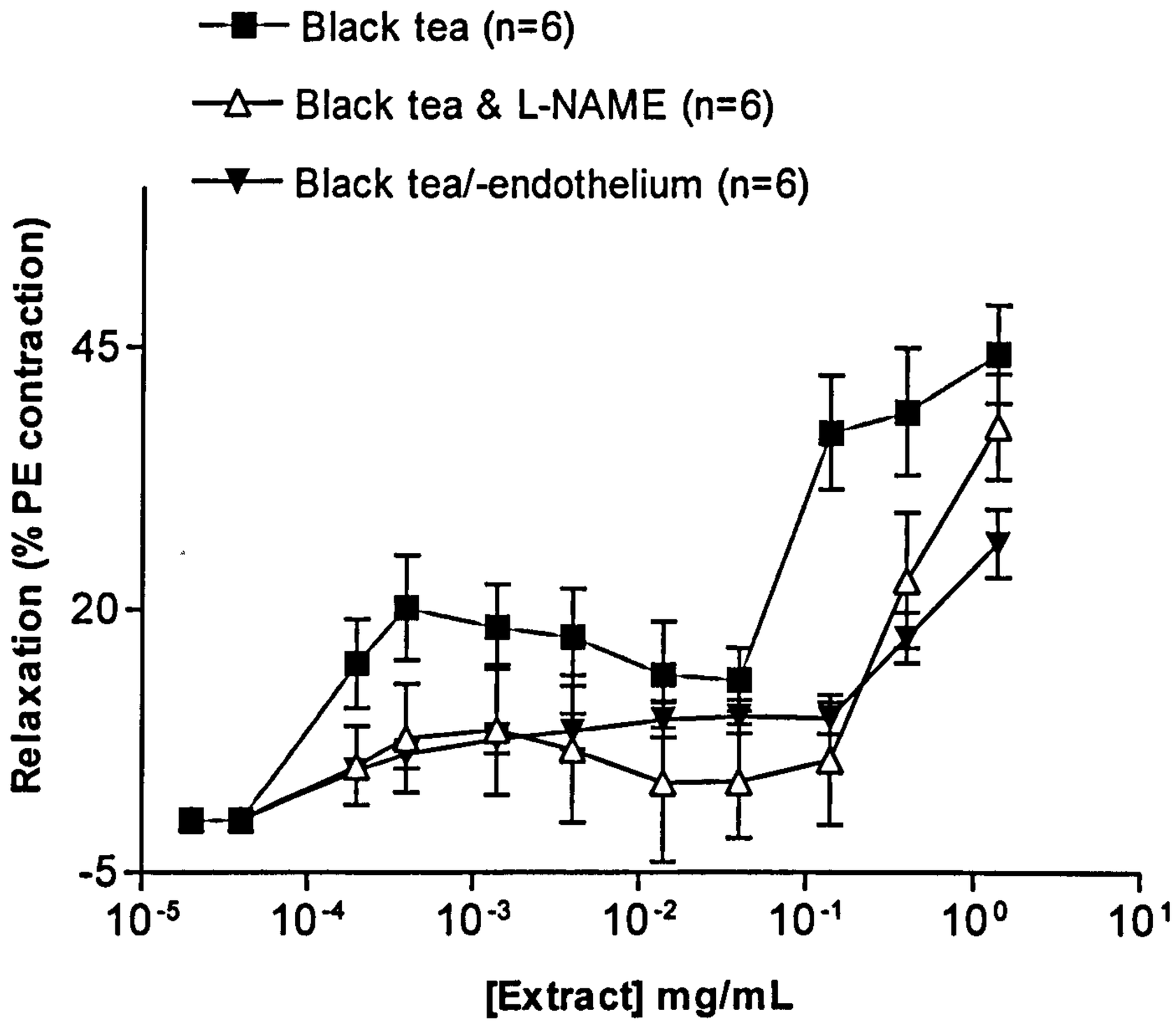
Graph 16 Green tea extract induced vasorelaxation in the presence of L-NAME and following the removal of the endothelium

Values expressed as mean % relaxation ± standard error of mean.



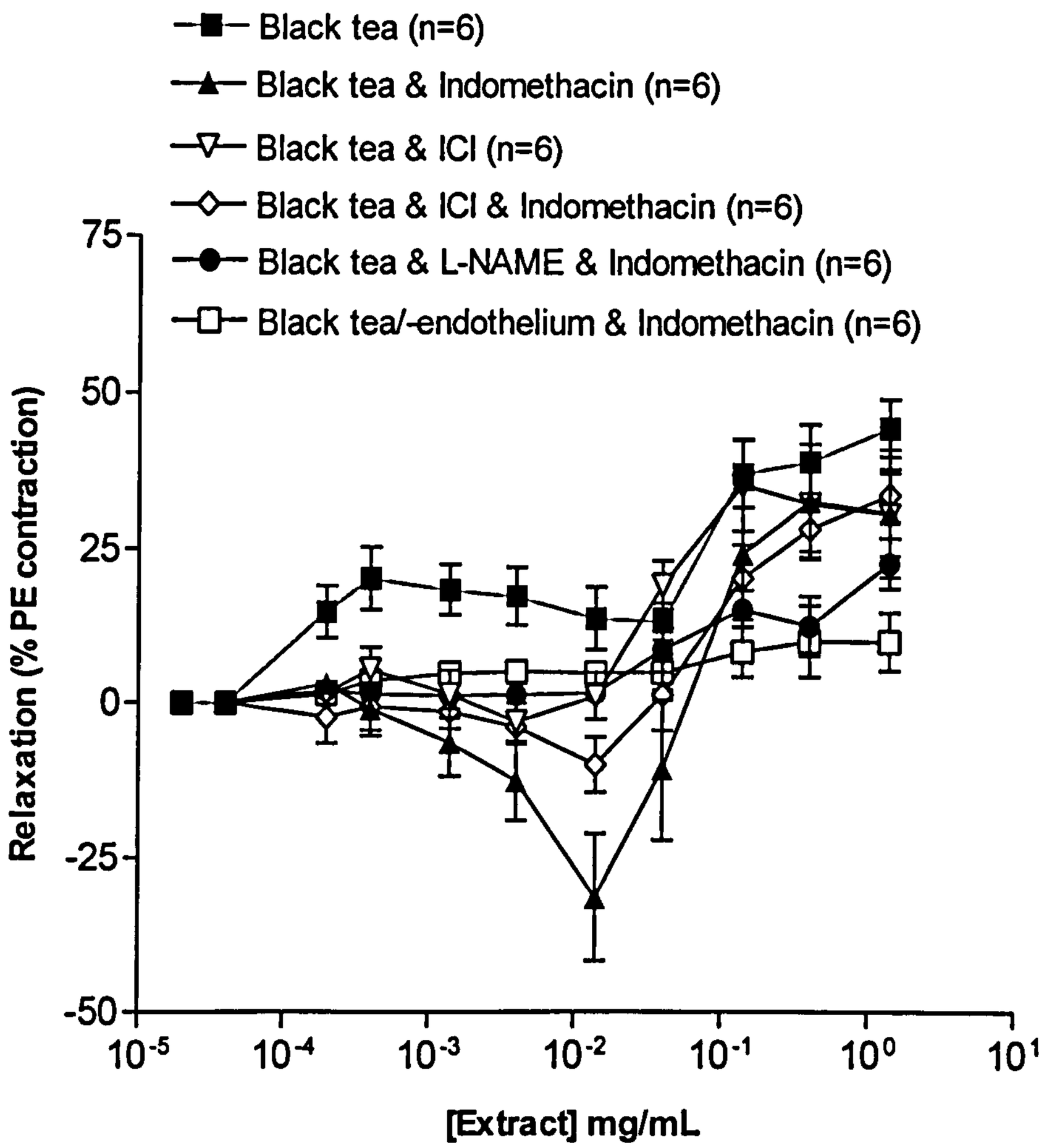
Graph 17 Green tea extract induced vasorelaxation, in the presence of L-NAME, indomethacin, ICI 192,605, & endothelial removal in combination

Values expressed as mean % relaxation ± standard error of mean.



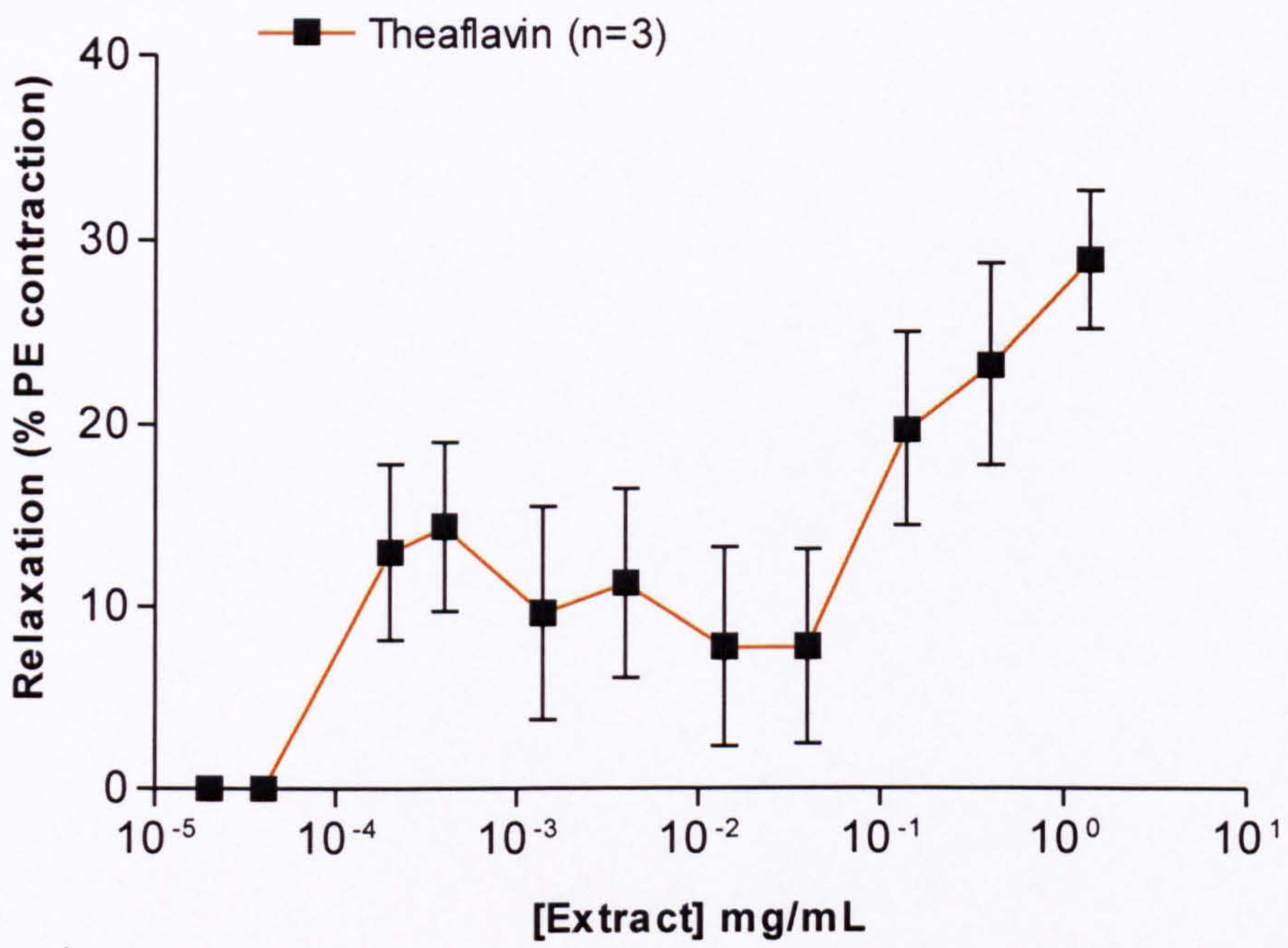
Graph 18 Black tea extract induced vasorelaxation, in the presence of L-NAME and following removal of the endothelium

Values expressed as mean % relaxation ± standard error of mean.



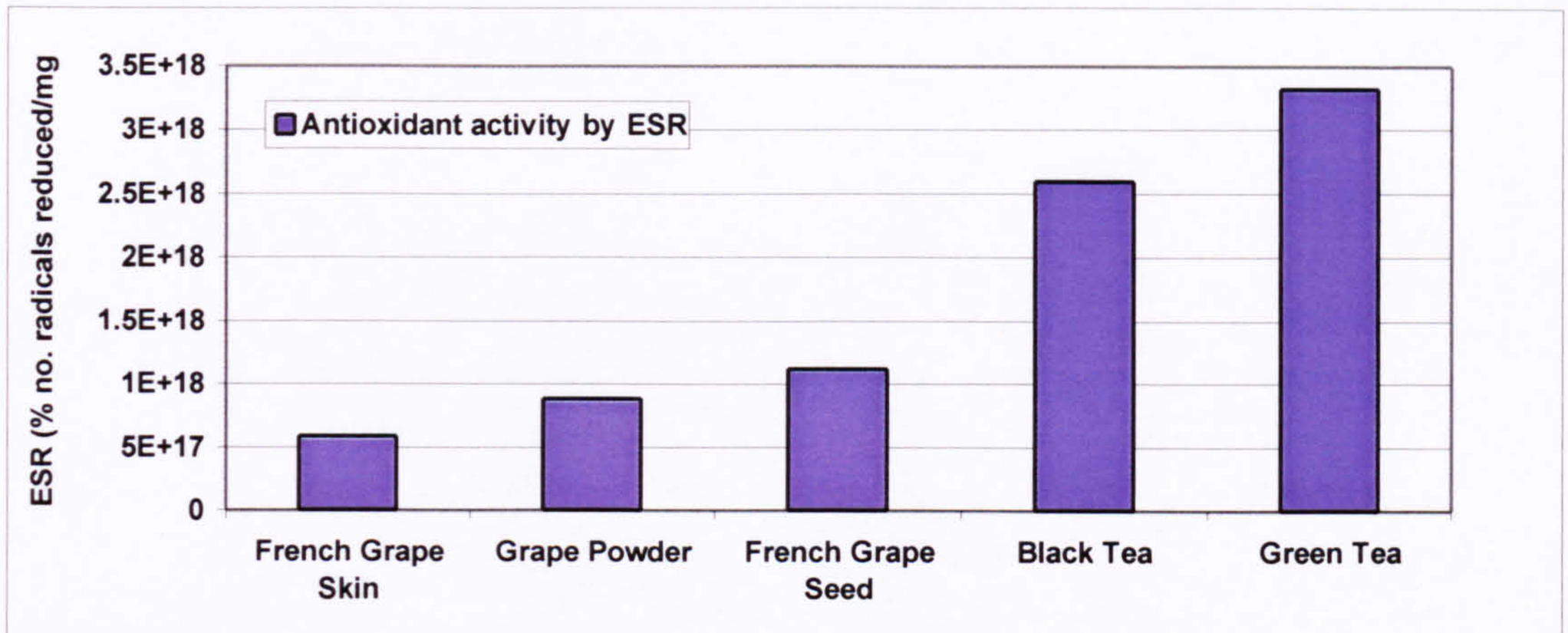
Graph 19 Black tea extract induced vasorelaxation, the presence L-NAME, indomethacin,ICI 192,605 & endothelial removal in combination

Values expressed as mean % relaxation ± standard error of mean.

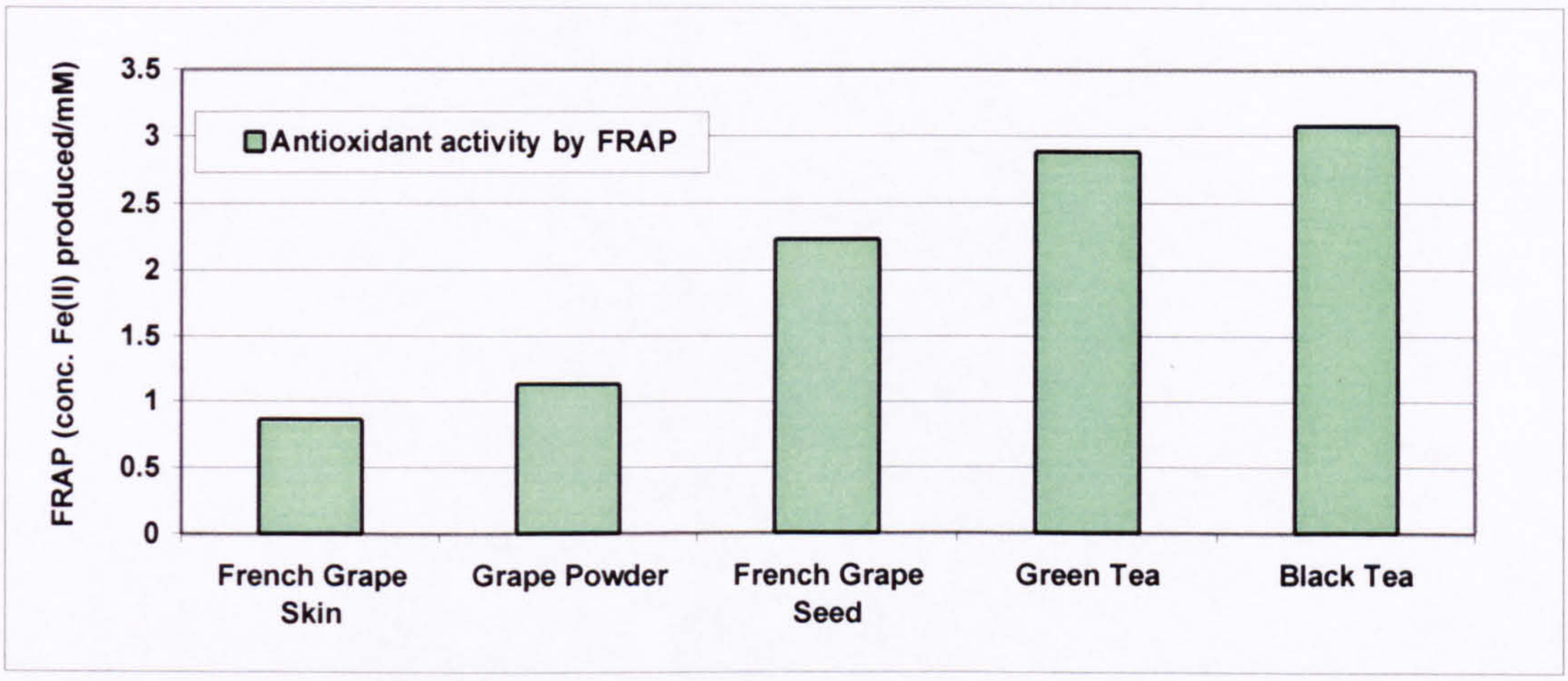


Graph 20 Vasorelaxation induced by theaflavin extract in preconstricted aortic vessels

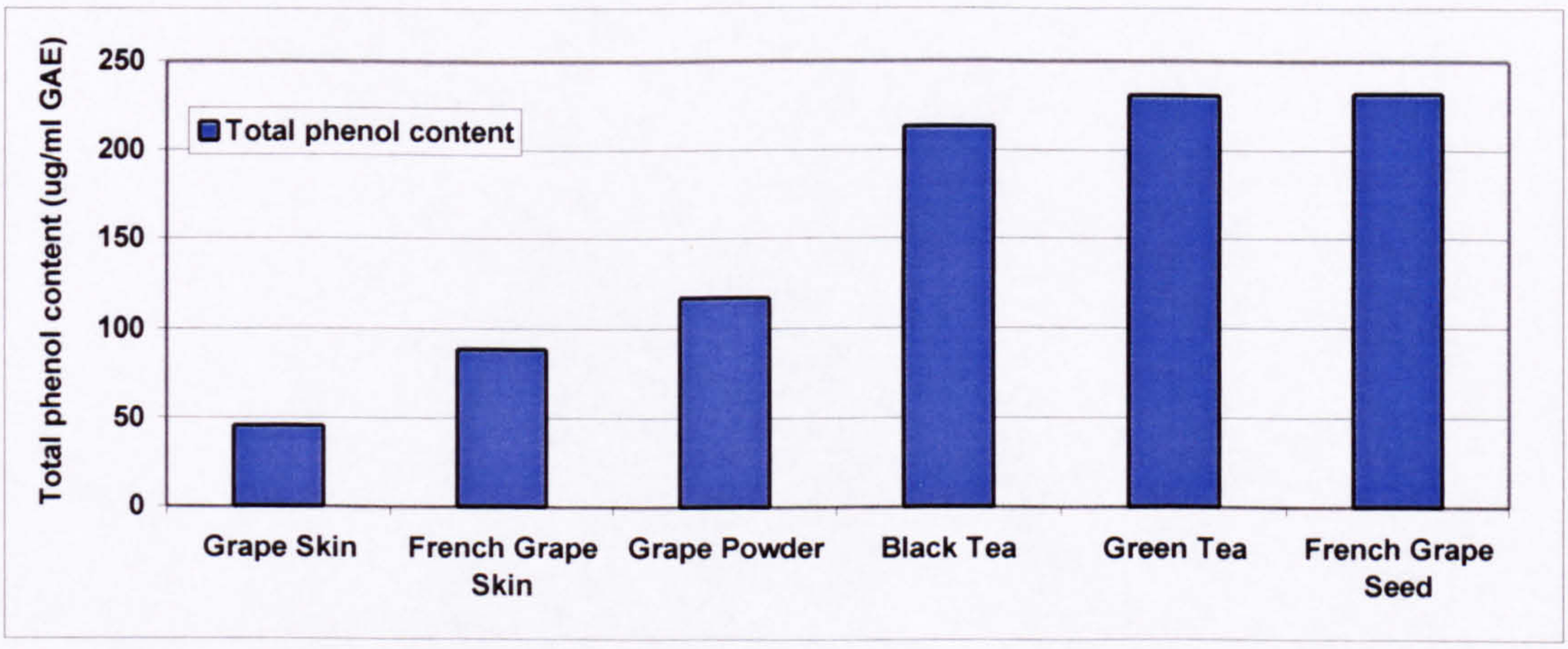
Values expressed as mean % relaxation ± standard error of mean.



Graph 21 Antioxidant activity of grape and tea fractions by ESR

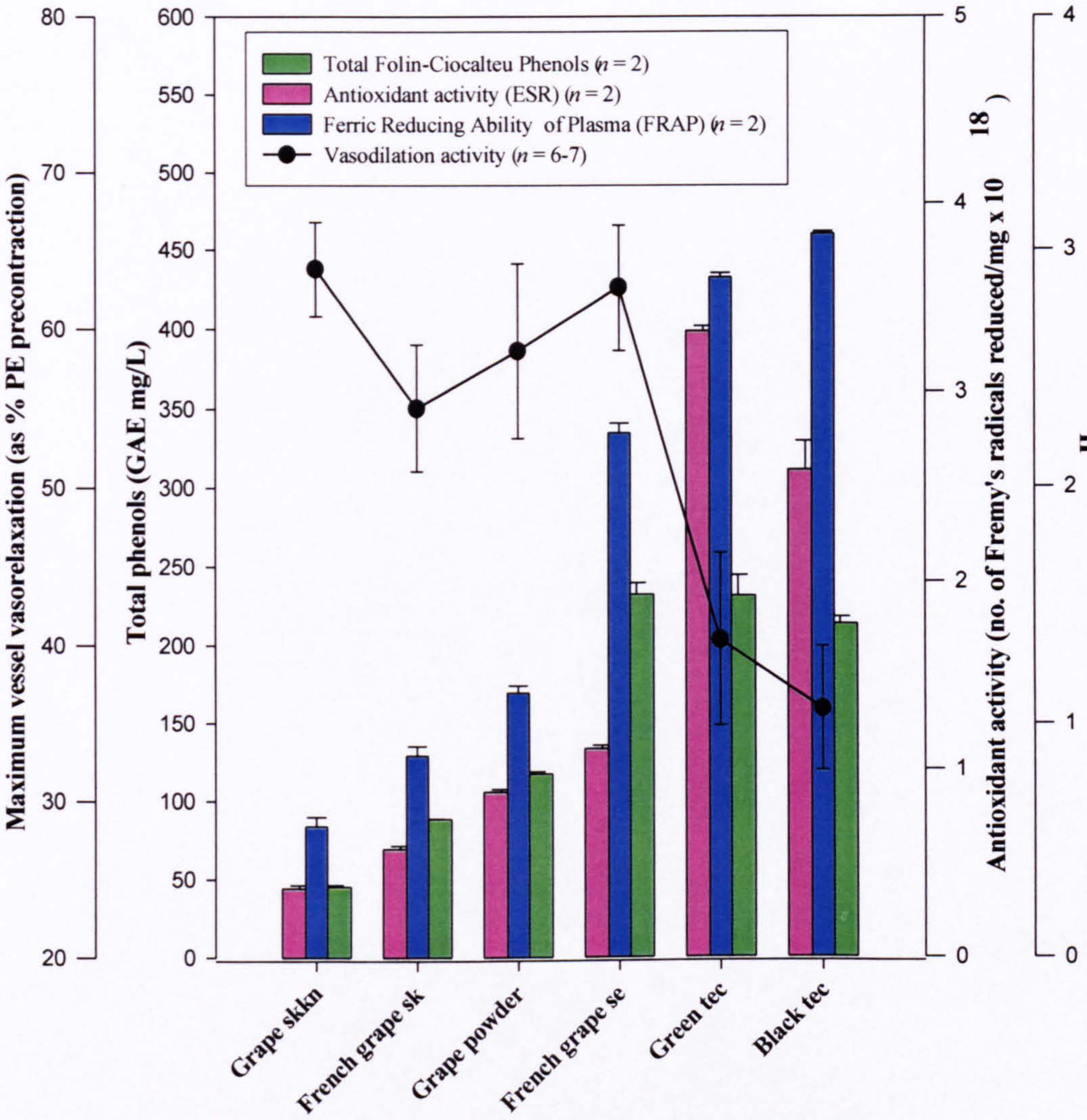


Graph 22 Antioxidant activity by FRAP analysis of grape and tea extracts



Graph 23 Total phenolic content of grape and tea extracts

All samples run in duplicate (n=2) and expressed as mean values. For individual assay values for each extract see Table 17.



Graph 23 Relationship between vasodilatory, antioxidant activity and total phenol content of grape and tea extracts.
All antioxidant assay values expressed as mean of n =2. See Table 17 for individual assay values.
Vasodilatory maximum relaxation values expressed as mean % relaxation +/- standard error of mean.
For individual extract maximum relaxation values see Table 15.

Chapter 4 Vasodilation and antioxidant activities of green tea epicatechins

4.1 Introduction

Tea, especially that of green tea, has received attention as an alleged protective agent against cardiovascular disease, stroke and several types of cancer. Tea, like red wine, is recognised for its high content of polyphenols, which account for more than 35% of its dry weight (Serafini *et al.,* 1996), and are believed to be responsible for its alleged cardio-protective effect *in vivo*, functioning principally as potent antioxidants and effective free radical scavengers, as discussed previously in Chapter 1.

Catechins are a group of natural polyphenols found in green teas usually accounting for 30 – 42% of the dry weight of the solids in brewed tea (Yang, 1999). Green tea is manufactured by drying fresh tea leaves, therefore its composition resembles that of the fresh tea leaf, containing the characteristic polyphenolic compound, (+)-catechin and its isomer (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-catechin gallate (CG) and (-)-epigallocatechin gallate (EGCG) (Balentine *et al.,* 1997). EGCG is the most abundant constituent of green tea and has to date received the most attention (Balentine *et al.,* 1997). *In vitro* and *in vivo* experiments have shown that catechins maybe potentially beneficial to human health. Green tea epicatechins have been previously shown to be effective antioxidants (Vinson *et al.,* 1995, 2000; Wiseman *et al.,* 1997; Gardner *et al.,* 1998), scavengers of free radicals (Salah *et al.,* 1995), possess anti-carcinogenic properties (Mukhtar *et al.,* 1992), be vasodilators *in vitro* (Huang *et al.,* 1998, 1999) and prevent platelet adhesion and aggregation (Vinson & Dabbagh, 1998; Mizugaki *et al.,* 2000) all mechanisms which may work in synergy to prevent or retard the development of CHD and several types of cancers.

To date, literature investigating the effect of green tea epicatechins on vascular function *in vitro* is scarce. Present evidence suggests that the vasodilator activity of green tea epicatechins occurs via endothelium-dependent and –independent mechanisms (Huang *et al.*, 1998). This has been attributed to the release of NO from vascular endothelium, the inhibition of calcium influx and the NO activation of Ca^{2+} sensitive K^+ channels (Huang *et al.*, 1999).

This study will determine the vasodilator effects of range green tea catechin and epicatechin derivatives *in vitro,* investigate their mechanisms and pathway of vasodilation in the cardiovascular system and relate their vasodilatory to their antioxidant activity and total phenolic content.

4.2 Materials & Methods

To investigate the vasodilatory and antioxidant activities of green tea epicatechins, several complementary methods were employed in the course of this study. These are explained in detail in Chapter 2.

4.2.1 Vasodilation activity

In brief, isolated rabbit aortic vessels were suspended in standard 10 ml organ baths (Figure 11), filled with Krebs buffer solution, continuously oxygenated with 16% O_2, 5% CO_2 and 79% N_2 and maintained at 37°C, to mimic the internal environment. Vessels were placed under an optimal resting tension of 2 g. Vessel contraction/relaxation was measured via an isometric force transducer.

After an equilibration period, vessels were twice maximally contracted with 50 mM KCl and washed out thoroughly with Krebs solution. To investigate the role of the endothelium in the vasodilation response, in some experiments the luminal endothelium was removed prior to the start of the experiment by the gentle mechanical rubbing of the vessels with ridged

forceps. After washing and returning to initial baseline tension, vessels with and without functional endothelium were precontracted submaximally with PE (10^{-7} M) and relaxed with ACh. Vessels were considered to be denuded of functional endothelium when there was no relaxation response to 1μM ACh (see Figure 13).

Once a stable plateau had been reached, CCRC's for the green tea epicatechin derivatives (10^{-8} – $3x10^{-4}$ M) were obtained. The mechanisms of vasodilation were investigated in the presence of the NO synthase inhibitor, L-NAME (100 μM), added to selected vessels 40 min prior to PE-induced tone.

4.2.2 Antioxidant activity

Antioxidant activity and phenolic and catechin content of the green tea epicatechins tested in this study, were determined using specific spectral and colorimetric assays discussed in detail in Chapter 2. In brief:

The plasma antioxidant capacity of the green tea epicatechins was estimated from its ferric reducing ability (FRAP) analysed at 593 nm. The development of an intense blue colour resulting from the conversion of a Fe(III)-2,4,6-Tri-2-pyr-idyl)-s-triazine (TPTZ) complex to Fe(II)-TPTZ being directly related to the amount of reductant present in each epicatechin sample. Results are expressed as the concentration of Fe(II) produced/mM (Benzie & Strain, 1996).

The total phenolic content of the green tea epicatechin derivatives was determined and expressed as gallic acid equivalents/mL (GAE) using the Folin-Ciocalteu method (Singleton & Rossi, 1965). Sample absorbance read at 765 nm.

The total catechin content of each green tea epicatechin derivative was determined using the method of Kivitis *et al* (1997). Total content is determined by the addition of a DMACA in a methanol/perchloric acid/water

mix to the epicatechin derivative. With the addition of the DMACA solution, samples are seen to develop highly specific colour complexes dependent on total catechin content. Absorbance was measured between 604 and 684 nm. Results are expressed as total catechins (g/L).

4.3 Results

4.3.1 Vasodilator effects of green tea epicatechins

The cumulative concentration-response curves (CCRC's) to each epicatechin derivative, from 10^{-8} to $3x10^{-4}$ M, in isolated rabbit aortic vessels preconstricted with PE, is shown in Graph 24. Time matched controls of each epicatechin derivative were also run to demonstrate the effect of each epicatechin on vascular tension as opposed to the natural relaxation response of each vessel with time (see Graph 3). Figure 16 displays a sample vasorelaxation trace of CCRC's to EGCG and CG *in vitro*. The effect of L-NAME and removal of the endothelium on the CG concentration-dependent vasorelaxation response curve is also shown in Figure 16. EGCG, ECG, EGC and CG all induced significant vasodilation in the aortic vessels. EC and catechin did not have a significant effect on vascular tension *in vitro*. EGCG induced the greatest maximum relaxation (40% of PE-induced tone, $P<0.01$), but CG was the most potent epicatechin derivative, inducing maximum vasodilation at $3x10^{-6}$ M ($P<0.01$), as compared to $3x10^{-5}$ to 10^{-4} M for all other epicatechins investigated. The maximum vasorelaxation values (as a % PE-induced tone) and the relaxation threshold values, the lowest concentration that induced a significant relaxation, reflecting differences in potency for each epicatechin and catechin derivative is shown in Table 18.

Table 18 Maximum % relaxation values and threshold concentration values for green tea epicatechin derivatives

Green tea Component	Max % Relaxation	Threshold conc. for relaxation	*n*
Epicatechin (EC)	4.9 ± 1.5^{ns}	0.086 ± 0.1 mM	6
Epicatechin gallate (ECG)	16.3 ± 9.3	0.053 ± 0.07 mM	6
Epigallocatechin (EGC)	17.6 ± 2.0	0.121 ± 0.1 mM	6
Epigallocatechin gallate (EGCG)	39.1 ± 8.6**	0.1 ± 0 mM	6
Catechin	8.6 ± 1.5^{ns}	0.12 ± 0.1 mM	6
Catechin gallate (CG)	21.0 ± 6.1	0.003 ± 0.02 mM**	5

Statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by a Dunnet's Multiple comparisons test **P<0.01, ***P<0.001, ns = not significant, as compared to relevant control. *n* = number of animals. Values expressed as mean % relaxation ± standard error of mean.

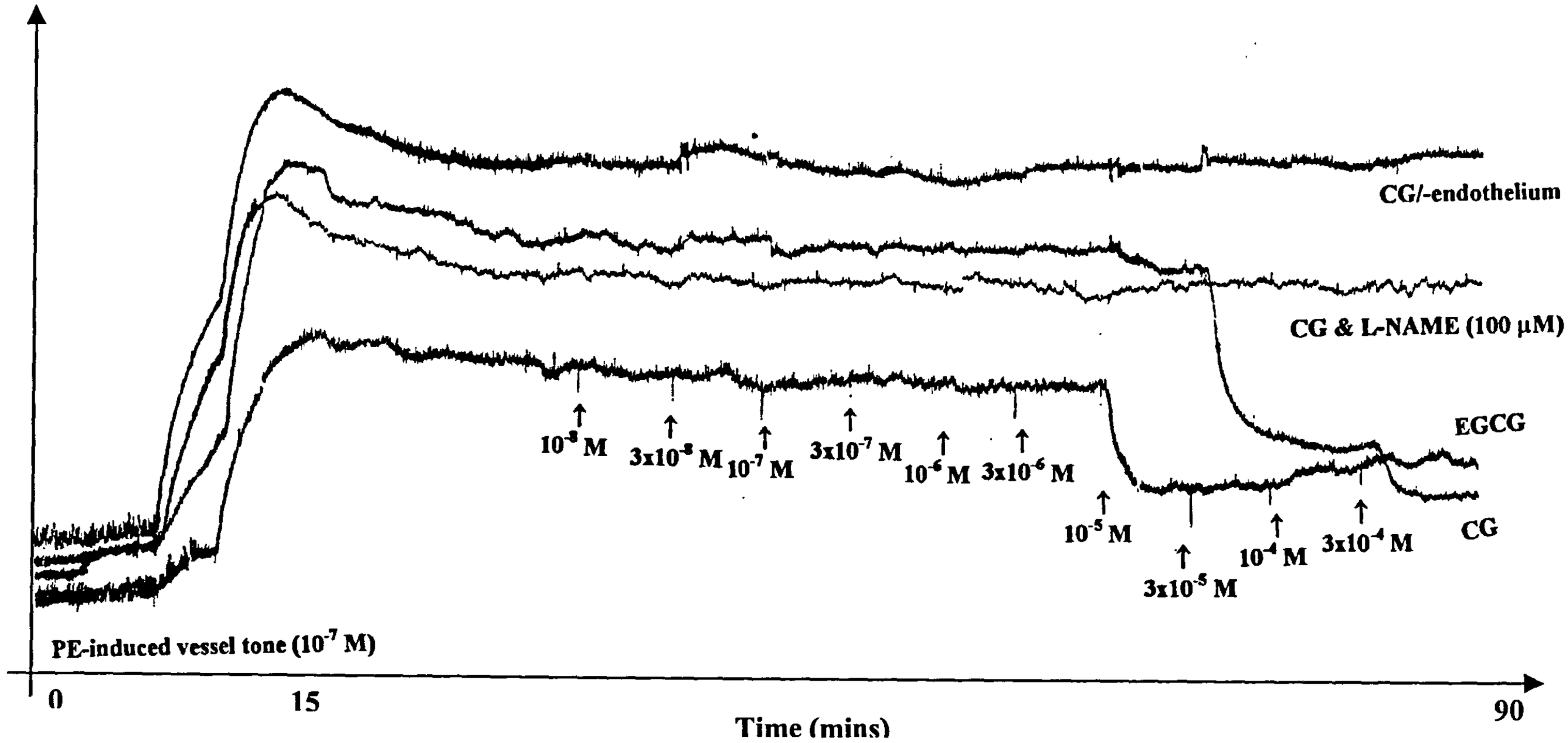


Figure 16 Trace of the CCRC induced by EGCG and CG *in vitro*

4.3.1.1 Epicatechin (EC)

The CCRC to epicatechin (EC) is shown in Graph 25. EC at concentrations ranging from 10^{-8} to $3x10^{-4}$ M had very little effect on vascular tension *in vitro*, inducing a maximum vasodilation at the highest concentration of EC added to the organ bath. L-NAME and removal of the endothelium had no further influence on the vasodilator effect of EC (Graph 25).

4.3.1.2 Epicatechin gallate (ECG)

Vasorelaxation with epicatechin gallate (ECG) in isolated rabbit aortic vessels is shown in Graph 26. The addition of ECG induced a minor vessel vasorelaxation and reversal back to baseline with increasing ECG concentrations. Maximum vasorelaxation was induced at 10^{-4} M producing a biphasic response curve. ECG induced a maximum relaxation value of 16% (Table 18). The presence of L-NAME and removal of the endothelium prior to ECG addition completely abolished the vasorelaxation response, indicating that vasorelaxation is occurring via NO mediated endothelium-dependent mechanisms.

4.3.1.3 Epigallocatechin (EGC)

The CCRC to epigallocatechin (EGC) induced vasorelaxation is shown in Graph 27. EGC induced slight vessel contraction with increasing concentration up to $3x10^{-5}$ M, as compared to the time control. Rapid maximum vasorelaxation was induced only at high vessel concentrations (10^{-4} M). EGC induced a maximum relaxation value of 17.5% of PE-induced tone (Table 18). The removal of the endothelium and the addition of L-NAME also abolished the vasorelaxation response induced by EGC, indicating NO and endothelium-dependent vasorelaxation.

4.3.1.4 Epigallocatechin gallate (EGCG)

Epigallocatechin gallate (EGCG) was identified to be the most effective derivative vasodilator ($P<0.01$) but not the most potent, that is inducing the highest maximum vasodilation value but not at the lowest concentration of all derivatives investigated. The CCRC to EGCG is shown in Graph 28. EGCG induced vessel contraction at 10^{-8} to $3x10^{-6}$, inducing maximum vessel vasodilation at 10^{-5} M. EGCG induced a maximum vasorelaxation value of 40% of PE-induced tone (Table 18). As with previous epicatechins, the presence of L-NAME and removal of the endothelium abolished the concentration dependent vasorelaxation response (Graph 28), indicating NO and endothelium-dependent vasorelaxation.

4.3.1.5 Catechin

Catechin induced vasorelaxation in isolated rabbit aortic vessels, like that of EC, had very little effect on overall vascular tone *in vitro* (Graph 29). Catechin induced a maximum vessel non-significant vasorelaxation value of 8.5% of PE-induced tone (Table 18). This minor degree of vasodilation was mediated via the release of NO and was dependent upon the presence of intact functional endothelium as the vasorelaxation response was abolished by the presence of L-NAME and removal of the endothelium prior the addition of catechin (Graph 29).

4.3.1.6 Catechin gallate (CG)

Finally, catechin gallate (CG) was identified to be the most potent of all the green tea epicatechin derivatives investigated in this study inducing vasorelaxation at a lower derivative concentration than all other catechin and epicatechins. CG induced maximum vessel vasorelaxation at $3x10^{-6}$ M (Graph 30) as compared to relaxation induced at $3x10^{-5}$ to 10^{-4} M by the other epicatechins examined. Lower concentrations of CG (10^{-8} – 10^{-6} M) had no effect on vascular tension *in vitro*. CG induced vasorelaxation up to 21% of

PE-induced tone (Table 18). As with all other epicatechin derivatives investigated in this study, the presence of L-NAME and the removal of functional intact endothelium abolished the vasorelaxation response (Graph 30), indicating NO and endothelium-dependent vasorelaxation.

4.3.2 Antioxidant activities of green tea epicatechins

To investigate the antioxidant ability and the total phenolic and catechin content of the green tea epicatechin derivatives, specific colorimetric and spectral assays were carried out. Antioxidant capacity was determined by FRAP analysis. Table 19 displays the values obtained by each individual epicatechin in each of the 3 assays and Graph 34 demonstrates their correlation to one another.

4.3.2.1 Total phenolic content

The total phenolic colorimetric assay is a non-specific test which identifies the presence of gallic acid groups, with all results being expressed as gallic acid equivalents (GAE) mg/L (Singleton & Rossi, 1965). EGCG is identified to have the highest total phenol content (Graph 31) with a content of 238 mg/L of total phenols (Table 19), followed closely by catechin, ECG and EGC. EC was identified to have the overall lowest phenol content with only 68.5 mg/L of total phenols present.

4.3.2.2 Total catechin content

The total catechin colorimetric assay is a specific test which identifies the presence of 1,3-dihydroxy-benzene structures. Catechins react with the DMACA solution (previously discussed, Chapter 2) to form highly specific colour condensation products, ranging from pale yellow to dark blue with increasing catechin content (Kivitis *et al.*, 1997). All results are expressed as total catechins present in each sample (g/L). EGC was had the highest total catechin content (197 g/L), followed closely by EGCG (160 g/L). EC and CG

had the overall lowest catechin levels, 66 g/L and 64 g/L total catechins respectively (Graph 32, Table 19).

4.3.2.3 Ferric reducing ability (FRAP)

Antioxidant capacity of each of the green tea epicatechin derivatives has been determined by the ferric reducing ability or "antioxidant power" of each derivative. Results are expressed as the concentration of Fe(II) produced by each green tea derivative during its reduction from Fe(III). EGCG was identified to have the greatest antioxidant activity as compared to the other epicatechin derivatives (Graph 33), with EC having the least activity. EGCG produced 2.6 mM Fe(II) produced as compared to only 0.65 mM Fe(II) produced by EC (Table 19).

Graphs 34 and 35 demonstrate the relationship between the FRAP-based antioxidant activity, total phenolic and catechin content and the vasodilatory ability of the green tea catechin and epicatechin derivatives. A significant positive correlation ($r = 0.93$, $P<0.01$, $P = 0.01$) between vasodilator activity and antioxidant activity by FRAP analysis was determined. The high r value of this relationship indicates that the two variables are truly related and increase together. However, no statistically significant correlation was demonstrated between vasodilatory activity of the green tea derivatives and total phenol ($r = 0.67$, $P = 0.14$) and catechin content ($r = 0.30$, $P = 0.57$).

4.4 Discussion

In terms of vasodilation induced in isolated rabbit aortic vessels, CG was identified to be the most potent derivative *in vitro* and EC to be the least. In all cases, vasorelaxation was seen to occur via the release of NO and was dependent upon the presence of intact functional endothelium. Green tea derivatives were also determined to be effective antioxidants with a potency directly correlated with their overall vasodilatory activity.

Table 19 Antioxidant activity and total phenol and catechin content of green tea epicatechin derivatives

	n	Total Phenol[a]	Total Catechin[b]	Ferric reducing ability[c]
EC	2	68.5 ± 1.3	66.1 ± 0.2	0.65 ± 0.2
ECG	2	170.8 ± 2.4**	94.5 ± 0.7**	1.36 ± 0.2*
EGC	2	177.4 ± 2.1**	197.0 ± 3.5**	1.61 ± 0.3*
EGCG	2	238.0 ± 3.9***	160.3 ± 1.6**	2.59 ± 0.1**
Catechin	2	190.8 ± 0.8**	162.2 ± 1.4**	1.36 ± 0.2*
CG	2	116.6 ± 0.6**	64.4 ± 1.8	1.32 ± 0.2*

EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; CG, catechin gallate

[a]Total phenol content of the tea derivatives as determined using the Folin-Ciocalteu method, expressed as mean mM gallic acid equivalents (GAE) mg/L. [b]Total catechin content expressed as mean g/L catechin. [c]Ferric reducing ability of plasma (FRAP) antioxidant capacity expressed as the mean concentration of Fe(II) produced/mM. All samples run in duplicate ($n = 2$) and expressed as mean values. Statistical analysis was carried out using an ANOVA followed by a Dunnet's Multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001 as compared to control.

4.4.1 Vasodilation activity

The flavan-3-ols are a subclass of flavonoids (Haslam, 1998). They range from the simple monomers (+)-catechin and its isomer (-)-epicatechin, to the oligomeric and polymeric proanthocyanidins (Balentine *et al.,* 1997). In addition to forming complexes with other catechins, they maybe hydroxylated to form gallocatechins such as EGC, and undergo esterification with gallic acid to form catechin gallates such as ECG and CG (Robb & Brown, 2001).

Red wine, previously discussed, is also rich in (+)-catechin and (-)-epicatechin (Burns *et al.,* 2000). Catechin and epicatechin derivatives are found primarily in the seeds of red grapes (Sato *et al.,* 1999). This fact may contribute to the high antioxidant and vasodilatory capacity of the French grape seed investigated in Chapter 3, as compared to the predominately anthocyanin containing grape skin extracts examined. Grapes also contain gallate esters and gallocatechins but only in low levels in red wine (Burns *et al.,* 2000) but may, according to the present study account for a small degree of vasodilation induced by red wines. Green teas contain (+)-catechin, EC, EGCG, EGC, ECG and CG (Balentine *et al.,* 1997). The major catechin in green teas is EGCG, which accounts for ~37% of the dry weight of green tea (de Pascual-Teresa *et al.,* 2000) and was in this study identified to be a very potent vasodilator and antioxidant. In the present study EC and (+)-catechin were devoid of significant vasodilator activity in the rabbit aorta. This finding is consistent with studies in the rat aorta where catechin and EC had very low vasodilator activity (Andriambeloson *et al.,* 1997; 1998).

EGCG, CG, EGC and ECG all induced vasodilator responses which were inhibited by the presence of the NO synthase inhibitor, L-NAME and removal of the endothelium, demonstrating that vasorelaxation with these catechins and epicatechins was mediated via the release of NO from vascular endothelium and is dependent on the presence of intact functional endothelium. These findings may account for some of the vasodilator activity of green tea but, as discussed previously (Chapter 3), cannot account for all

the dilator activity of the green tea, which also involves non-endothelial factors and prostaglandin-mediated effects. This study has also shown CG to be a very potent vasodilator *in vitro* but also to have low antioxidant activity and low overall phenol content, a pattern opposite to that identified for the other epicatechins investigated. The grape based extracts investigated in Chapter 3 had comparable vasodilatory and antioxidant responses to that of CG. CG is found in low levels in red wine, derived primarily from the grape seeds (Burns *et al.*, 2000) but which has also been identified in the grape skin. CG may therefore, be in part accountable for the potent vasodilation induced by both grape skin and seed extracts. Epicatechin derivatives have also been determined to be vasodilators in rat mesenteric arteries with EGCG being the most effective dilator. Relaxation occurred via endothelium-dependent mechanisms and was mediated via the release of NO.

4.4.2 Antioxidant activity

The previous chapter demonstrated that vasodilation induced by grape and tea based extracts was inversely correlated to their overall antioxidant activity, with grape extracts being good vasodilators but poor antioxidants as compared to tea extracts. This chapter on the other hand has demonstrated that catechins and epicatechins derived from green tea, and which are also present to a smaller extent in black tea, are vasodilators and antioxidants, a feature that is positively correlated. A non-significant relationship was observed between increasing total catechin and phenolic content and increasing vasodilatory ability.

The differences in the statistical results presented between the vasodilatory and antioxidant activities of the grape and tea based extracts of the previous chapter as compared to that of the green tea epicatechin and catechin derivatives presented in this study may be due to genuine differences in the basic chemical composition of the compounds investigated as same standardised methodology was used in both sets of experiments. The grape and tea extracts investigated contained a range of active components in

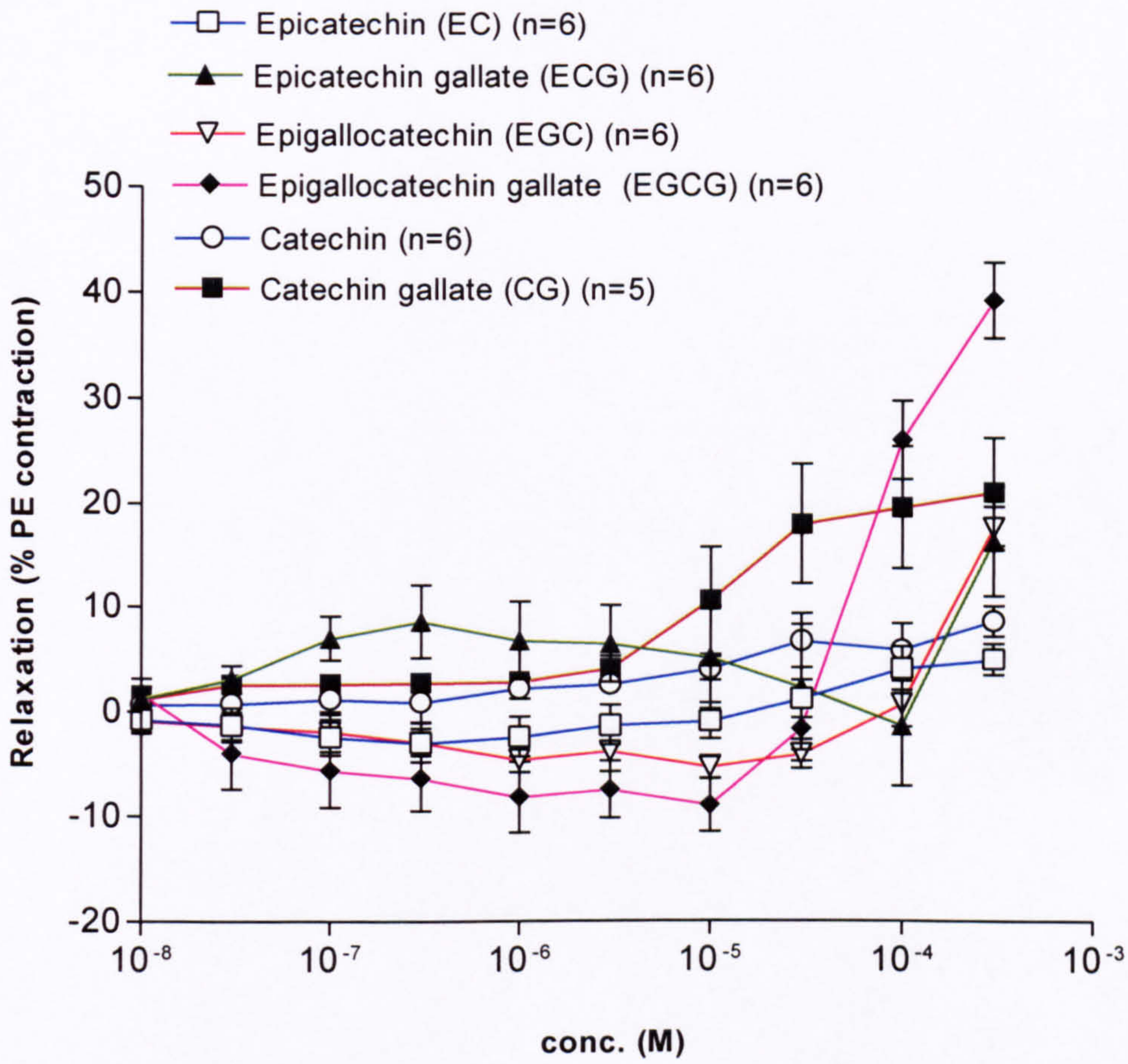
varying concentrations which may have interacted together to produce cumulative effects as compared to the purified epicatechin standards of a known concentration which could be directly compared. Contamination of the extracts is another factor which may have had an overall affect on the results and which could not be ruled out.

This study has also demonstrated that the antioxidant activity of the selected catechins and epicatechins vasodilatory and antioxidant activities of the grape and tea based extracts as compared to that of the green tea epicatechin and catechin derivatives range in the order magnitude of EGCG>EGC>ECG≥Catechin>CG>EC. EGCG was found to be the most significantly ($P<0.01$) effective antioxidant of all the derivatives tested. EGCG was identified to be the most powerful antioxidant in both aqueous and organic media in similar studies (Salah *et al.*, 1995; Gardner *et al.*, 1998). These studies did not investigate the antioxidant activities of CG and catechin.

4.4.3 Conclusion

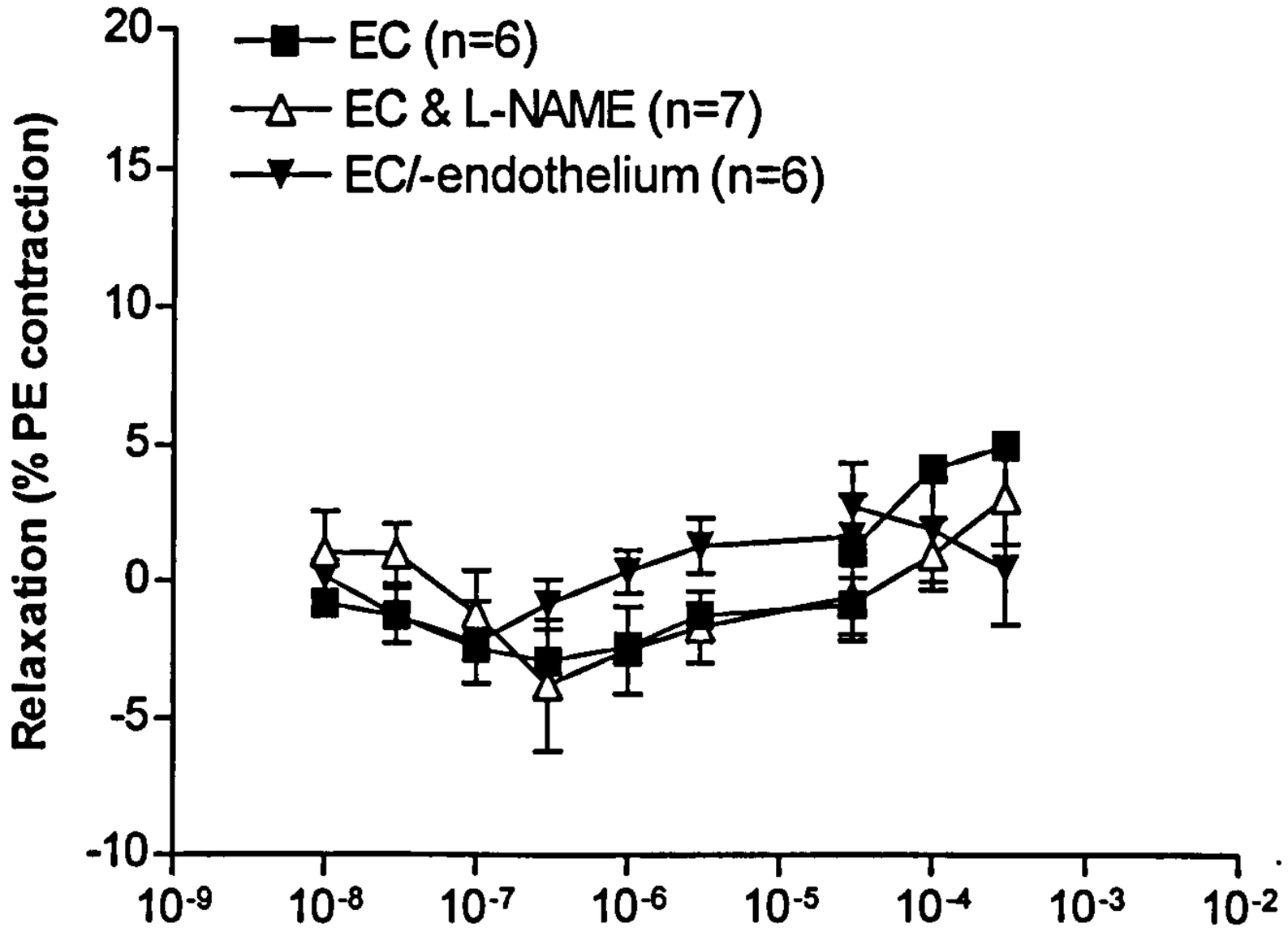
In conclusion, this study has determined and demonstrated that a wide-range of epicatechins and catechins present in green tea induce vasorelaxation in isolated rabbit aortic vessels and are effective antioxidants, a relationship that is statistically significant. Their overall total catechin and phenolic contents are not significantly related to their vasodilatory ability. It seems likely that the possible cardio-protective effects of catechins and epicatechins in green tea are due to the cumulative effects of these compounds and other components present which have not yet been identified working in synergy.

4.5 Graphs



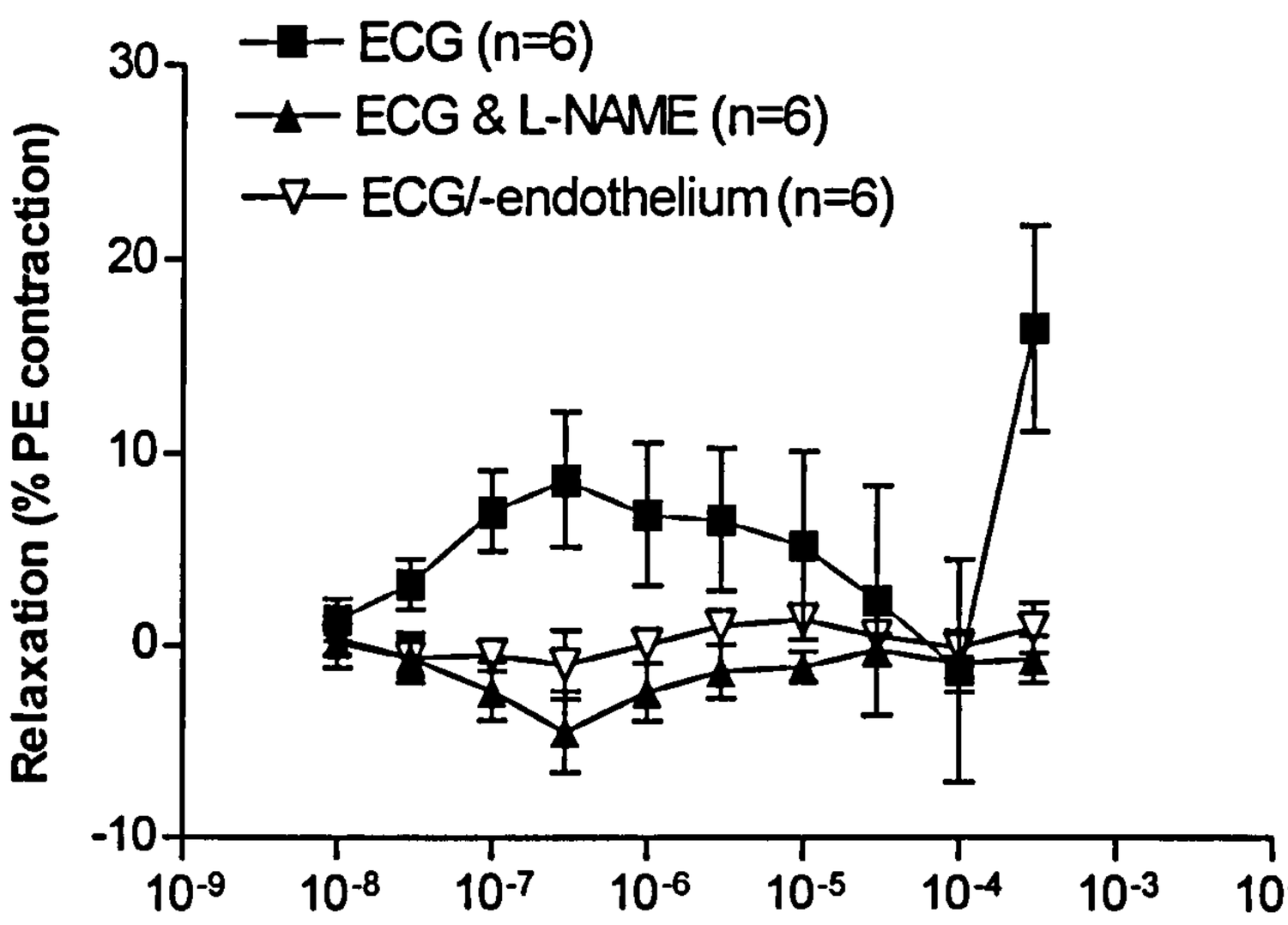
Graph 24 Vasorelaxation induced by green tea epicatechin and catechin derivatives in preconstricted rabbit aortic vessels

Values expressed as mean % relaxation ± standard error of mean.
See Table 18 for individual derivative maximum relaxation values.



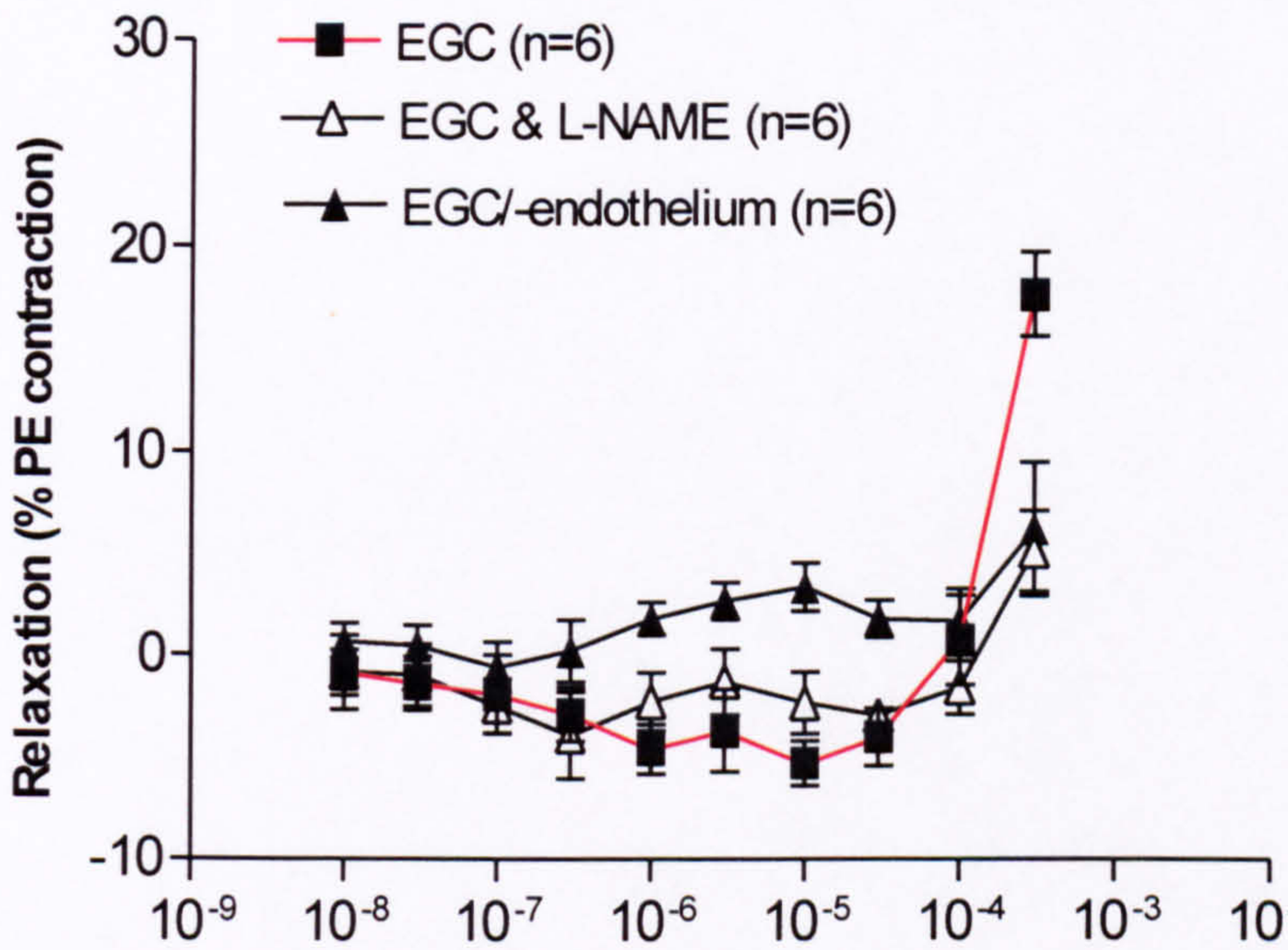
Graph 25 Epicatechin (EC) induced vasorelaxation in the presence of L-NAME and after removal of the endothelium.

Values expressed as mean % relaxation ± standard error of mean.



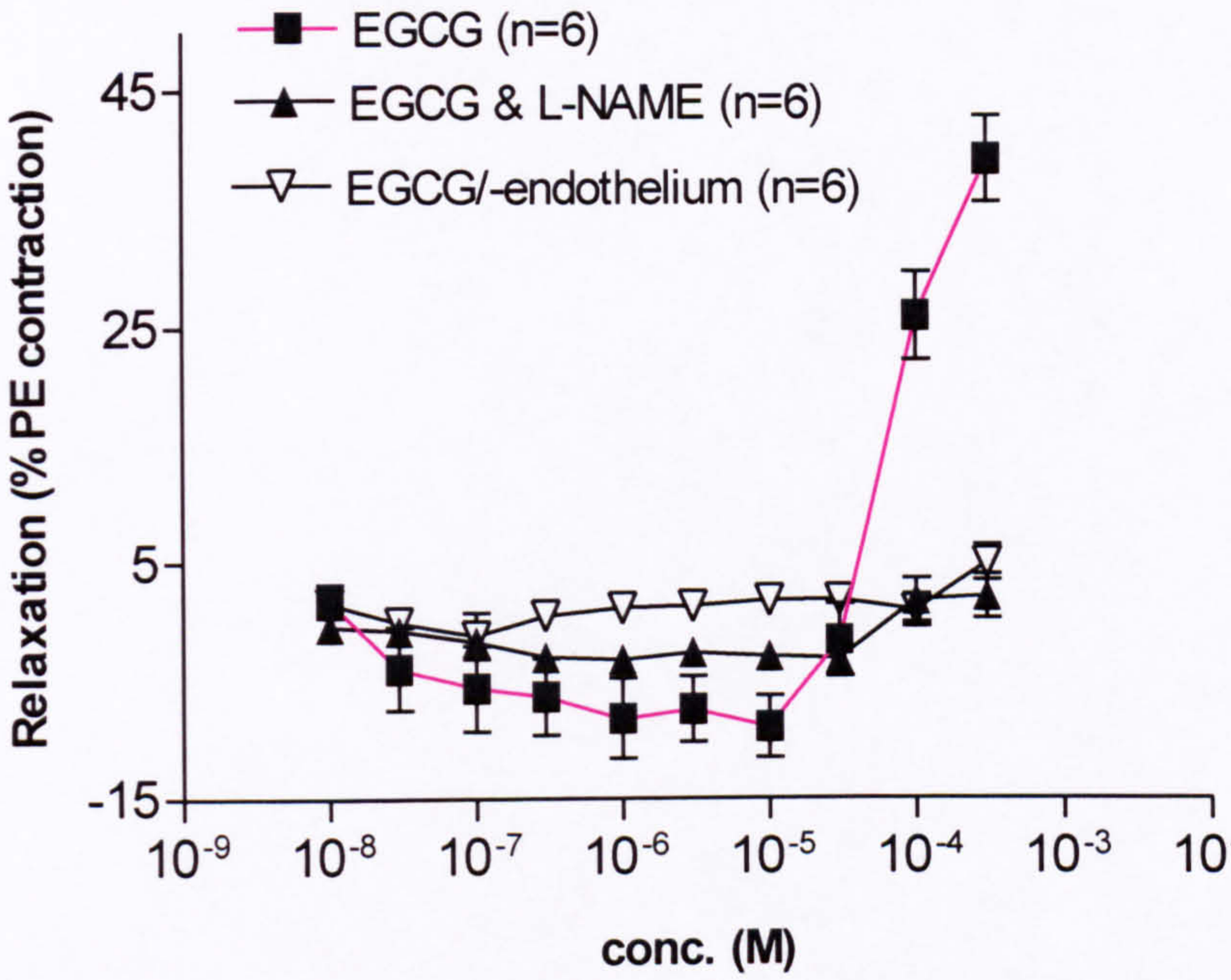
Graph 26 Epicatechin gallate (ECG) induced vasorelaxation in the presence of L-NAME and after the removal of the endothelium

Values expressed as mean % relaxation ± standard error of mean.



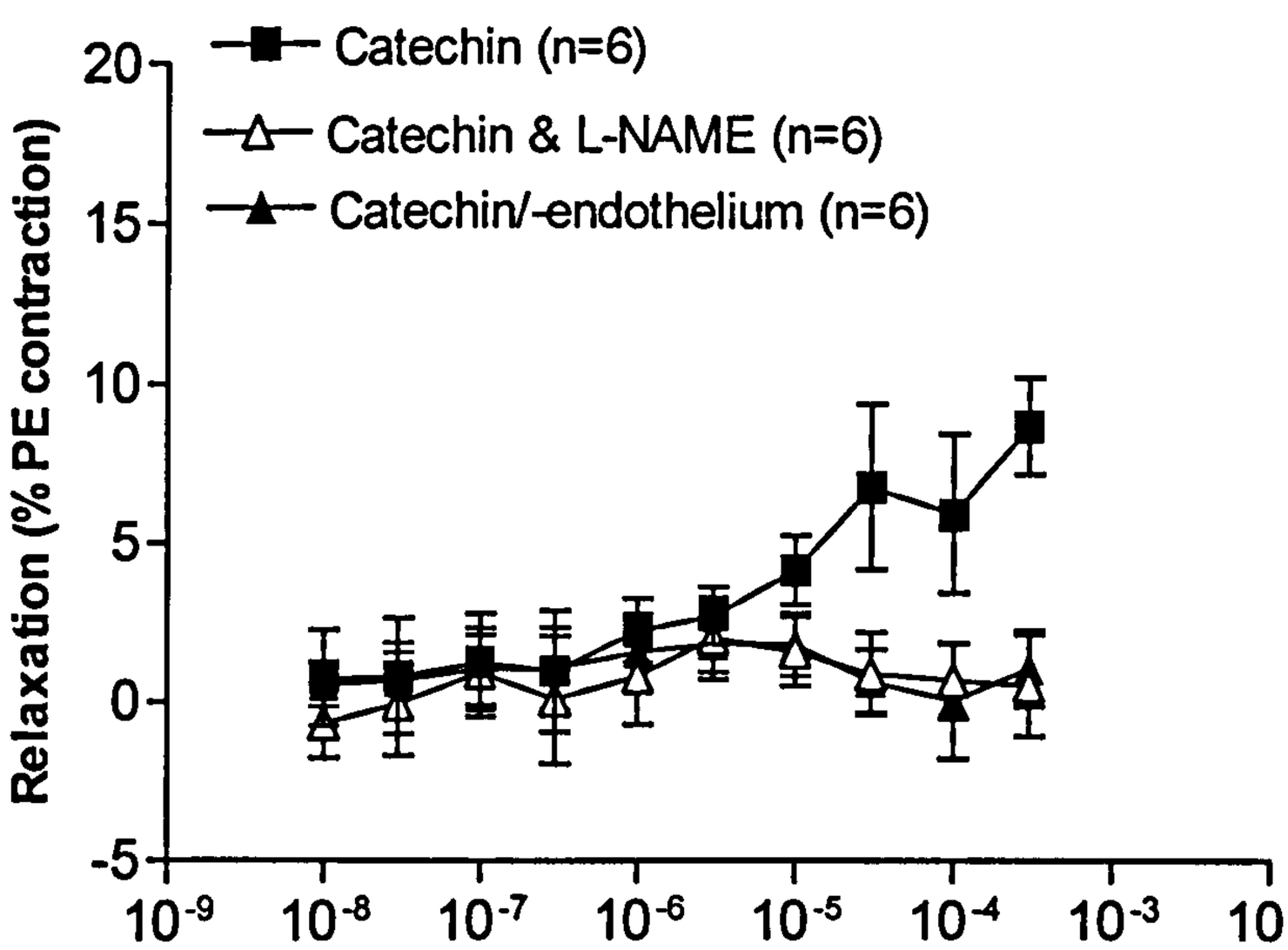
Graph 27 Epigallocatechin (EGC) induced vasorelaxation in the presence of L-NAME and after the removal of the endothelium

Values expressed as mean % relaxation ± standard error of mean.



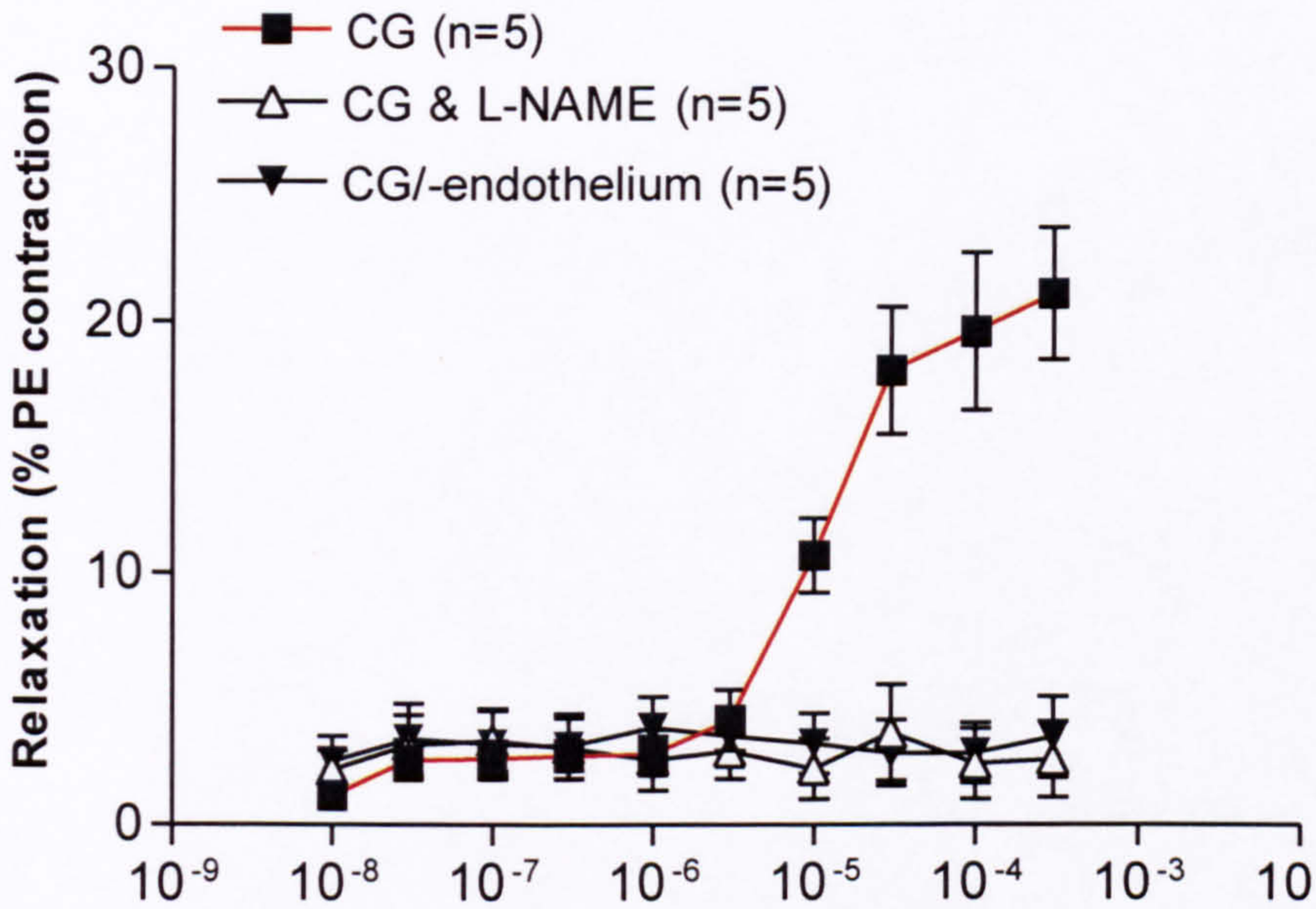
Graph 28 Epigallocatechin gallate (EGCG) induced vasorelaxation in the presence of L-NAME and after removal of the endothelium

Values expressed as mean % relaxation ± standard error of mean.



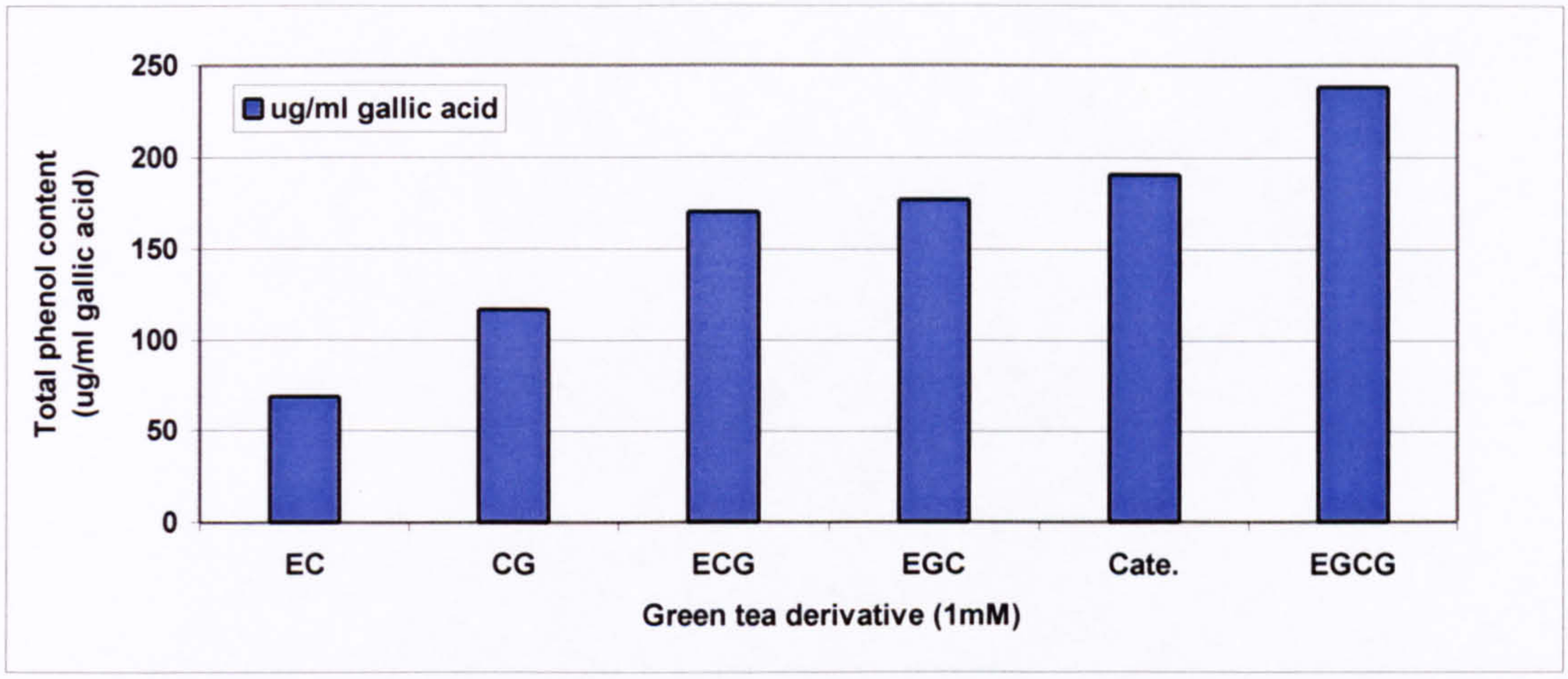
Graph 29 Catechin induced vasorelaxation in the presence of L-NAME and after the removal of the endothelium

Values expressed as mean % relaxation ± standard error of mean.

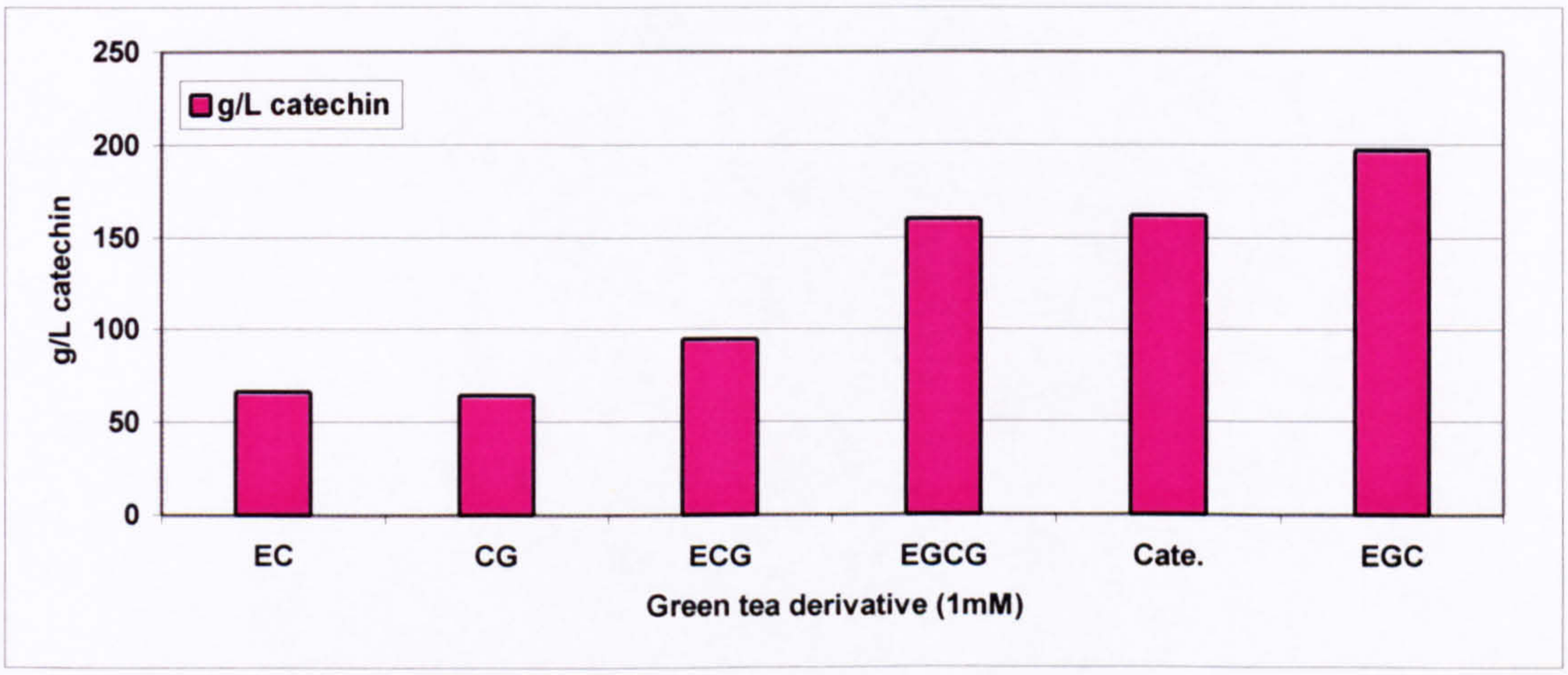


Graph 30 Catechin gallate (CG) induced vasorelaxation in the presence of L-NAME and after removal of the endothelium

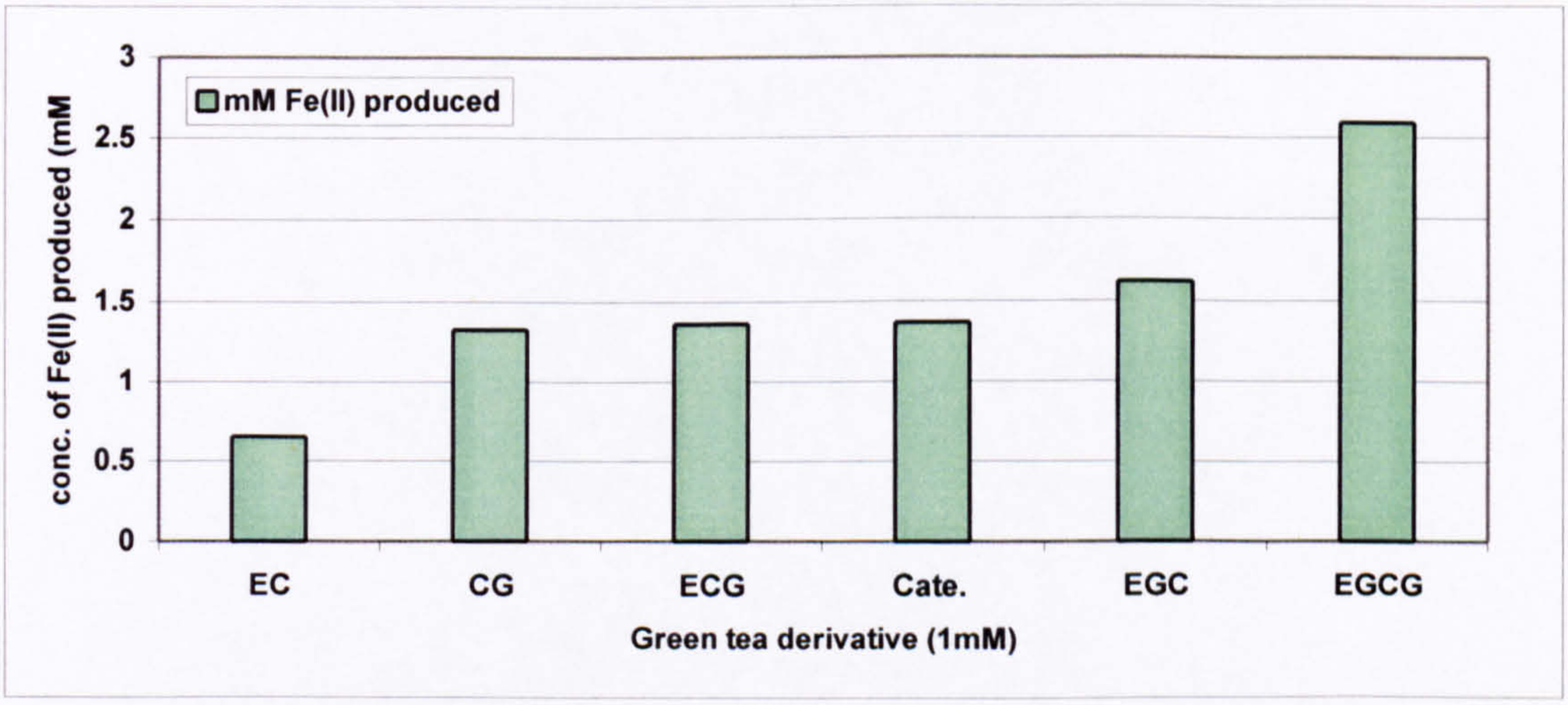
Values expressed as mean % relaxation ± standard error of mean.



Graph 31 Total phenolic content of green tea epicatechin derivatives

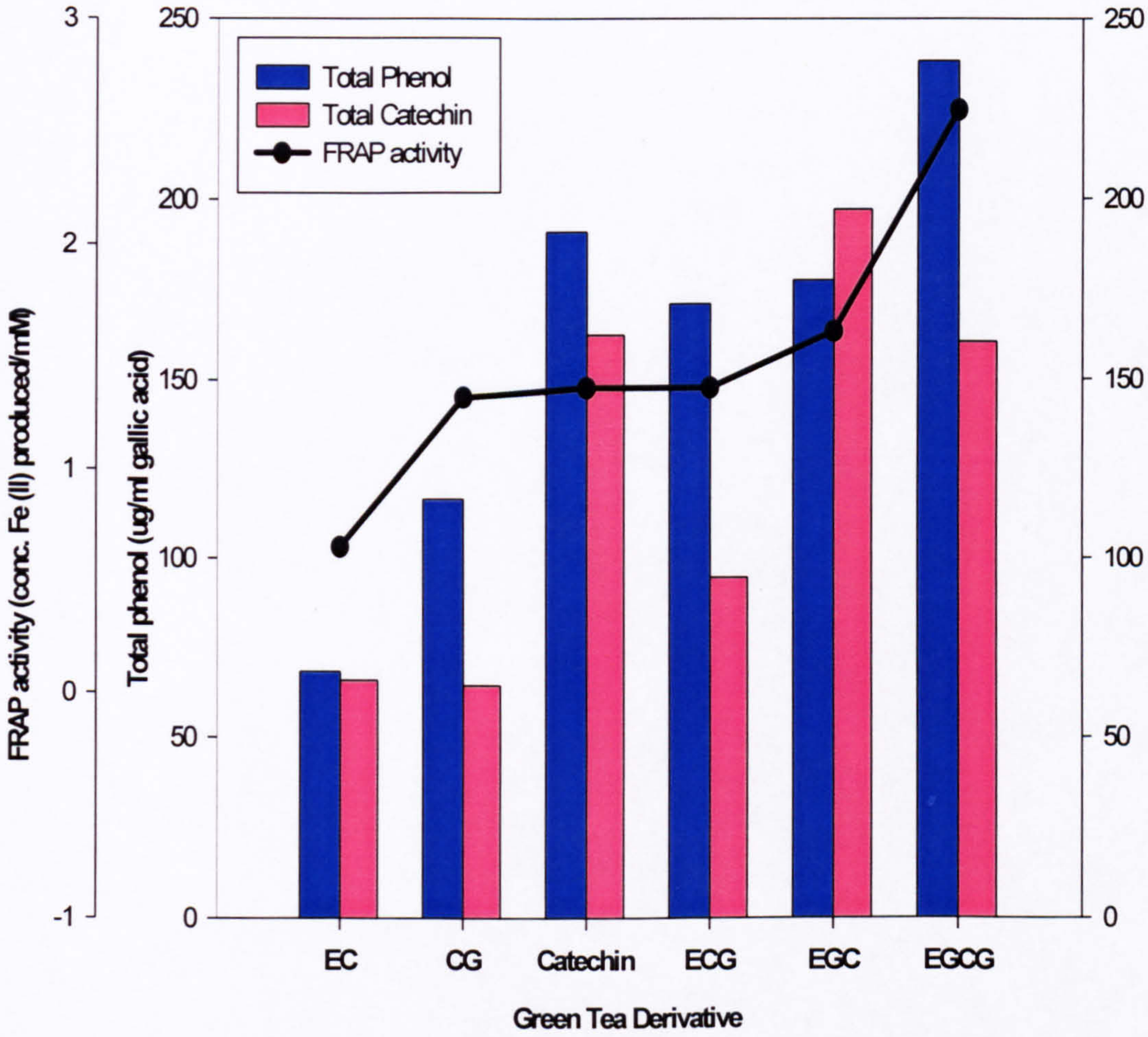


Graph 32 Total catechin content of green tea epicatechin derivatives



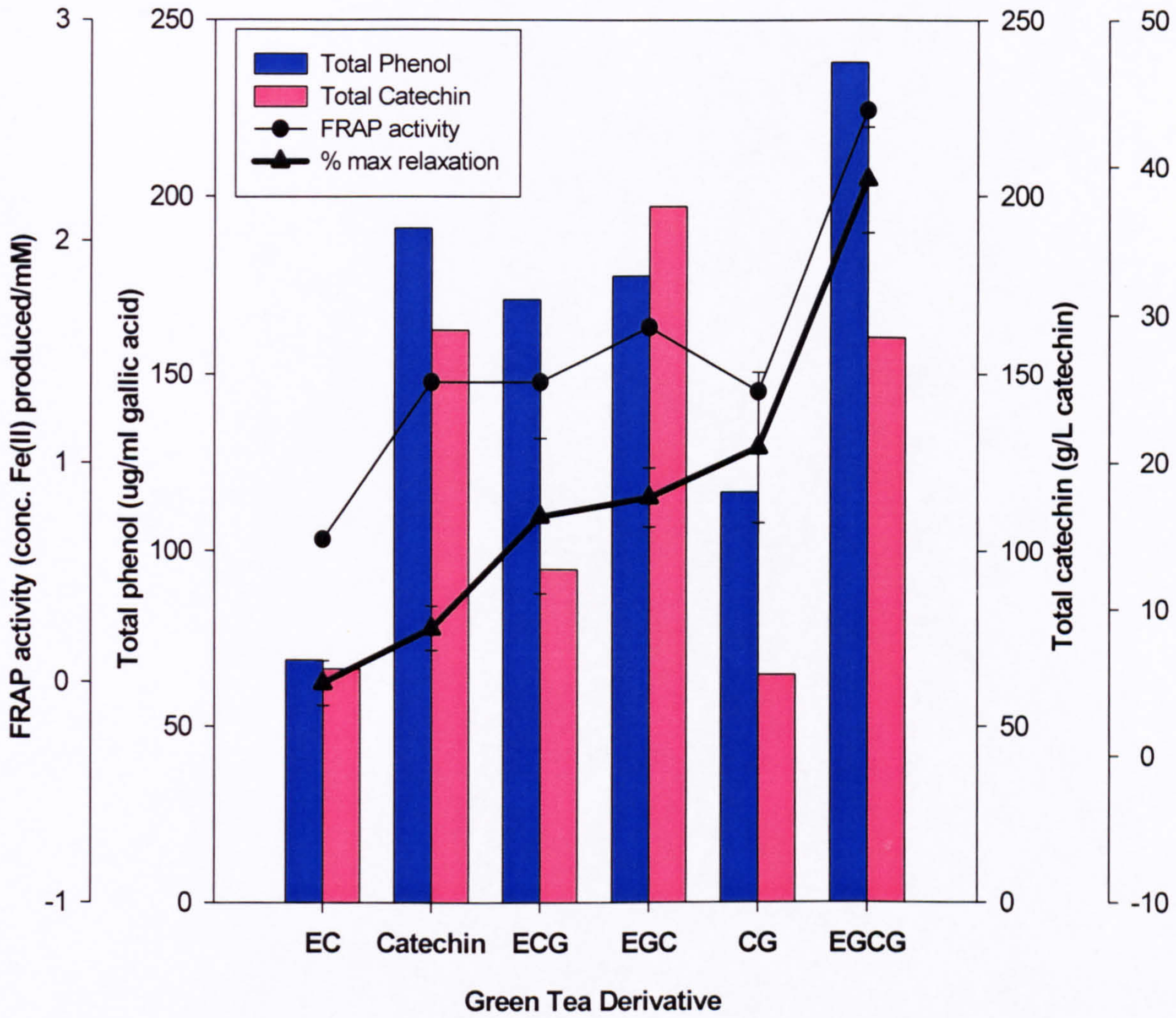
Graph 33 Antioxidant activity of green tea epicatechin derivatives

All samples run in duplicate (n =2) expressed as mean values. For individual derivative assay values see Table 19.



Graph 34 Relationship between total phenolic and catechin content and antioxidant activity of green tea epicatechin derivatives as ranked by FRAP activity.

All samples run in duplicate (n =2) and expressed as mean values.
For individual derivative assay values see Table 19.



Graph 35 Relationship between vasodilatory activity and total phenolic content, total catechin content and antioxidant activity of green tea epicatechin derivatives as ranked by % maximum vasodilatory activity.

All vasodilatory maximum relaxation values expressed as mean +/- standard error of mean of n = 5-6.For individual maximum relaxation values see Table 18.

FRAP antioxidant activity and total phenolic and catechin content samples run in duplicate (n=2) and expressed as mean values. For individual derivative assay values see Table 19.

Chapter 5 Identification of the major phenolics present in green & black teas

5.1 Introduction

Brewed tea and chocolate are two of the few food products known to contain significant levels of catechins (Vison, 1998). Comparable concentrations have not been reported in any other food stuffs (Bronner & Beecher, 1998). As a result, tea is one of the major dietary source for this potentially important group of compounds. The four main catechins or flavan-3-ols, -(-)epicatechin (EC), -(-)epicatechin gallate (ECG), -(-)epigallocatechin (EGC) and -(-)epigallocatechin gallate (EGCG), constitute about one third of the dry weight of green tea (Balentine *et al.,* 1997). Of the approximately 2.5 million metric tons of dried tea manufactured annually, only 18% is consumed as green tea but over 80% is consumed as black tea (Yang & Landau, 2000), with black tea consumption contributing 82% of the flavonols and flavones in the diet (Graham, 1992). During the fermentation of green tea to black tea, catechins are oxidised by polyphenol oxidase to form the reddish-brown well-characterised theaflavins and thearubigens, and the poorly described theaflagallins, theasinensins (bisflavonols) and theacitrins (Beecher *et al.,* 1999). Consequently, the catechin content of black tea is low, representing only 30% of total dry weight, as compared to that encountered in green tea (90%). Theaflavins account for about 10-15% of the dry weight of black tea with the balance of catechins forming the undefined high molecular weight thearubigens (1 – 40 kDa) (Beecher *et al.,* 1999). These are a class of heterogenous polyphenolic condensation products believed to be responsible for the dark colour of black tea (Robb & Brown, 2001).

The measurement and identification of polyphenols in tea has employed a variety of analytical techniques. For standard analysis, high performance liquid chromatography (HPLC) equipped with a reversed-phase (RPLC) column and an ultraviolet visible (UV-Vis) detector has become the instrumental method of choice for catechin detection and quantification (Beecher *et al.,* 1999). In 1976,

Hoeffler & Coggon, published the first quantitative analysis of catechins from tea extracts using a C18 HPLC column (Robb & Brown, 2001). Since 1976, several chromatographic techniques have been used to separate, characterize and quantify the individual polyphenols in teas. To date, only a very small number of analytical techniques have been established for the analysis of theaflavins and thearubigens (Lee & Ong, 2000).

Recent advances in instrumentation have permitted the characterization of polar molecules, such as polyphenols and catechins from tea via the use the mass spectroscopy (MS). Mass spectroscopy, includes the techniques of electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and thermospray ionisation (TSI), are characterised by the formation of ions from solutions that are sprayed from a thin capillary at high voltages (Dalluge & Nelson, 2000). Direct analysis of infusions of green tea with ESI operated in the negative ionisation mode have met with success in terms of the identification of prominent polyphenols and other minor components (Poon, 1998). However, following the evaluation of several ionisation procedures for MS of the predominant catechins in tea it was concluded that the best results were obtained when individual compounds were separated by HPLC prior to mass spectroscopic analysis (Miketova *et al.*, 1998). This method is seen to give the most useful qualitative results and the most accurate analytical data.

Estimation of the "total" polyphenol content of teas has relied on colorimetric procedures, such as the Folin-Denis or Prussian Blue (Balentine *et al.*, 1997) assays, based on "reducing ability" or redox reactions mostly specific to phenolic groups, or to the Folin-Ciocalteu assay based on the identification of gallic acid groups (Singleton & Rossi, 1965). In spite of the recent advances in instrumental techniques, these non-specific methods are still important for estimating the amounts of non-characterised polyphenols in tea and other foods (Beecher *et al.*, 1999). Likewise, another indirect approach to the estimation of the polyphenol content of teas is the measurement of the free radical scavenging activity, similar to that of the total phenol content as it is also based on redox potentials. Several techniques have been employed to measure radical scavenging

activity, these include electron spin resonance (ESR) (Gardner *et al.*, 1998) and the ferric reducing ability of plasma (FRAP) (Benzie & Strain, 1996, 1997). These methods are based on the ability of tea extracts or individual compounds to scavenge or reduced free radicals in the aqueous phase. Finally, total polyphenols in teas can also be estimated by use of the rapid and sensitive method for catechin identification in plasma and aqueous solutions. Catechins form a coloured complex, which can then be quantified colorimetrically (Kivitis *et al.*, 1997).

Previous work carried out with red wine has shown that by using preparatory fractionation HPLC systems (Labarbe *et al.*, 1999) and small-scale liquid/liquid extraction (C18 Sep-Pak cartridge) fractionation (Oszmianski *et al.*, 1987; Ghiselli *et al.*, 1989; Sun *et al.*, 1998), we are able to exploit differences in the solubility of different chemical components present in wine, it is possible to separate and collect different fractions as they elute off the column over a pre-set time-scale, resulting in each fraction having a different composition (Sun *et al.*, 1998). This then allows the identification of the compounds present in each fraction and the biological activity of each individual sample to be obtained.

In this final study, samples of brewed green and black tea will be fractionated by preparative HPLC with the polyphenolics, specifically catechins, present in each sample being quantified. The identification of the fractions high in antioxidant activity and total phenolic and catechin content will also be determined. The effect of green tea fractions on vascular tension *in vitro* is also investigated.

5.2 Materials & Methods

In order to identify the catechins and polyphenols present in fractionated samples of green and black tea and their activities *in vitro*, several complementary methods were employed in the course of this study. These methods are explained in detail in Chapter 2. In brief:

5.2.1 Pre-fractionation tea preparation

An initial amount of 1g of Tetley's coarse grain green tea (B-50048) and dry medium ground black tea (U-1704) was brewed in 100 mL of boiling water to allow the full infusion of the tea leaves into solution.

5.2.2 Fractionation methodology

A preparative HPLC system was used for a large-scale fractionation based on differences in polarity of the various green and black tea catechins. The HPLC system (Figure 12) used to fractionate both tea samples and their aid their identification using HPLC MS-MS in explained in detail in Chapter 2.

5.2.2.1 Green Tea

A sample green tea was eluted by a 7 – 30% gradient of acetonitrile in 2% formic acid and held at 30% for 5 min. Operating at 20 mL min^{-1}, fifty successive fractions were collected every 30 seconds from an injection of 10 mL of green tea, with each fraction containing 10 mL of eluent. Four identical 10 mL injection system runs were carried out, with a total pool of 40 mL of each fraction being collected.

5.2.2.2 Black Tea

A sample of black tea was run over a 5 – 15 % gradient of acetonitrile in 2% formic acid over the initial 20 minutes, then 15 – 35 % over the remaining 20 – 30 minutes. Operating at 10 mL/min, sixty successive fractions were collected every 30 seconds from an injection of 10 mL of black tea, with each fraction containing 5 mL of eluent.

5.2.3 Mass spectroscopy analysis

The phenolic profile of selected green and black tea fractions was analysed by high performance liquid chromatography – mass spectroscopy – mass spectroscopy (HPLC-MS-MS). The mobile phase was a 5 – 30% linear gradient over 60 min of acetonitrile in 1% formic acid eluted at a flow rate of 1 mL min^{-1}. Short isocratic runs (75% B) were used to analyse the preparative HPLC tea fractions. In all cases, 100 μL of each selected fraction was analysed in both positive and negative ion mode (scanning from 100.0 – 2000.0 amu), using data dependent MS/MS. Column eluent was directed to a UV-vis 6000 photodiode array detector operating at wavelengths 250 - 700 nm.

5.2.4 Antioxidant activity

The antioxidant activity and phenolic and catechin of the green and black tea fractions analysed in this study, were determined using specific spectral and colorimetric assays discussed in detail in Chapter 2.

5.2.4.1 ESR activity

Total antioxidant activity of each green tea fraction was determined using ESR spectroscopy, where the ability of the extracts to reduce radicals in the aqueous phase was estimated by adding the extract to an equal volume of a 1 mM Fremy's salt free radical. Results were standardised using trolox.

5.2.4.2 FRAP activity

Plasma antioxidant capacity was also estimated from the green and black tea fractions ferric reducing ability (FRAP) at 593 nm. The development of an intense blue colour resulting from the conversion of a Fe(III)-2,4,6-Tri-2-pyr-idyl)-s-triazine (TPTZ) complex to Fe(II)-TPTZ being directly related to the amount of reductant present in each tea fraction. Results are expressed as the concentration of Fe(II) produced/mM (Benzie & Strain, 1997).

5.2.4.3 Total phenol content

The total phenolic content of each green and black tea fraction was determined and expressed as gallic acid equivalents (GAE) using the Folin-Ciocalteu method of Singleton & Rossi (1965). All fractions were analysed at 765 nm.

5.2.4.4 Total catechin content

The total catechin content of each green and black tea fraction was determined using the method of Kivitis *et al* (1997). Total content is determined by the addition of a DMACA in a methanol/perchloric acid/water mix to the tea fraction. With the addition of the DMACA solution, samples are seen to develop highly specific colour complexes dependent on total catechin content. Absorbance was measured between 604 and 684 nm. Results are expressed as total catechins (mg/L).

5.2.5 Vasodilatory activity: green tea fractions

Vasodilatory studies were also carried out with the green tea fractions to investigate their vascular tension *in vitro*. This method is previously discussed in detail in Chapter 2.

In brief, isolated rabbit aortic vessels were suspended in standard 10 mL organ baths (Figure 11), filled with Krebs buffer solution, continuously oxygenated with 16% O_2, 5% CO_2 and 79% N_2 and maintained at 37°C, to mimic the internal environment. Vessels were placed under an optimal resting tension of 2g. Vessel contraction/relaxation was measured via an isometric force transducer.

After an equilibration period, vessels were twice maximally contracted with 50 mM KCl and washed out thoroughly with Krebs solution. After washing and returning to initial baseline tension, vessels were precontracted submaximally with PE (10^{-7} M) and relaxed with ACh to ensure the presence of functional and intact

endothelium (see Figure 13). Matched experimental time controls were run for all fractions investigated.

After washing and returning to starting baseline tension, aortic rings with functional endothelium were submaximally precontracted with PE (10^{-7} M). Once a stable plateau had been reached and was maintained, 20 μg mL^{-1} (twice 10 μg mL^{-1} aliquots) of each green tea fraction concentrate were added to the organ bath to induce vasodilation in the aortic ring segments. All results were calculated and expressed as the % vasorelaxation induced by each green tea fraction following PE-induced tone in 2 vessels ($n = 2$).

5.3 Results

5.3.1 Green Tea Fractionation

Following the large-scale prep fractionation of green tea (B-50048), the antioxidant activity of each fraction was determined by ESR and FRAP analysis. The total phenolic and catechin content of each fraction was also determined. Results are shown in Graphs 36 to 39. Values for each individual assay carried out are shown in Table 20.

5.3.1.1 Spectral & colorimetric assays

Green tea fractions 19, 27 and 28 were all found to be consistently higher in their antioxidant capacity analysed by ESR, reducing 94.7, 82.5 and 50.1 x 10^{18} radicals/L, respectively (Graph 36, Table 20). The overall pattern of large-scale fractionation was seen to be variable in activity throughout the 50 fractions, with the main area of antioxidant activity being confined to the middle band of tea samples, fractions 18 to 29. Poor antioxidant activity was observed with samples at the end of the fractionation range, fractions 40- to 50.

Similar activity was recorded when samples were analysed using their ferric reducing ability an as index of their antioxidant capacity (Graph 37, Table

20). FRAP analysis identified high antioxidant activity in green tea fractions 8, 18, 19, 27 and 28, with fraction 27 having the overall highest activity recorded.

As before, fractions 19, 27 and 28 were found to have the highest total phenols and catechins present (Graphs 38, 39). Fraction 27 was found to have the highest phenolic content followed by fraction 19, with 453 μg/mL and 309 μg/mL GAE (Table 20), respectively. On the other hand, fraction 19 followed by fraction 27 was found to have the highest catechin content with 634 mg/L and 474 mg/L of catechin present in each sample (Table 20), respectively. Spectrally the antioxidant assays, ESR and FRAP, were positively related to both total phenolic and total catechin contents, with the same fractions being higher in both colorimetric assays as compared to remaining samples. In all assays, the fractions at the beginning and end of the fraction range were found to have both poor antioxidant activity and low total phenolics and catechins present.

The antioxidant assays, ESR and FRAP were found to be highly correlated ($r = 0.86$, $P<0.0001$) when analysed using Pearson's correlations. Both antioxidant tests produced very similar patterns of activity when analysing the green tea fractions (Graph 40) indicating that the two tests of antioxidant capacity can produce comparable results. The relationship between the total phenolic and catechin contents and antioxidant activities by ESR and FRAP are shown in Graph 41. ESR-based antioxidant activity was highly and positively correlated to both total phenolic ($r = 0.88$, $P<0.001$) and catechin content ($r = 0.94$, $P<0.0001$) by multivariate correlation analysis. The higher r value reported by the total catechin assay on analysis indicates that the catechin content of the green tea fractions has a greater effect on ESR determined antioxidant activity than phenol content. On the other hand, both total phenol ($r = 0.80$, $P<0.0001$) and total catechin ($r = 0.87$, $P<0.001$) contents were also highly correlated to antioxidant activity by the FRAP assay, with total phenol content having a greater effect on activity than catechin content. Overall total catechin and phenol contents of the green tea fractions were also highly correlated ($r = 0.89$).

Table 20 Green tea fractionation assay mean values table

Fraction number	Total Phenol[a]	Total Catechins[b]	AOX capacity[c]	FRAP activity[d]
1	29.1	-8.6	0.95	-0.15
2	29.5	-8.6	1.66	-0.05
3	20.7	-8.6	-0.5	-0.10
4	43.1	-4.2	7.88	-0.04
5	36.3 1	-7.5	3.29	0.05
6	34.8 4	-7.5	4.25	0.15
7	33.4	-8.2	6.08	0.68
8	100.2	-7.8	11.4	2.90
9	40.2	-6.0	5.74	0.29
10	28.4	-7.6	3.57	0.21
11	18.1	-7.5	4.67	0.14
12	60.2	29.9	23.9	1.66
13	44.8	5.0	18.0	0.67
14	38.1	-4.9	7.72	0.23
15	34.3	-7.2	3.83	0.39
16	39.5	-3.5	6.02	0.30
17	54.8	10.3	9.35	1.10
18	82.8	24.3	33.8	2.39
19	308.7	634.5	94.6	3.87
20	42.8	26.3	28.9	0.76
21	47.5	8.6	11.2	1.47
22	71.6	22.4	17.9	0.81
23	71.0	4.1	13.6	0.77
24	21.3	-8.6	2.11	0.69
25	39.0	-2.1	11.1	0.56
26	74.8	20.7	31.6	0.90
27	452.8	474.6	82.8	4.01
28	290.7	356.5	50.1	3.20
29	96.9	41.9	23.6	0.92
30	47.8	7.0	1.66	0.42
31	46.1	0.08	12.4	0.38
32	39.8	-5.6	8.4	0.23
33	33.7	-6.1	6.7	0.14
34	41.4	-6.02	7.24	0.11
35	40.7	1.7	10.22	0.17
36	41.9	0.11	9.63	0.30
37	38.4	-5.9	11.0	0.22
38	229.0	60.6	18.9	1.88
39	139.2	43.3	17.4	1.84
40	43.7	0.6	8.56	0.44
41	44.0	-3.5	9.4	0.27
42	55.4	-6.2	7.66	0.31
43	35.7	-7.4	5.10	0.16
44	41.9	-7.5	4.67	0.19
45	21.9	-7.9	4.10	0.09
46	31.1	-5.9	4.45	0.12
47	33.7	-3.2	4.17	0.18
48	30.7	-7.3	4.98	0.12
49	31.9	-7.7	4.79	0.10
50	28.2	-8.1	8.60	0.16

[a]Total phenol data expressed as mean μg/ml gallic acid equivalents (GAE). [b]Total catechins expressed as mean mg/L catechin. [c]Antioxidant (AOX) activity by ESR, expressed as number mean of radicals reduced/L x 10^{17}. Ferric reducing ability (FRAP) expressed as the mean concentration of Fe^{II} produced (mM). All samples run in duplicate from pooled samples from 4 fractionations.

5.2.1.2 Vasodilatory activity

The vasodilatory activity of each green tea fraction in rabbit aortic vessels *in vitro* was determined. Time controls for each fraction run to demonstrate the individual effect of each fraction on vascular tension. In general, fractions with high antioxidant activity were able to relax preconstricted aortic vessels (Graph 42). Vasodilation activity of the fractions was found to correlate with their antioxidant activity by ESR ($r = 0.28$, $P>0.05$) by multivariate correlation analysis. Fractions induced an initial period of transient vasodilation with the first 10 μg mL^{-1} aliquot of green tea fraction concentrate added to the bath, followed by period of maintained vasodilation with the second 10 μg mL^{-1} aliquot (Figure 17). Fraction 8 induced the greatest degree of vasodilation *in vitro* (Graph 42).

Figure 17 displays an example of a vasorelaxation trace induced by fractions 27 and 28 following PE-induced tone. Fraction 27 induced a period of vasodilation with the first fraction addition followed by a period of rapid vessel reversal and contraction with the second concentrate addition. On the other hand on addition of the second fraction concentrate fraction 28 induced a period of rapid maintained vasorelaxation (Figure 17).

5.2.1.3 Mass spectrometry analysis

Mass spectrometry (MS) detection has been employed in this study to aid catechin and polyphenol identification. The phenolics present were determined by co-chromatography with authentic standards. Compounds were identified on the basis of their retention times, absorbance spectra and MS-MS fragmentation patterns.

Figure 18 displays an HPLC chromatographic traces for the green tea sample, displaying absorption at 280 nm, the specific wavelength for identification of catechins (Finger *et al.*, 1992) and 365 nm the specific wavelength for identification of flavonols (Finger *et al.*, 1992). Table 21 identifies each component of the green tea sample by peak number from its HPLC trace retention

time. These chromatographic traces display the complex nature and large number of compounds present in green tea. EGCG, the main epicatechin in present in green tea, was the main peak identified with absorbance at 280 nm (peak 6). A catechin trimer (4-8 linkage) was also identified (peak 8) which is composed of 3 catechin units linked together. The main flavonol identified was a quercetin-hexose-rhamnose molecule (peak 10), composed of a quercetin molecule with the addition of a hexose sugar unit and a rhamnose sugar unit. These compounds were identified on the basis of the molecular ion weight, fragmentation pattern and absorbance spectra for each individual compound.

Table 21 Identification of the main phenolics in green tea by HPLC MS-MS

Peak No.	Component	Retention Time (R_t mins)
1	Epigallocatechin (EGC)	13.05
2	Catechin	15.17
3	Chlorogenic acid	16.75
4	Caffeine	17.90
5	Epicatechin (EC)	20.45
6	Epigallocatechin gallate (EGCG)	21.80
7	Cyanidin	28.82
8	Catechin trimer	31.07
9	Rutin	32.80
10	Quercetin-hexose-rhamnose	33.65
11	Quercetin-3-glucoside	34.93
12	Kaempferol-3-glucose	40.00
13	Quercetin	53.88

Sample run on a 5-30% acetonitrile/1% formic acid gradient over 60 mins in positive ion mode.

On analysis of the results fraction 27 displayed high antioxidant activity, high phenolic and catechin content levels and subsequent low vasodilatory activity, the opposite pattern to the results in Chapter 4 for green tea catechins and epicatechins.

Fraction 27 was therefore investigated by HPLC MS-MS to determine the phenolics present. The presence of caffeine and EGCG were identified in this fraction by their MS-MS fragmentation patterns and co-chromatography with standards (Figure 19 & 20). On calculating peak area, both EGCG and caffeine were found to be equal in size.

The positive identification of EGCG was made by its MS-MS of its M^- molecular ion *m/z* 459.0 amu, with fragments at *m/z* 289.0, representative of a catechin/epicatechin unit, and the neutral loss of 170.0, representative of a gallate unit (Figure 19). EGCG had a lamba maximum (λ_{max}) of 272 nm (Table 22) identified by its PDA spectra.

The positive identification of caffeine was made by its MS-MS of *m/z* 195.0 amu, with molecular ion fragment at 138.0 (Figure 20) and PDA spectra with a λ_{max} of 261 nm (Table 22).

The confirmed positive identification of EGCG and caffeine in green tea fraction 27 was carried out using authentic standards of both compounds.

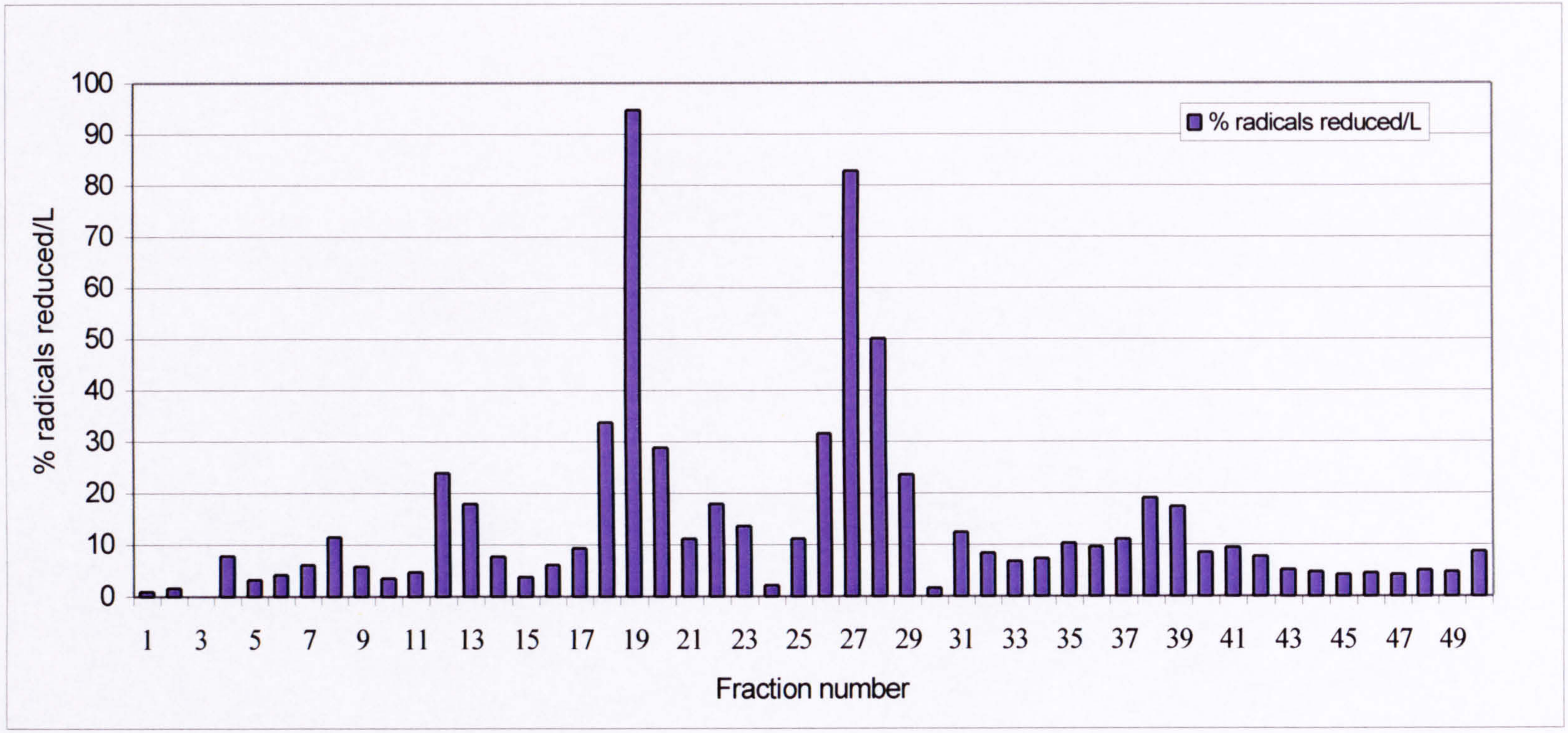
Table 22 MS-MS of the molecular ions in green tea fraction 27

Component	R_t (min)	M^+ (*m/z*)	MS^2 fragment (*m/z*)	λ_{max}
EGCG	21.80	459.0	289, 151, 139	272
Caffeine	17.90	195.0	138	261

Sample run on a 5-30% acetonitrile in 1% formic acid gradient over 60 mins in positive ion mode.

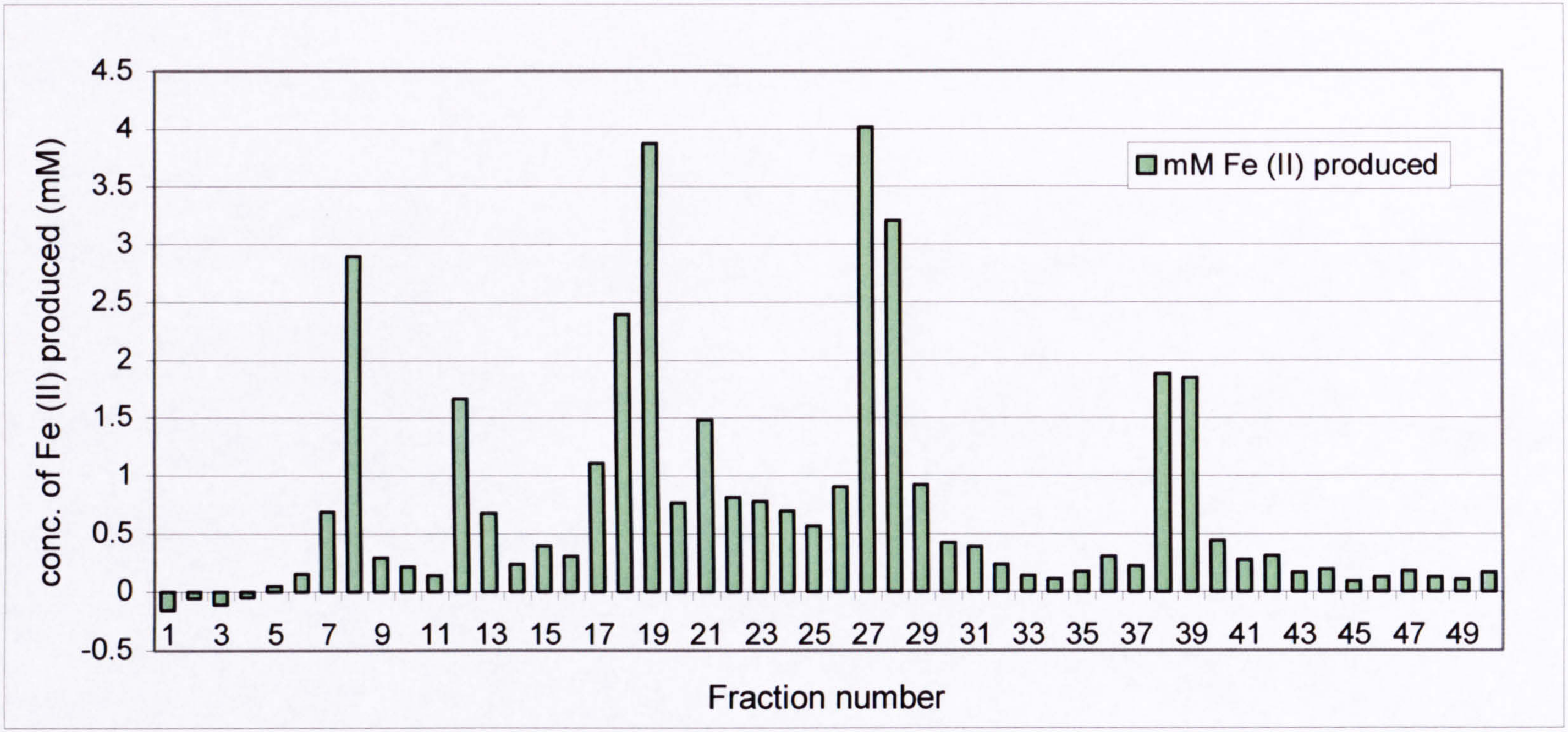
Due to time limitations, the MS-MS analysis of other green tea fractions of interest was not carried out.

5.3.3 Green Tea Fractionation: Graphs & Figures



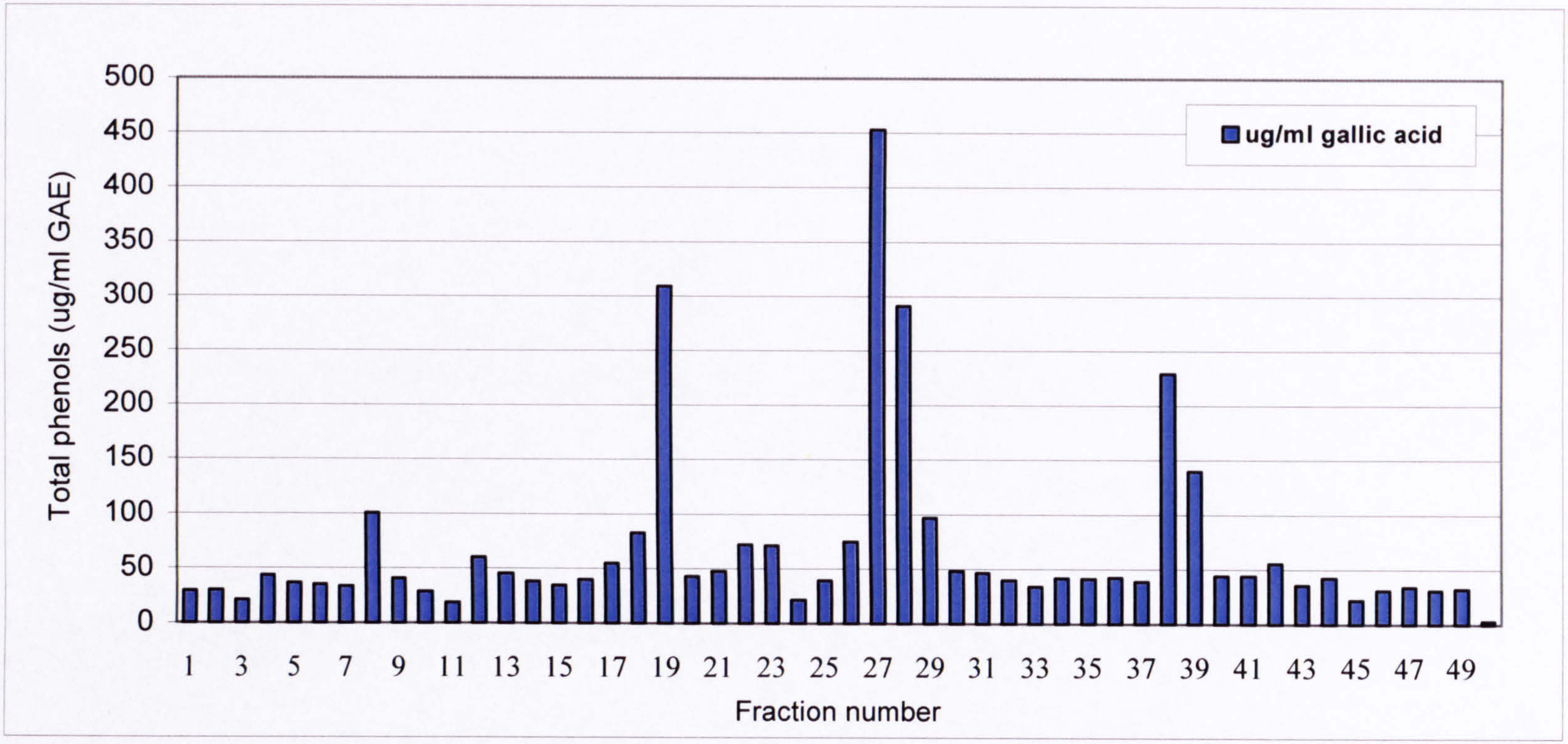
Graph 36 Antioxidant activity of green tea fractions by ESR

All samples run in duplicate (n =2) and expressed as mean number of radicals reduced/L x 10^{17}. See Table 20 for individual values.



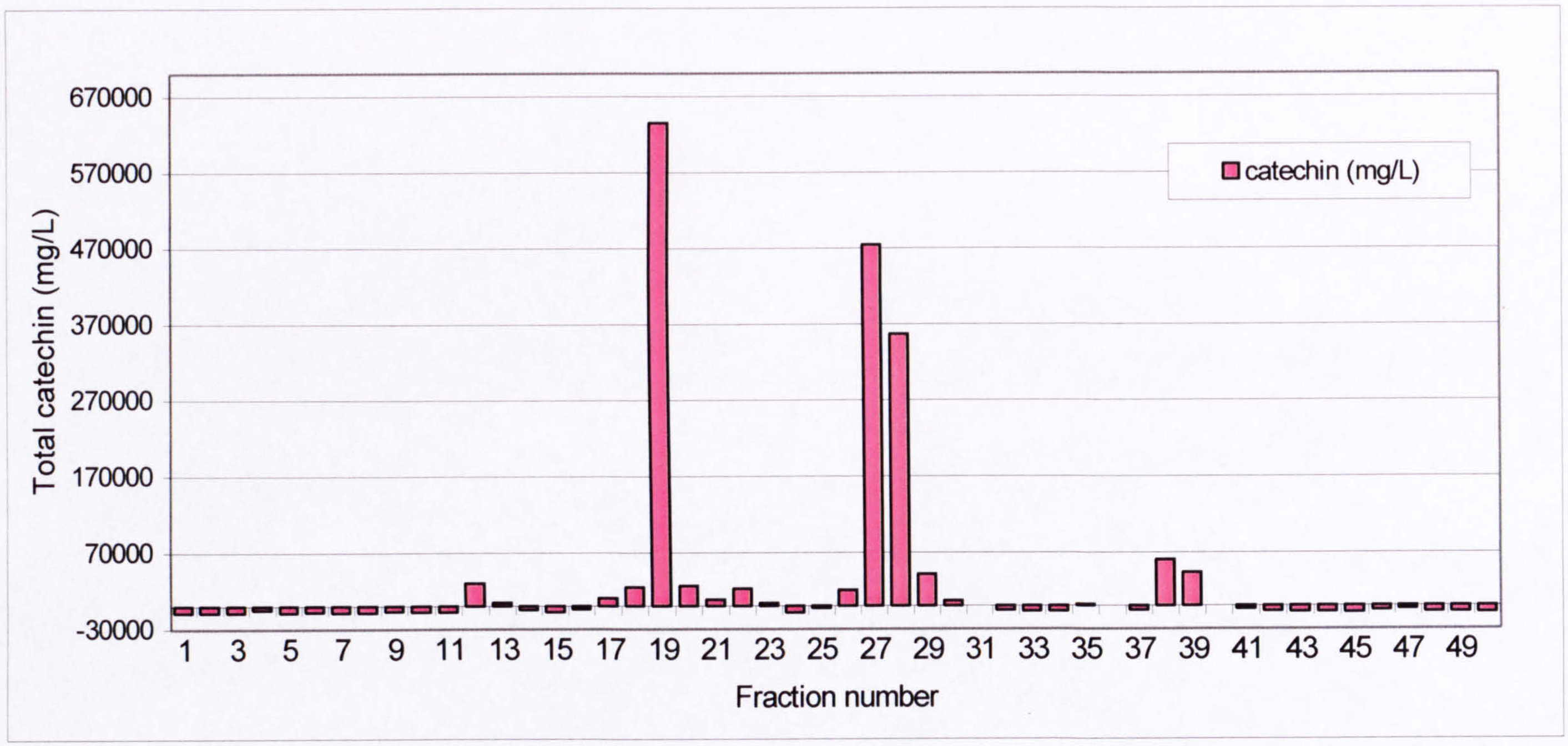
Graph 37 Antioxidant activity of green tea fractions by FRAP analysis

All samples run in duplicate (n =2). Values expressed as mean concentration of Fe^{II} produced (mM). See Table 20 for individual values.



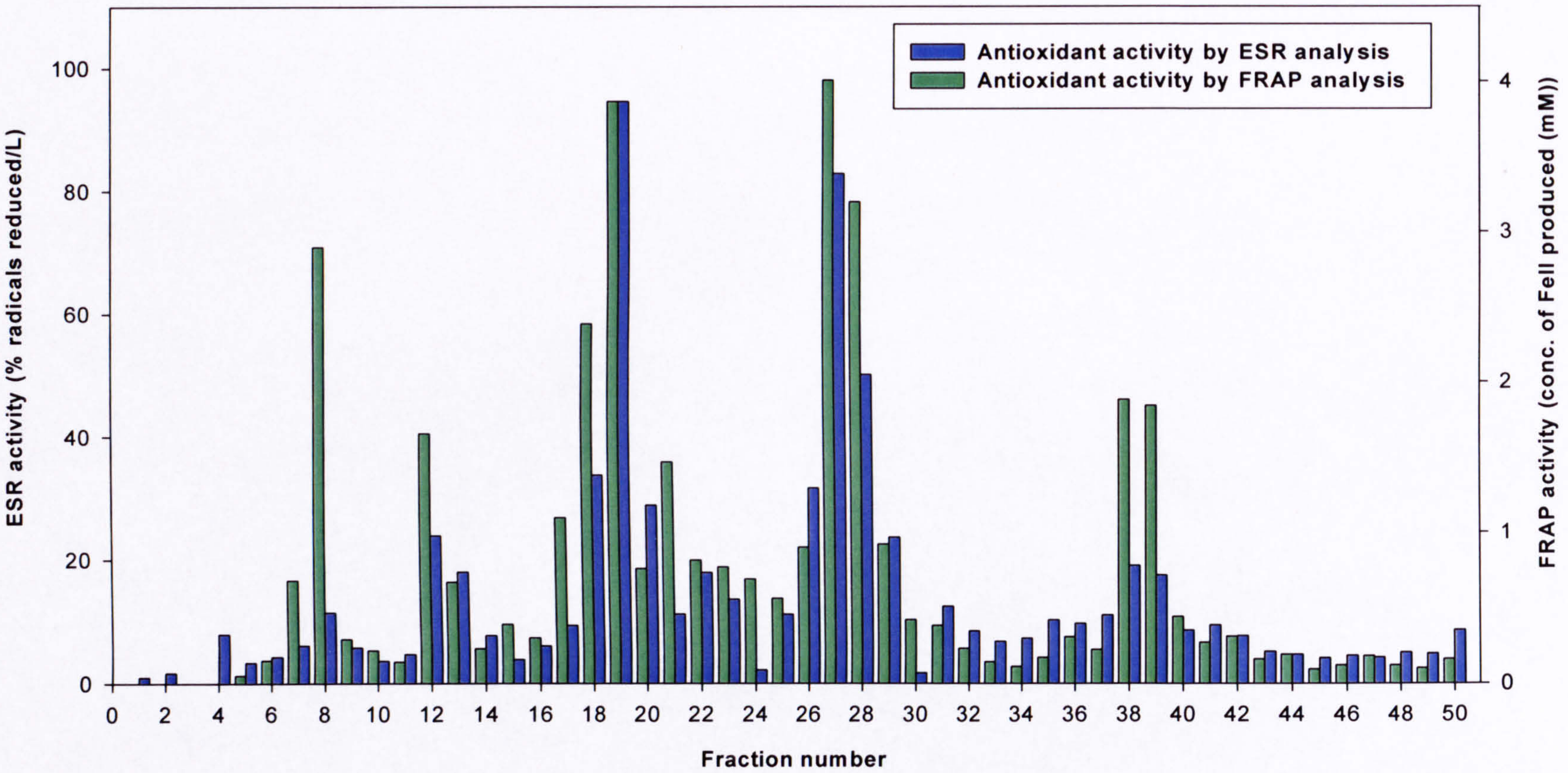
Graph 38 Total phenol content of green tea fractions

All samples run in duplicate (n =2). Values expressed as mean µg/ml gallic acid equivalents (GAE). See Table 20 for individual values.



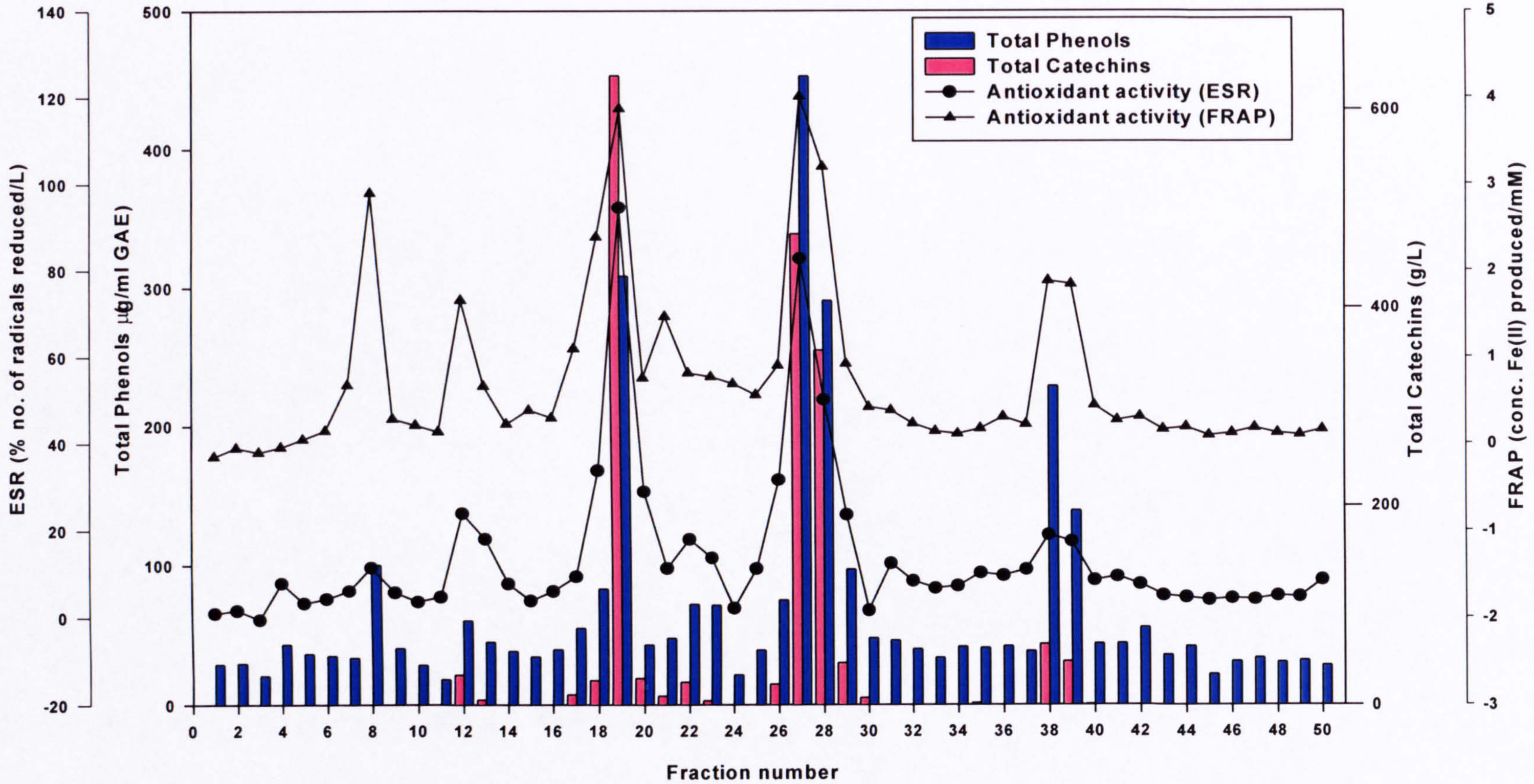
Graph 39 Total catechin content of green tea fractions

All samples run in duplicate (n =2). Values expressed as mean mg/L catechin. See Table 20 for individual values.



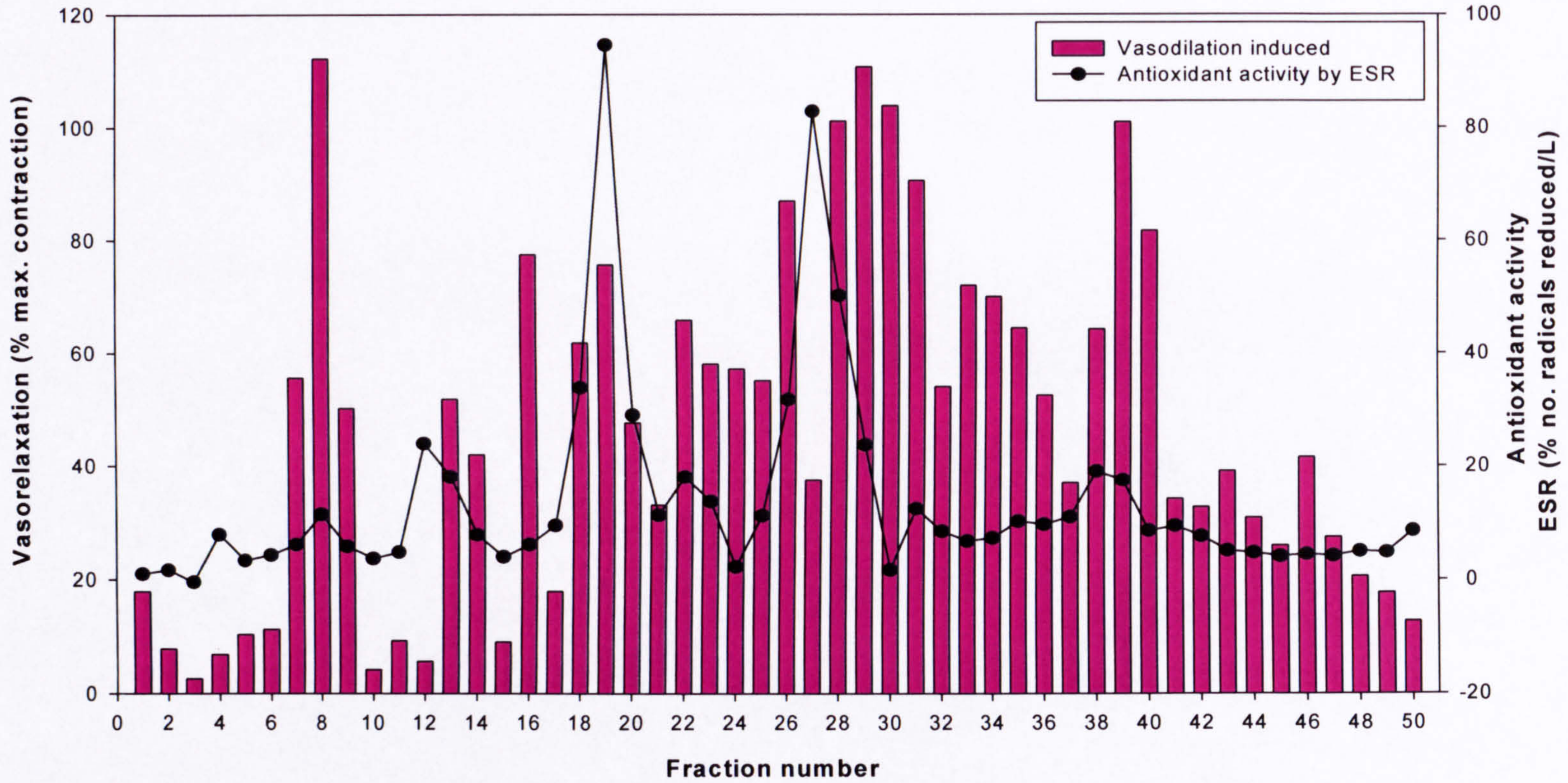
Graph 40 The antioxidant activity of green tea fractions by ESR and FRAP

All samples run in duplicate (n =2) and expressed as mean values. See Table 20 for individual values.



Graph 41 Total phenolic and catechin content and antioxidant activity of green tea fractions

All samples run in duplicate (n =2) and expressed as means values. See Table 20 for individual values.



Graph 42 Antioxidant capacity and vasodilatory activity of green tea fractions

All samples run in duplicate (n =2) and expressed as means values.

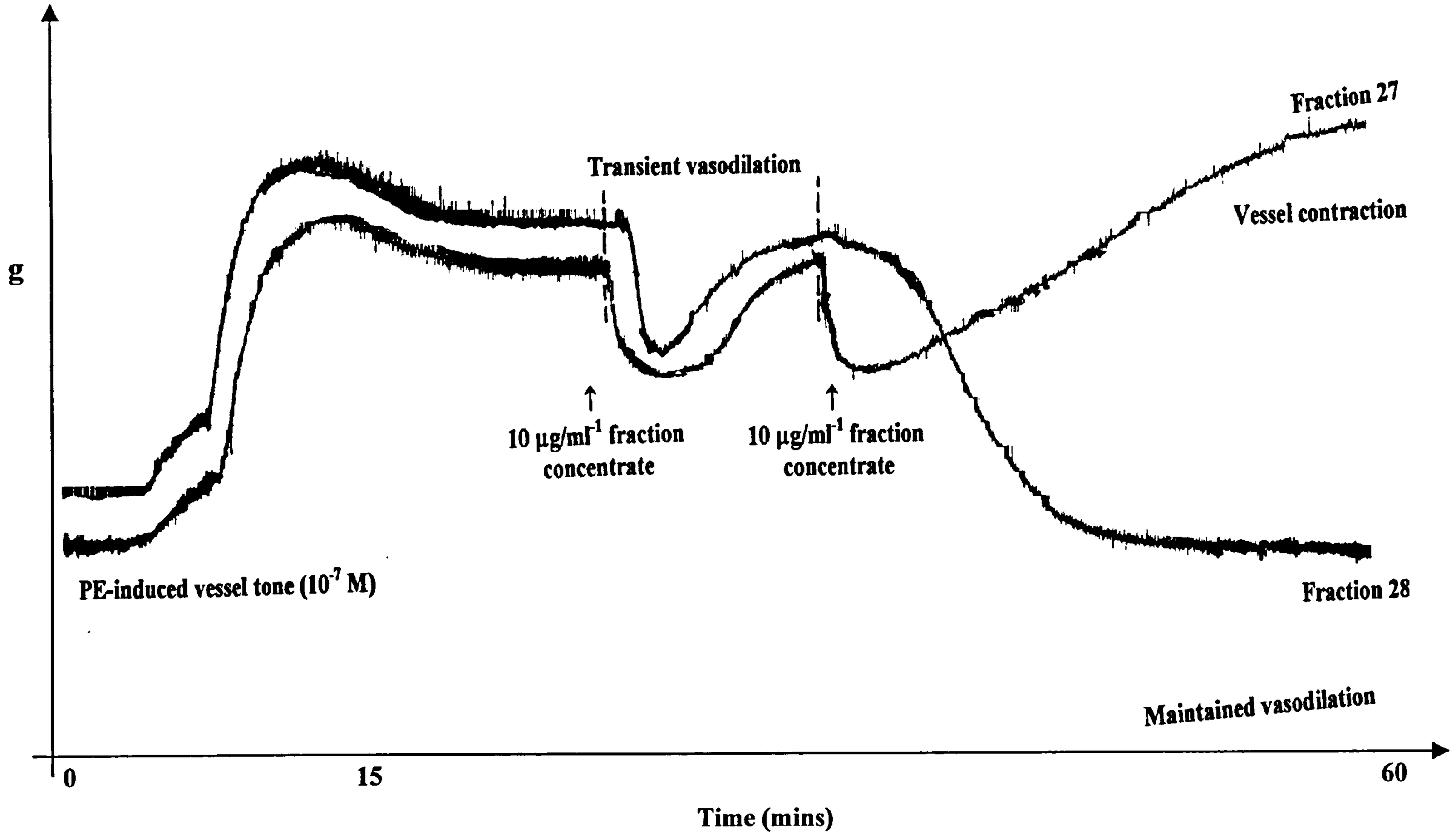


Figure 17 Vasodilation induced by green tea fractions 27 & 28

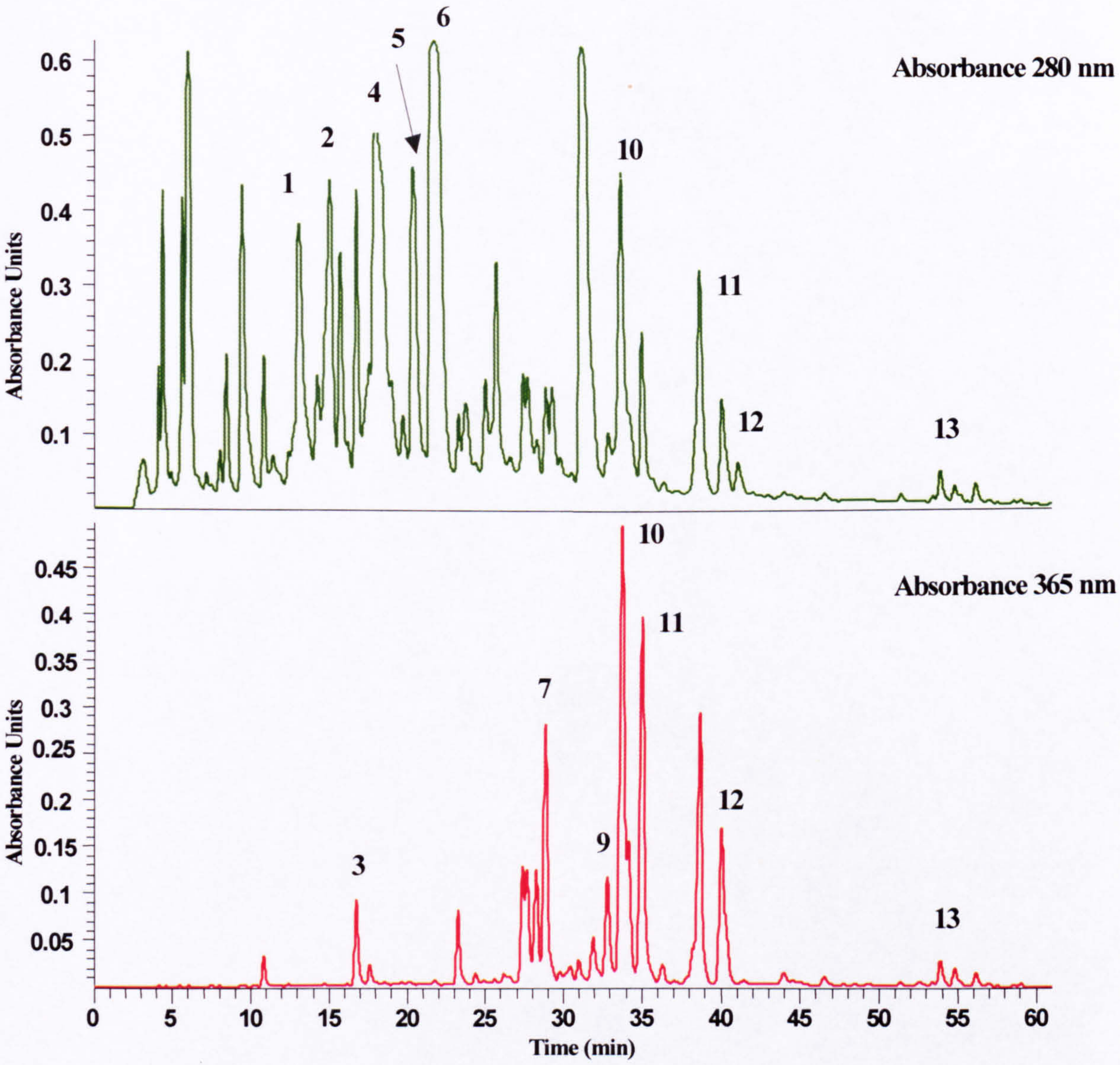


Figure 18 Green tea HPLC chromatographic traces

(For peak identities see Table 21)

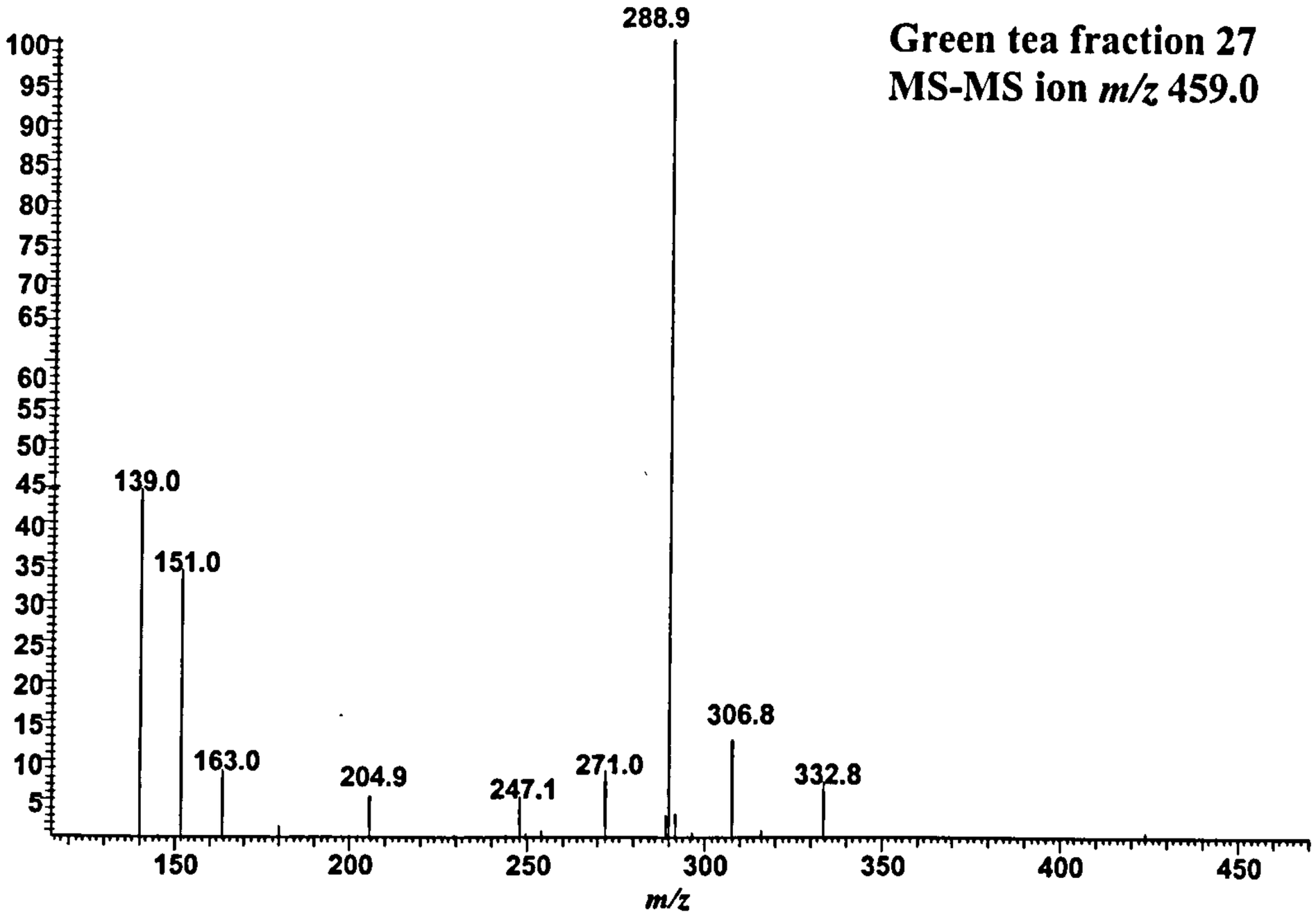


Figure 19 Mass spectrum of EGCG molecular ion M⁻ 459.0

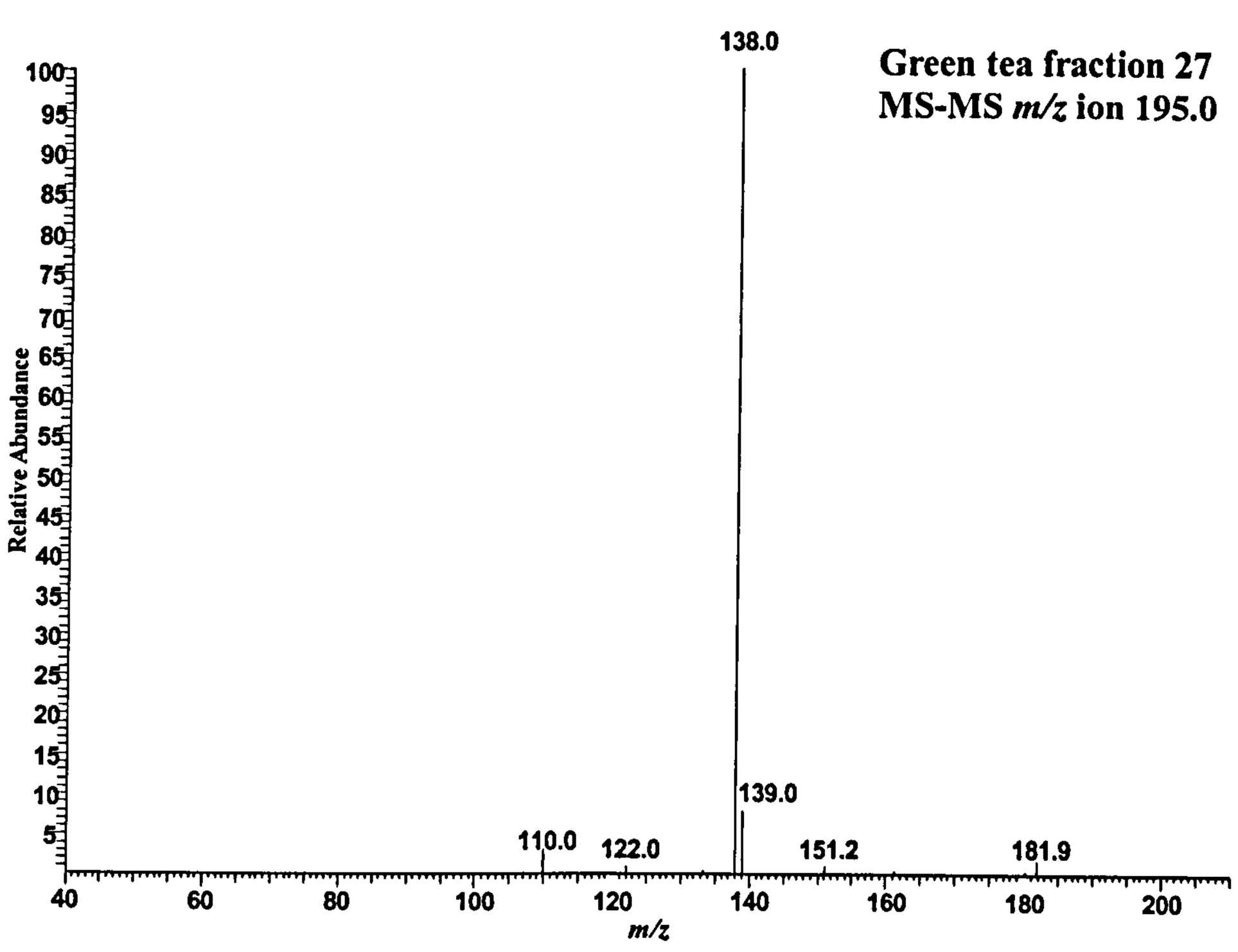


Figure 20 Mass spectrum of caffeine molecular ion M⁻ 195.0

5.3.3 Black Tea Fractionation

Following the large-scale prep fractionation of black tea (U-1704), the antioxidant activity of each fraction was determined by FRAP analysis. The total phenolic and catechin content of each fraction was also determined. Results are shown in Graphs 43 to 45. Values for each individual assay carried out are shown in Table 23 & 24.

5.2.1.4 Spectral & colorimetric assays

The antioxidant activity of the black tea fractions showed little variability across the range of fractions (Graph 43) as determined by their ferric reducing ability as an index of their antioxidant capacity (FRAP analysis). Fractions 3 & 4, and 17-21 had high antioxidant activity, with an overall decrease in activity thereafter. Fraction 21 was found to have the highest overall activity producing 0.6 mM of Fe(II) (Table 23). A slight increase in antioxidant activity is evident with fractions at the end of the collection range, fractions 57-60. The antioxidant activity of the black tea fractions was not investigated using ESR. Previous analysis with the green tea fraction demonstrated that ESR and FRAP produce highly correlated and equivalent results.

The total phenolic content of the black tea fractions produced a similar content pattern as that of the FRAP antioxidant activity. As before, fractions 18 – 21, were found to be consistently higher than surrounding fractions allowing for a peaked effect with a reduction in total phenolic content with increasing fraction number, with the exception of fraction 28 which had the overall highest content (Graph 44) containing 68 μg/mL GAE as compared to fraction 18 which contained 65 μg/mL GAE (Table 23).

The catechin assay displayed the specific separation in catechin content (Graph 45). A gradual increase in content up to fraction 18 was evident. Fractions 18 –22 and 30 – 35 were found to have a high total catechin content with a decrease in content with surrounding fractions. Fraction 21 had

Table 23 Black tea fractionation assay mean values table (fractions 1-40)

Fraction	Total Phenol[a]	Total Catechin[b]	FRAP ability[c]
1	34.6	-0.5	0.03
2	47.7	0.44	0.20
3	55.2	2.07	0.45
4	52.7	2.64	0.53
5	43.6	1.37	0.19
6	40.8	2.20	0.16
7	45.5	2.28	0.19
8	44.9	2.93	0.25
9	44.9	2.12	0.20
10	52.4	3.13	0.22
11	44.9	3.68	0.25
12	44.0	3.60	0.23
13	47.7	6.61	0.32
14	51.1	6.74	0.37
15	52.1	4.59	0.35
16	55.5	5.31	0.40
17	54.6	8.60	0.44
18	67.7	13.5	0.55
19	65.8	11.6	0.51
20	60.5	8.26	0.47
21	64.9	14.3	0.60
22	59.0	12.5	0.59
23	56.1	6.94	0.44
24	48.3	3.34	0.35
25	46.8	2.82	0.33
26	46.8	4.07	0.34
27	50.8	3.29	0.35
28	68.3	3.83	0.36
29	48.6	3.99	0.40
30	37.6	7.54	0.46
31	39.1	12.1	0.39
32	28.8	11.3	0.38
33	33.5	8.31	0.36
34	36.3	13.2	0.46
35	30.7	11.3	0.39
36	30.1	5.48	0.32
37	30.7	4.23	0.27
38	27.6	2.39	0.26
39	34.5	2.60	0.27
40	31.0	2.98	0.32

[a]Total phenol data expressed as mean μg/mL gallic acid, [b]Total catechins expressed as mean g/L catechin, [c]Ferric reducing ability expressed as the mean concentration of Fe(II) produced (mM). All samples run in duplicate.

Table 24 Black tea fractionation assay mean values table (fractions 41-60)

Fraction	Total Phenol[a]	Total Catechins[b]	FRAP ability[c]
41	38.2	3.15	0.34
42	31.3	2.77	0.31
43	30.7	2.28	0.28
44	31.0	3.64	0.31
45	31.3	2.55	0.34
46	33.5	2.44	0.27
47	27.3	2.22	0.24
48	25.7	4.56	0.23
49	28.5	4.78	0.25
50	28.5	3.91	0.22
51	30.1	4.45	0.27
52	27.9	4.61	0.28
53	30.4	4.61	0.27
54	24.5	3.53	0.28
55	23.8	3.36	0.26
56	35.4	3.80	0.29
57	29.1	6.52	0.35
58	29.8	6.63	0.37
59	32.3	6.52	0.39
60	25.4	6.14	0.35

[a]Total phenol data expressed as mean μg/mL gallic acid, [b]Total catechins expressed as mean g/L catechin, [c]Ferric reducing ability expressed as the mean concentration of Fe(II) produced (mM). All samples run in duplicate.

the highest overall catechin content, with 14.3 g/L of catechin present (Table 23). As with the FRAP analysis, an increase in total catechin content is evident with fractions 57-60.

The relationship between the total phenolic and catechin contents and antioxidant activities by FRAP of the black tea fractions is shown in Graph 46. FRAP-based antioxidant activity was highly and significantly correlated with both total phenolic (r = 0.50, P<0.001) and total catechin content (r = 0.75, P<0.0001) by Pearson's correlation analysis. The higher r value reported by the total catechin assay on analysis indicates that the catechin content of the black tea fractions has a greater effect on overall on antioxidant activity than phenol content, and has a stronger relationship with the FRAP assay. Total catechin and phenol contents of the black tea fractions were also positively correlated (r = 0.26, P<0.05).

5.2.1.5 Vasodilatory activity

Vasodilation studies with the black tea fractions were not carried out.

5.2.1.6 Mass spectrometry analysis

Catechin and polyphenol identification with the black tea fractions was determined by HPLC MS-MS. The phenolics present in the tea sample were determined by co-chromatography with authentic standards. Compounds were identified on the basis of their retention times, absorbance spectra and MS-MS fragmentation patterns. Figure 21 displays the HPLC chromatographic trace for the black tea sample, displaying absorption at 280 nm and 365 nm.

A number of compounds were identified in the black tea sample following co-chromatography with standards. Table 25 identifies each component of the black tea sample by peak number from the HPLC traces by retention time. These chromatographic traces display the complex nature and large number of compounds present in black tea. The small number of peaks not identified with retention times between 40 to 45 minutes in present in both

HPLC traces (Figure 21), are thought to correspond to the presence of theaflavins which are known to elute after the flavonols (Lee & Ong, 2000). The poor HPLC baseline with the black tea chromatography is attributed to the presence of the high molecular weight thearubigens being present throughout the tea sample which do not separate or chromatograph well.

Table 25 Identification of the main phenolics in black tea by HPLC MS-MS

Peak No.	Component	Retention Time
1	Epigallocatechin (EGC)	13.62
2	Chlorogenic acid	16.52
3	Epicatechin (EC)	19.83
4	Epigallocatechin gallate (EGCG)	21.43
5	Catechin trimer	25.85
6	Myricetin glucoside	27.28
7	Cyanidin	28.72
8	Rutin	32.50

Black tea sample run on a 5-30% acetonitrile/1% formic acid gradient over 60 mins in positive ion mode.

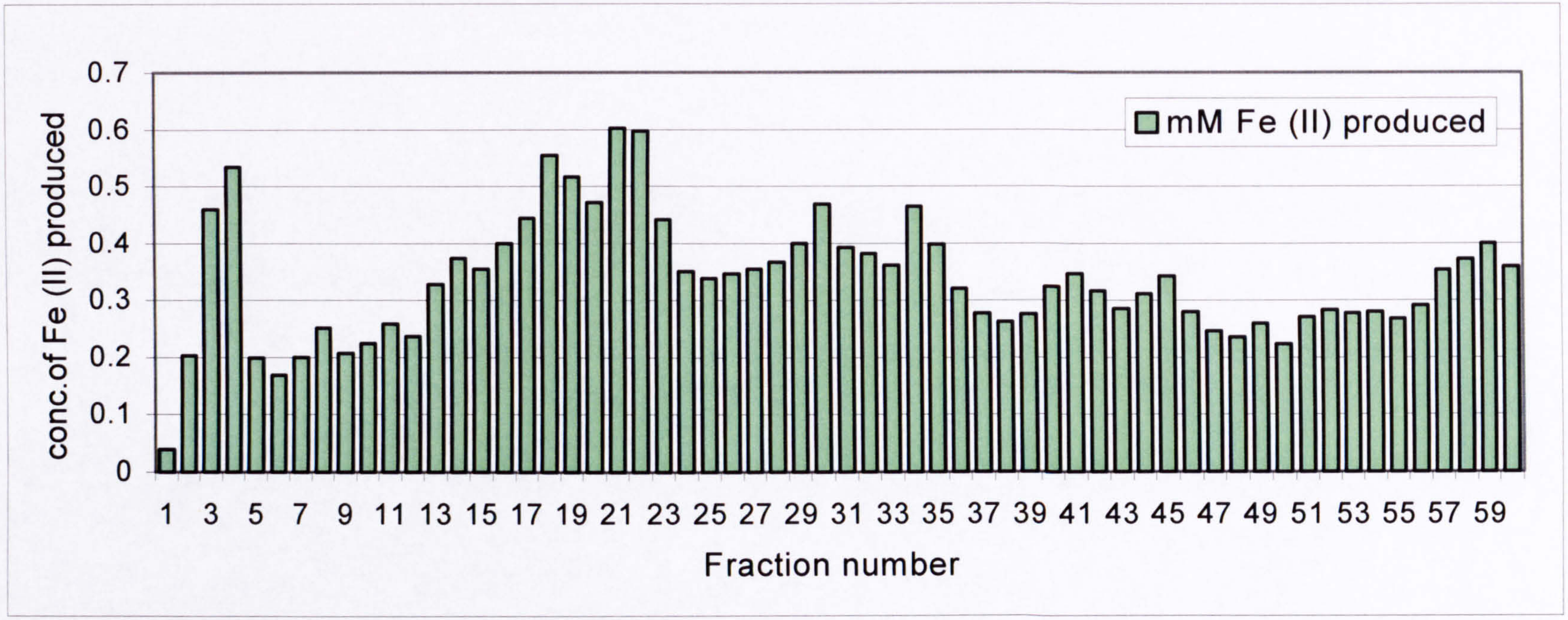
Additional work with other tea samples using co-chromatography with a standard tea extract of theaflavins (Sigma-Aldrich, Dorset, England), retention times and elution order identified the presence of the 4 main theaflavins in black tea (Figure 22, Table 26) using MS-MS their molecular ions and fragmentation patterns (Figures 23 - 26).

Table 26 Identification by HPLC MS-MS of the theaflavins in black tea

Peak No.	Component	R_t (min)	M^+ (*m/z*)
1	Theaflavin	50.88	563
2	Theaflavin-3-gallate	53.55	715
3	Theaflavin-3′-gallate	55.17	715
4	Theaflavin-3,3′-digallate	55.83	867

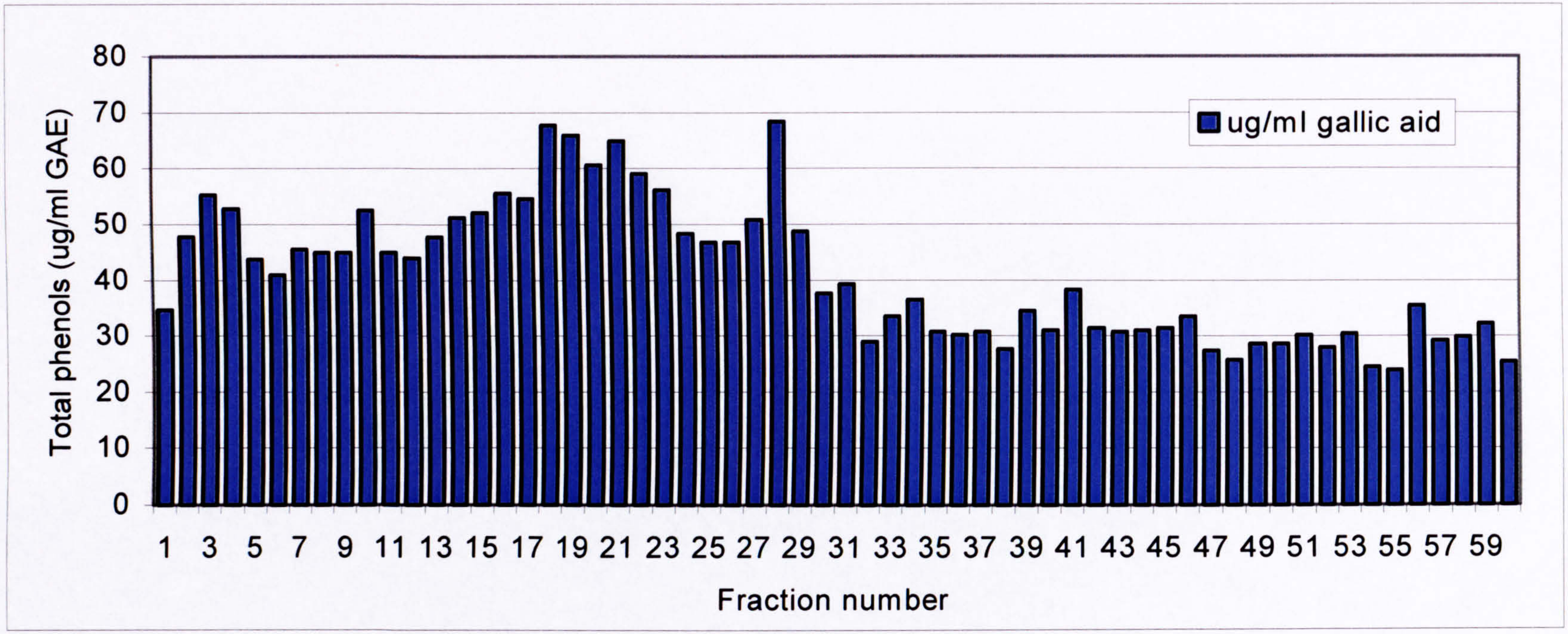
Peak 1 had a M^- of m/z 563 amu, the molecular ions of peaks 2 and 3 were 152 amu greater, an increase which corresponds to the addition of a gallate group. The molecular ion of peak 4 was 304 amu greater than peak 1, twice 152 amu, corresponding to the addition 2 gallate groups.

5.3.4 Black tea fractionation: Graphs & Figures



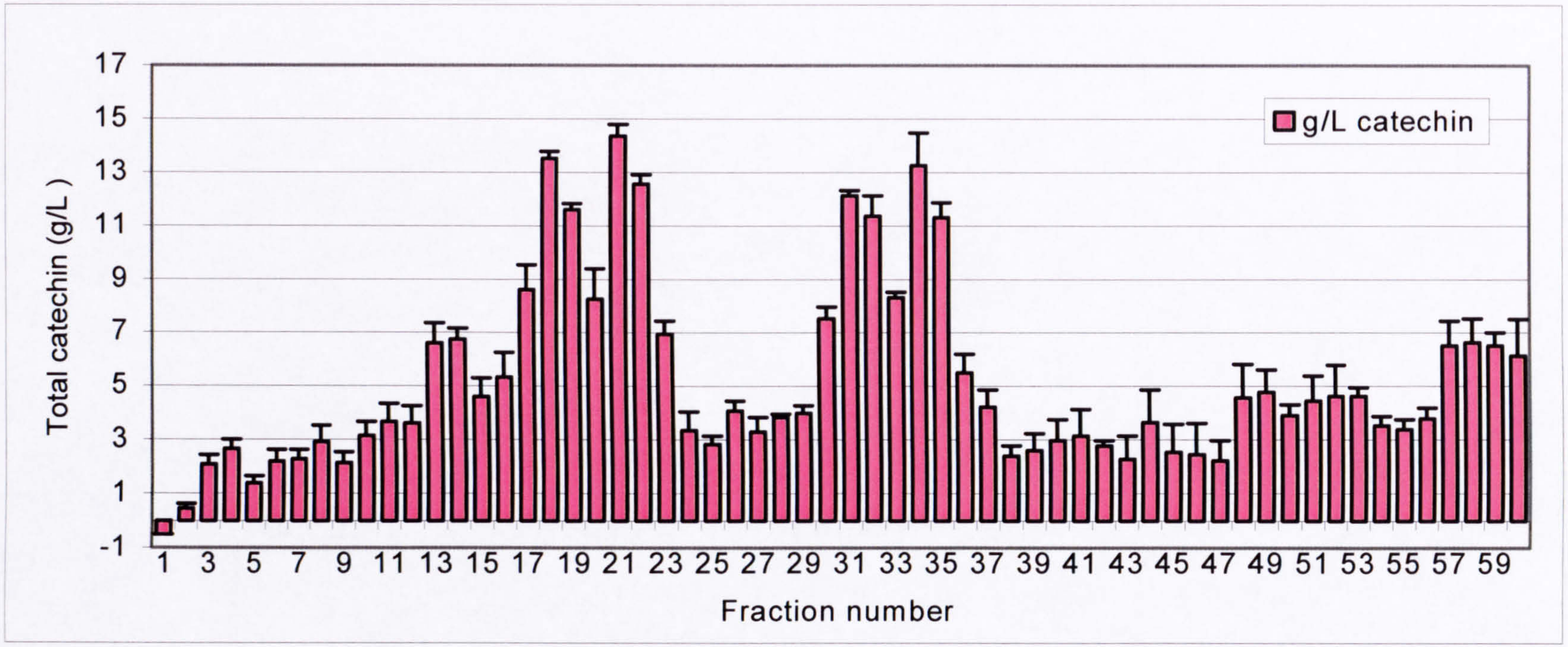
Graph 43 FRAP antioxidant activity of black tea fractions

All samples run in duplicate (n =2). Values expressed as mean concentration of Fe(II) produced (mM). See Tables 23 & 24 for individual assay values.



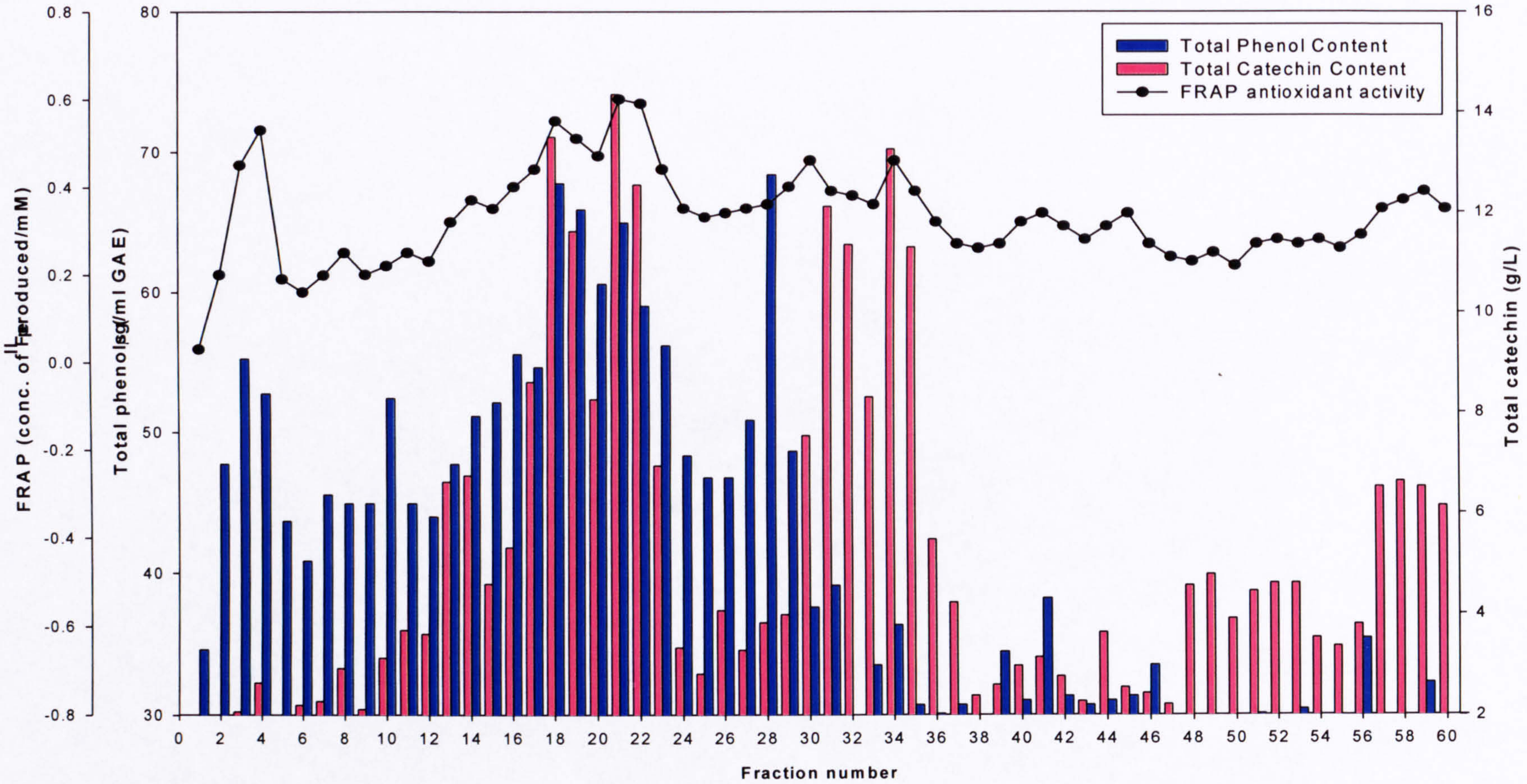
Graph 44 Total phenolic content of black tea fractions

All samples run in duplicate (n =2). Values expressed as mean μg/mL gallic acid equivalents (GAE). See Tables 23 & 24 for individual assay values.



Graph 45 Total catechin content of black tea fractions

All samples run in duplicate (n =2). Values expressed as mean g/L catechin. See Tables 23 & 24 for individual assay values.



Graph 46 Antioxidant activity and total phenolic and catechin content of black tea fractions

All samples run in duplicate (n =2) and expressed as means. See Tables 23 & 24 for individual assay values.

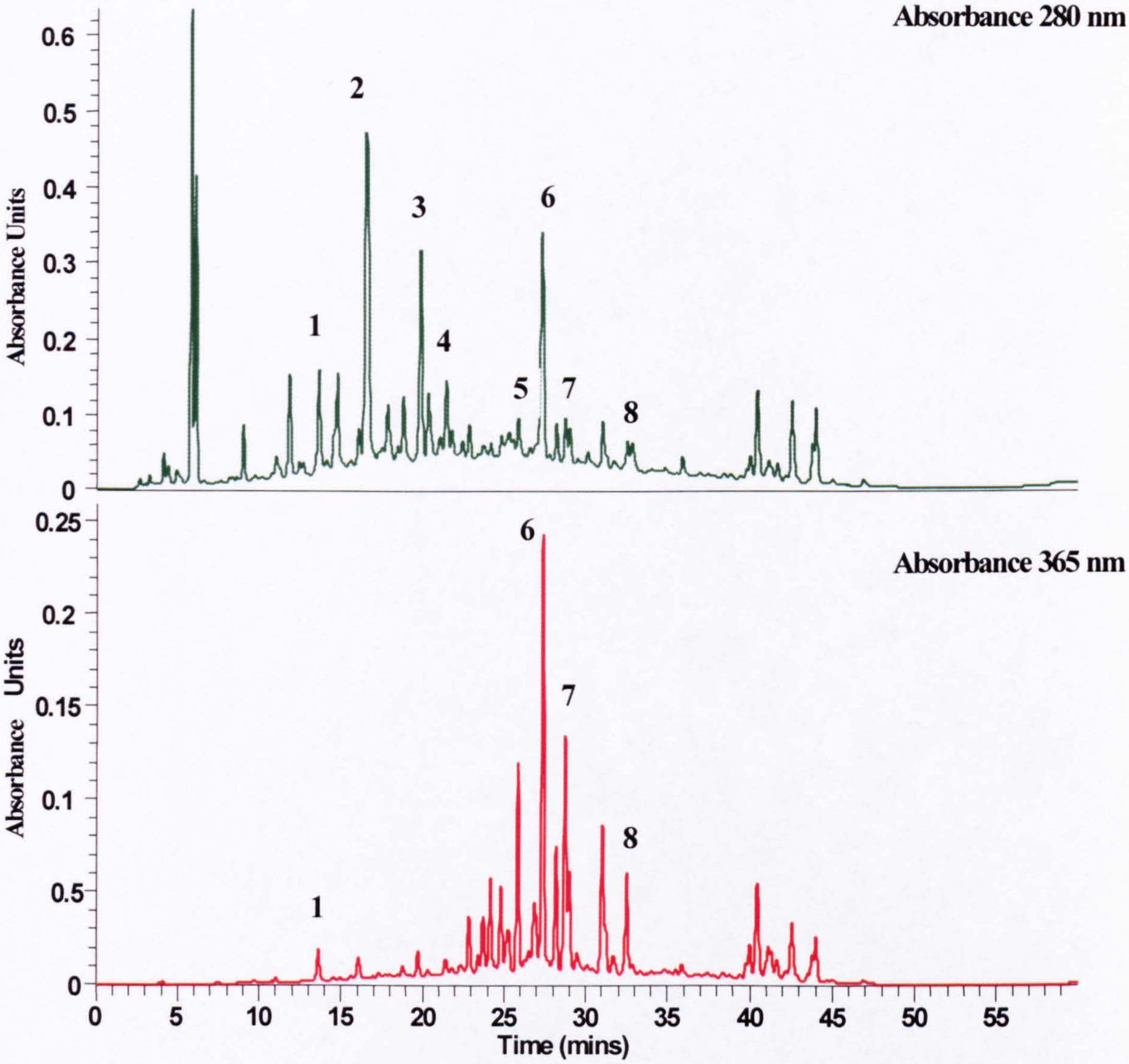


Figure 21 Black tea HPLC chromatographic traces

(For peak identities see Table 25)

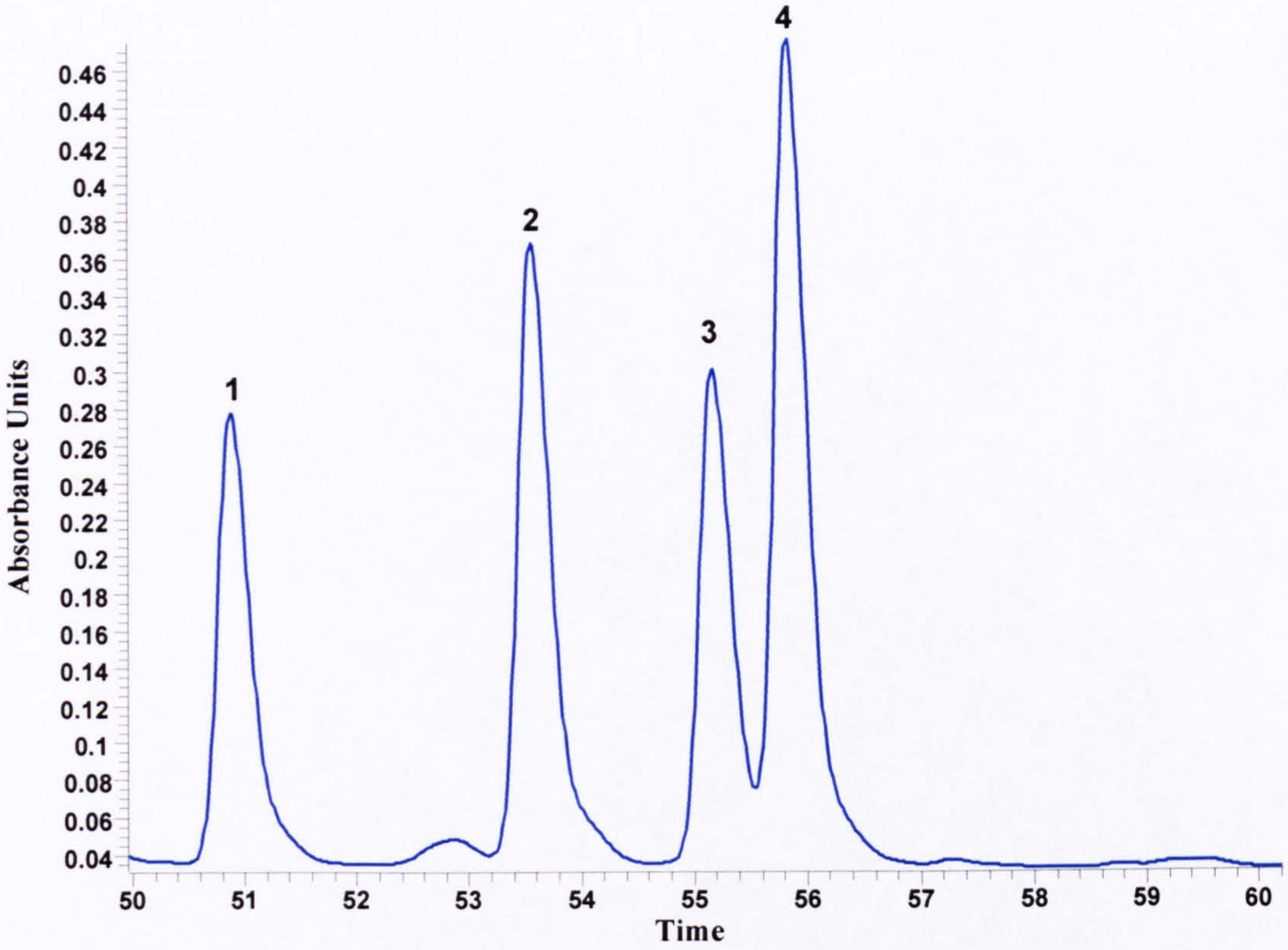


Figure 22 HPLC chromatographic trace of the theaflavins present in black tea

(For peak identities see Table 26)

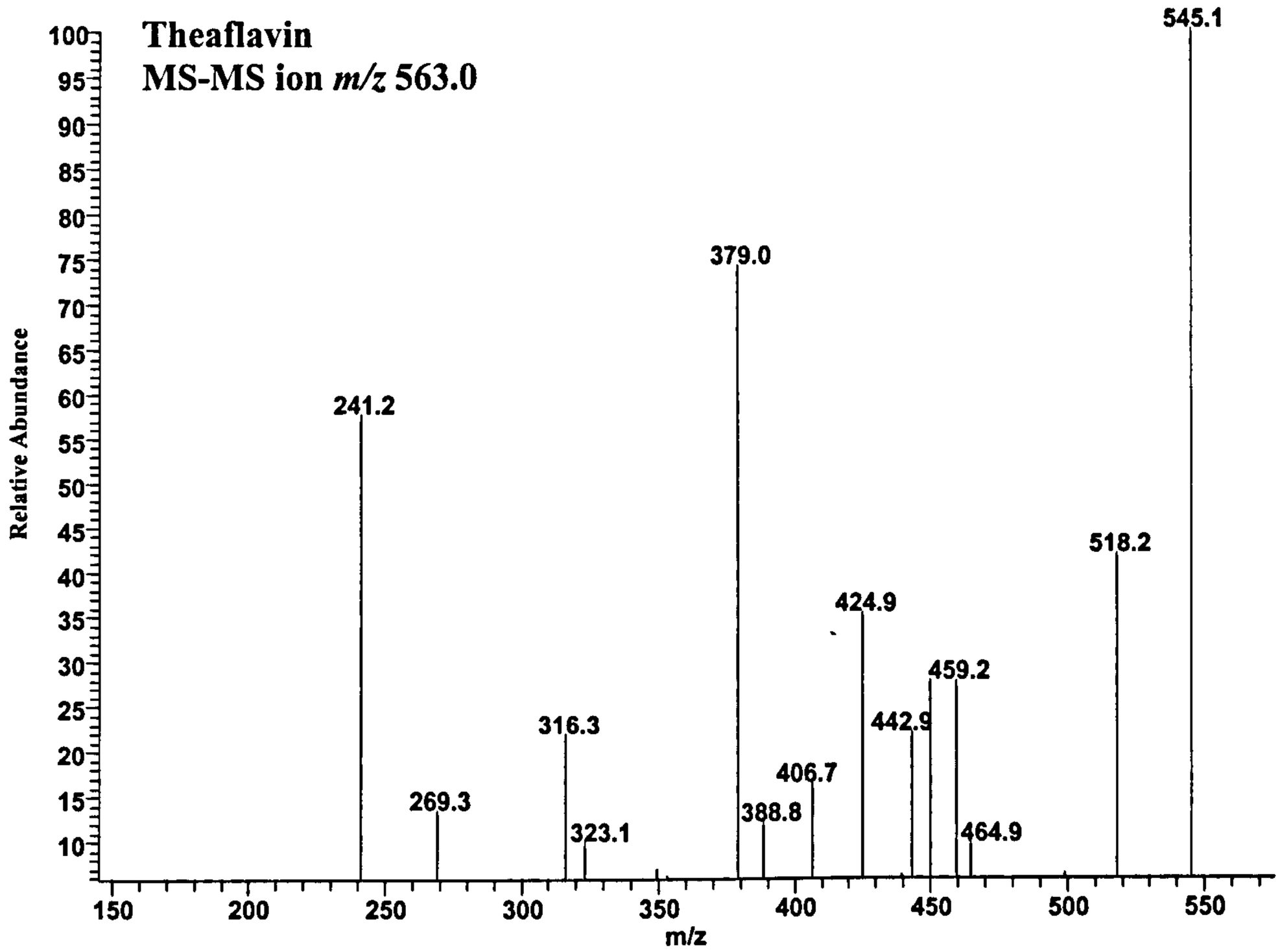


Figure 23 Mass spectrum of theaflavin molecular ion M^- 563.0

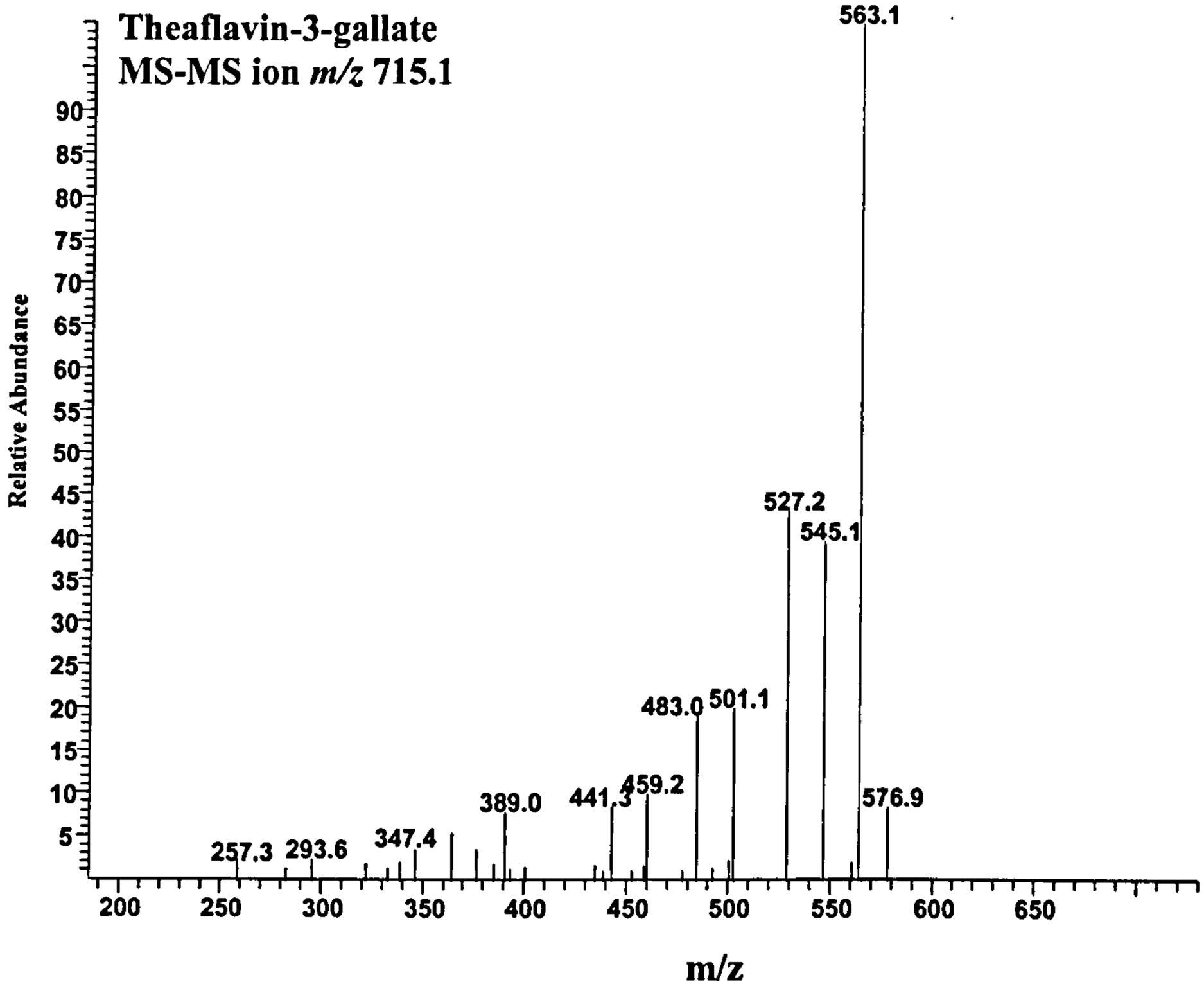


Figure 24 Mass spectrum of theaflavin-3-gallate molecular ion M˙ 715.1

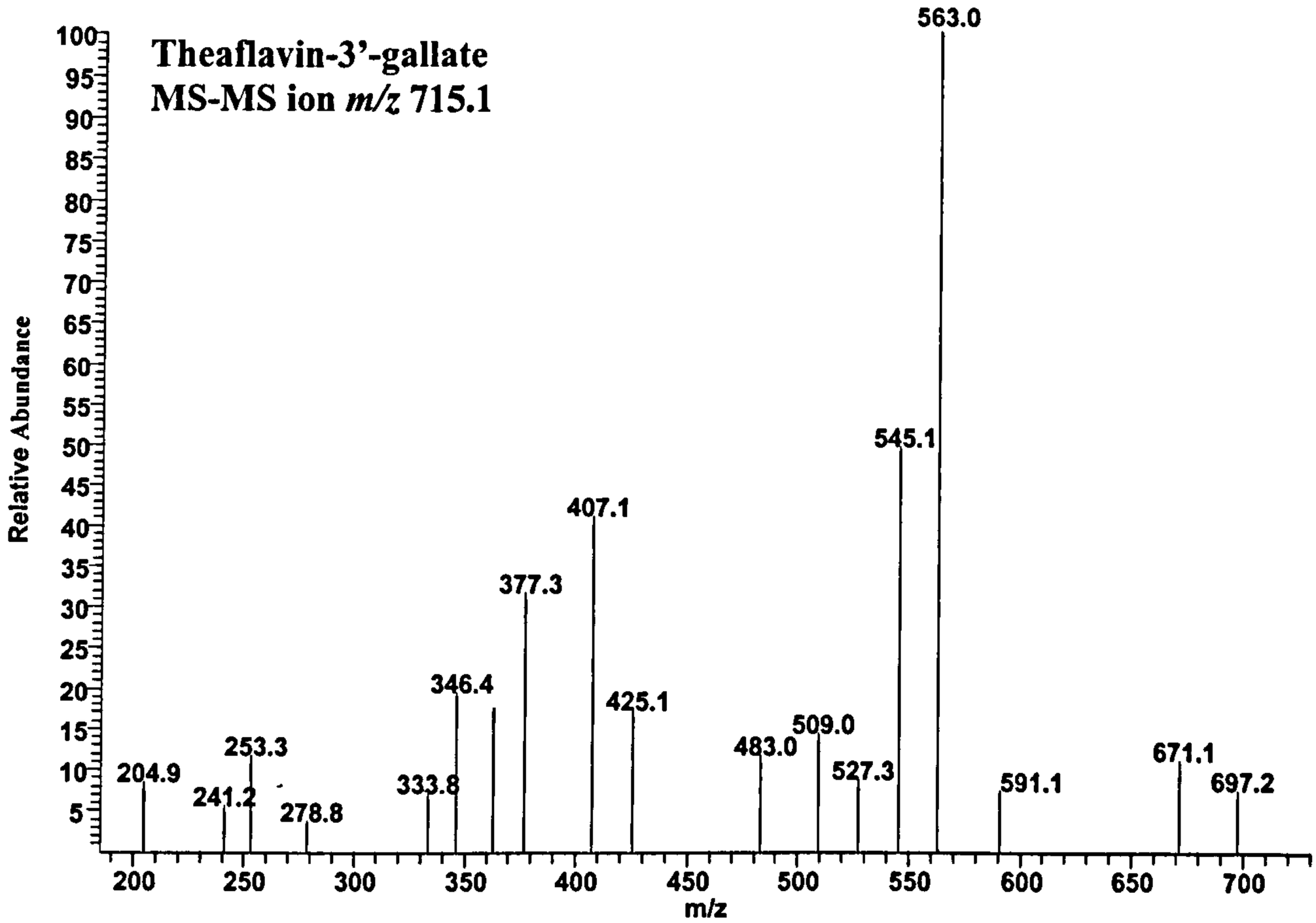


Figure 25 Mass spectrum of theaflavin-3'-gallate molecular ion M˙ 715.1

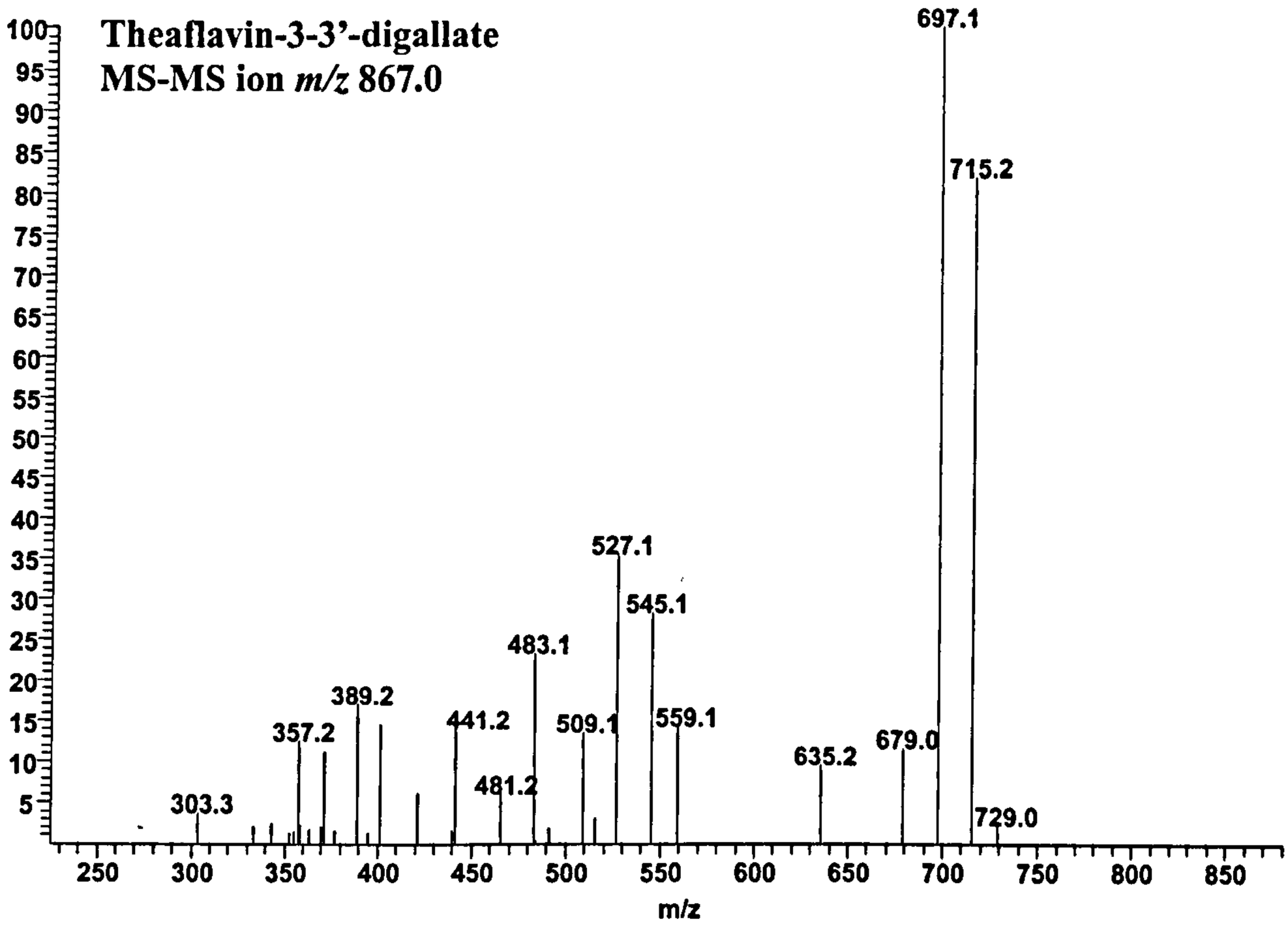


Figure 26 Mass spectrum of theaflavin-3-3'-digallate molecular ion M˙ 867.0

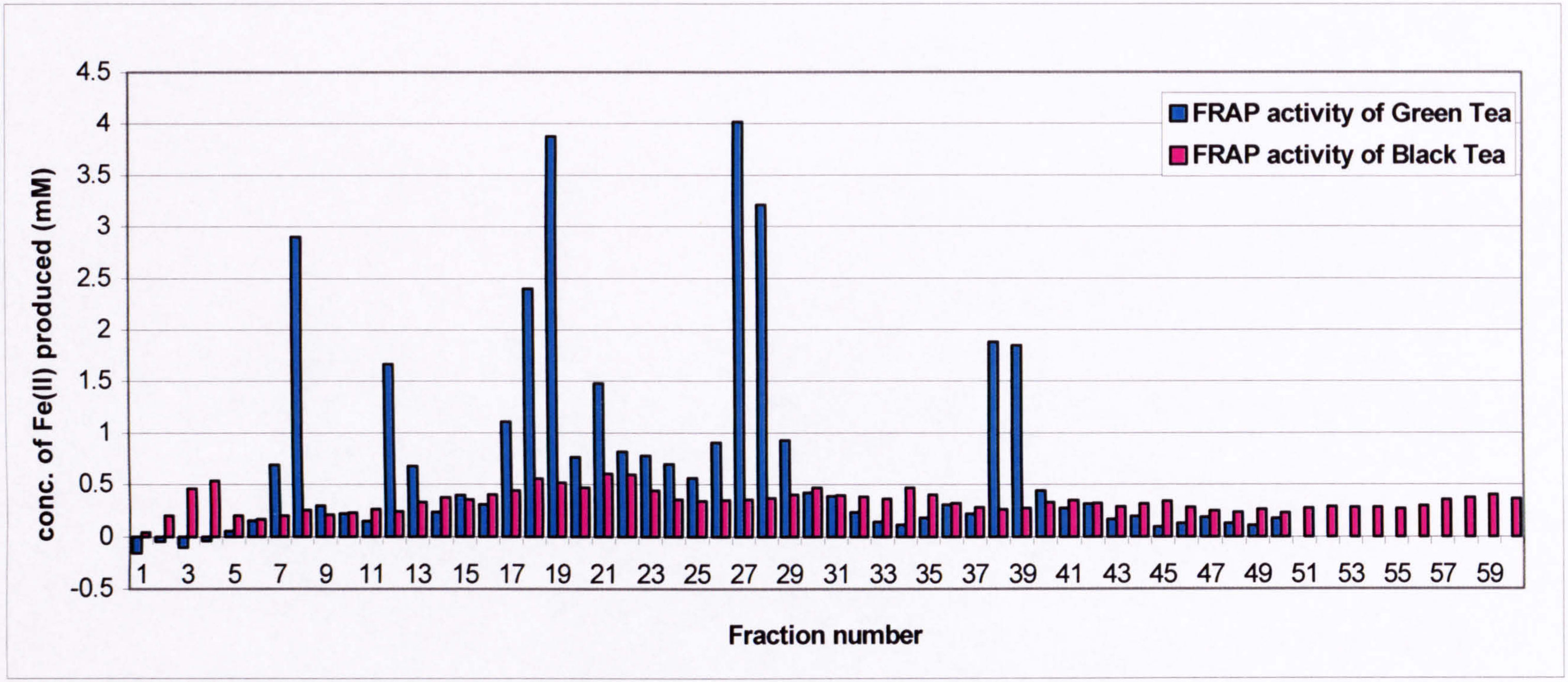
5.3.5 Comparison of the fractionation of green & black teas

In order to investigate differences between the 2 types of tea which were previously fractionated, comparisons of the fractionation results from the FRAP antioxidant assays and total phenolic and catechin contents were carried out. Comparison results for the green and black teas are shown in Graphs 47 to 49.

Green tea fractions are found to be consistently higher in both activity and content than the black tea fractions. Green tea fractions were found to be significantly ($P<0.001$) more effective antioxidants than corresponding black tea fractions, being in general about five times for effective free radical scavengers (Graph 46). The concentration of both phenols and catechins present in the black tea fractions were significantly lower ($P<0.0001$) (5 to 10 times lower) than that recorded for the green tea fractions (Graphs 48, 49). Differences in overall content and activity of the tea fraction may account for the differences in the effects of green and black tea *in vivo*, with green tea having a consistently higher *in vivo* effect.

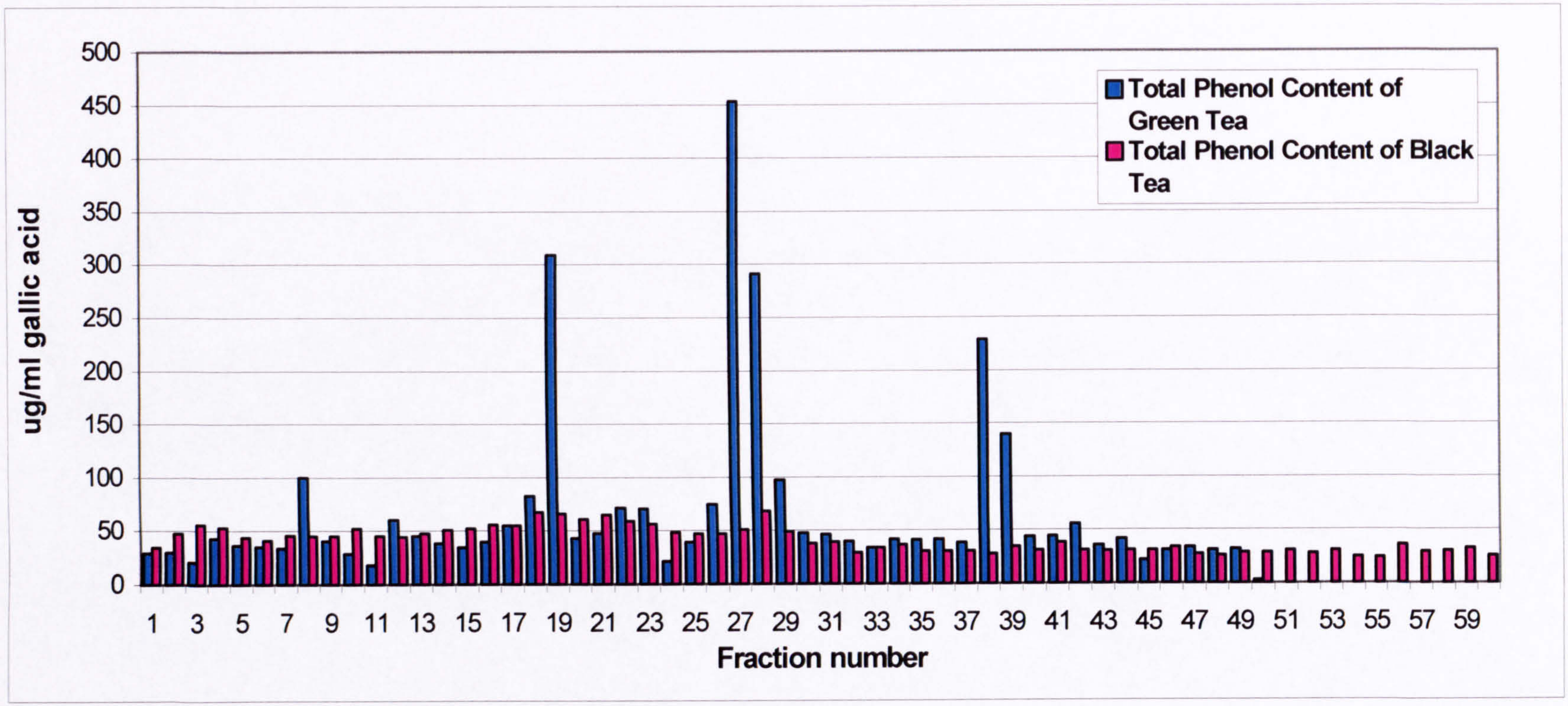
The use of mass spectrometry also identified differences between the chemical composition of each tea type.

5.3.6 Green & black tea fractionation: Graphs



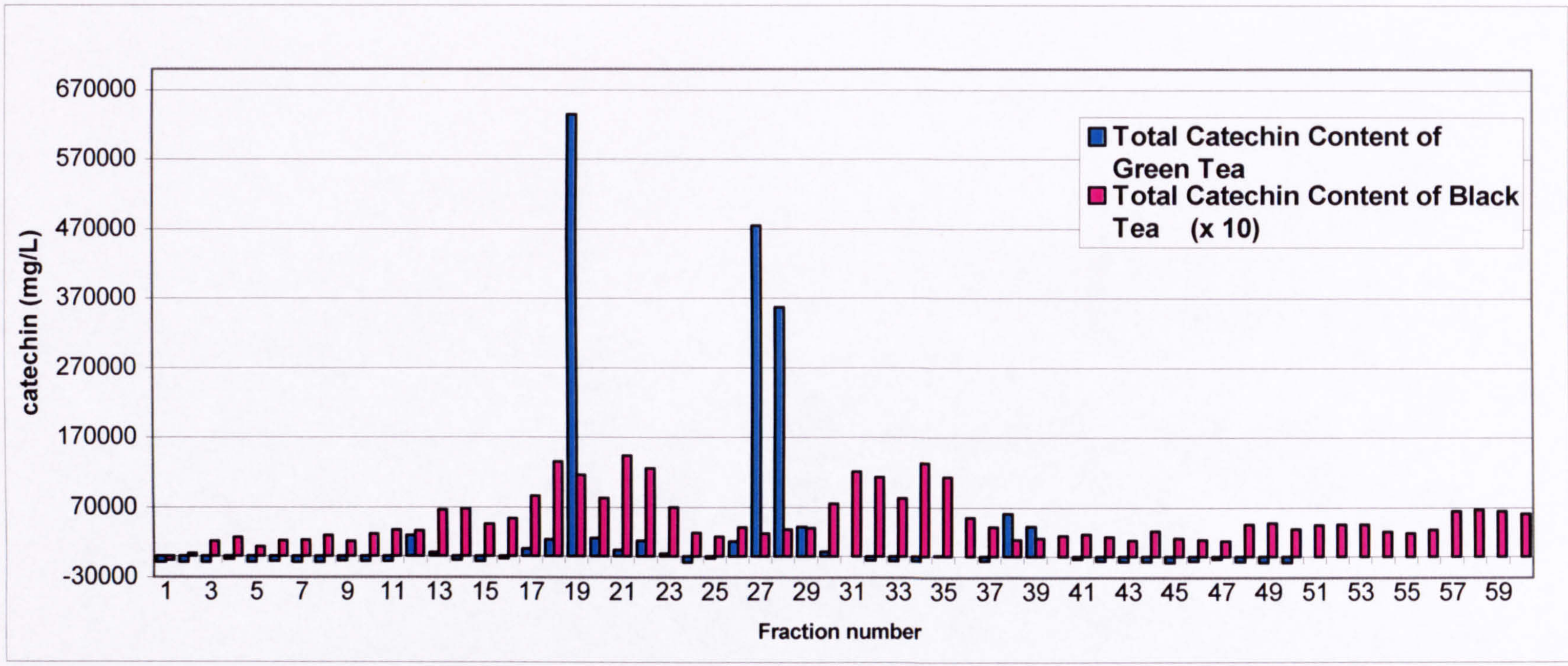
Graph 47 The antioxidant ability of green and black tea fractions analysed by the FRAP assay

All samples run in duplicate (n =2). Values expressed as mean concentration of Fe(II) produced (mM). See Tables 20, 23 & 24 for individual assay values.



Graph 48 Total phenolic content of green and black tea fractions

All samples run in duplicate (n =2). Values expressed as mean µg/mL gallic acid. See Tables 20, 3 & 24 for individual assay values.



Graph 49 Total catechin content of green and black tea (x10) fractions

All samples run in duplicate (n =2). Values expressed as mean g/L catechin. See Tables 20, 23 & 24 for individual assay values.

5.4 Discussion

The present study has demonstrated the pattern of separation, based on differences in polarity and identification using MS-MS, of the main catechins and polyphenols present in green and black teas following large-scale preparatory fractionation. This study has further determined by the use of complementary methods, the antioxidant activity, total phenolic and catechin content of each fraction, highlighting a significant positive association of increasing antioxidant capacity with increasing phenol and catechin content. Likewise, in the case of the green tea fractions, indicating a general correlation between increasing antioxidant capacity and vasodilator activity *in vitro*.

Phenols in tea are believed to be responsible for its subsequent antioxidant activity. The pure catechins and phenolics found in tea are more powerful than the antioxidant vitamins C, E and β-carotene in an *in vitro* lipoprotein oxidation model (Vinson *et al.*, 1995). Comparison of the tea fractions in this study indicate that the catechins and theaflavins contribute to the teas' antioxidant characteristics (Vinson & Dabbagh, 1998). Green and black tea extracts have also been reported to have 3.5 times greater antioxidant activity than the traditional vitamins (Langley-Evans, 2000). Data on the levels of total phenolics in this study were obtained by the relatively non-specific Folin-Ciocalteu method calibrated with gallic acid (Singleton & Rossi, 1965). Green tea was found to have a higher total phenol content as compared to black tea fractions, which may account for the lower antioxidant capacity of the black tea fractions determined using the FRAP assay. In all 3 assays investigating black tea fractionation, fractions 57-60 at the end of the collection range increased in both activity and content. This feature of the fractionation corresponds to the presence of theaflavins, the high molecular weight products with previously described comparable antioxidant potency to green tea catechins (Leung *et al.*, 2001) which are generally considered to be more effective components for the inhibition of carcinogenesis (Lee & Ong, 2000).

The antioxidant properties of polyphenols have been proposed to underlie their apparent health benefit. The results displayed in this study show the antioxidant effectiveness of green and black tea fractions. Green tea fractions were found to be about 5 times more active as antioxidants as compared to black tea fractions. A large number of *in vitro* studies have shown that tea catechins are absorbed by humans. Studies have shown that both green (He & Kies, 1994; Serafini *et al.*, 1996; Benzie *et al.*, 1999; Sung *et al.*, 2000) and black (Langley-Evans, 2000) tea consumption may lead to subsequent increases in plasma antioxidant capacity.

HPLC is extensively used for the identification and quantification of polyphenols. The main catechins and epicatechins can be routinely detected in green tea using UV at 280 nm (Finger *et al.*, 1992), with catechin and epicatechin known to be naturally fluorescent with excitation at 280 nm and emission at 310 nm. In black tea, catechin and epicatechin are present at much lower levels and due to UV sensitivity, fluorescence detection is preferred (Blacklock, personal communication). The use of both isocratic and gradient solvent systems have been used for the analysis of catechins from teas. Mobile phases are generally based on methanol or acetonitrile and contain acid (Robb & Brown, 2001). The presence of acid is necessary not only for complete separation of the catechins but also for optimal separation efficiency. Although catechins are extremely stable at pH <4, stability is pH dependent in the range of pH 4-8: the lower the pH, the greater the stability. Catechins instantly degrade at pH levels above 8 (Finget *et al.*, 1992). Column temperature can affect catechin separations. Temperatures ranging from 20-50°C have been employed to get good separations (Beecher *et al.*, 1999). The HPLC system used in this study was run using acetonitrile and formic acid as the mobile phase, with a of pH 5 and a column temperature of 40°C, to endure the optimal conditions for resolution and sensitivity of detection for the effective separation of the catechins present in each sample. This study has shown the distinct composition of green and black teas and the differences between them. EGCG was identified in both tea samples, being the main peak identified in green tea and being present in lower levels in black tea.

MS detection has also been employed for improved catechin identification and quantification. Thermospray, electrospray and plasmaspray interfaces to the liquid chromatograph have all been used (Dalluge & Nelson, 2000). Using MS, the flow rate and temperature of the inlet tube have all to be optimised for each class of compounds. Good sensitivity is obtained for compounds which ionise efficiently (Dalluge & Nelson, 2000). However, in a tea extracts, a large number of compounds are present, which ionise at different efficiencies, thus optimisation is more difficult (Miketova *et al.*, 1998). Two recent studies of the mass spectrometric characteristics of the polyphenolic extract of green tea have shown that liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) is a powerful tool for the identification of the components present in the tea mixture (Miketova *et al.*, 1998; Dalluge & Nelson, 2000). The phenolic content of the teas in this study was determined by LC-MS-MS and co-chromatography with authentic standards and standard tea mixtures. It must however be noted that very few of the teas are commercially available in a purified form. The present study has identified the presence of 3 of the 4 main epicatechins present in green tea, EC, EGC and EGCG in our fractionated green tea sample, with additional catechin trimers also identified. The presence of large amounts of caffeine was also identified. This may account for the poor vasodilator ability of green tea fraction 27, which was found to have potent antioxidant capabilities and high phenolic and catechin content. The identification of caffeine in green tea may also account for the contractile effects of green tea shown in previous chapters.

Theaflavins are catechin-based trimers (Robb & Brown, 2001). Individual theaflavin standards are not commercially available and additional work with a tea standard theaflavin mixture allowed the identification of theaflavins on the basis of elution order, retention time, MS-MS data and current related literature (Lewis *et al.*, 1998, Lee & Ong, 2000). The theaflavins are known to elute after the flavonols (Finger *et al.*, 1992; Lee & Ong, 2000) in black tea and are absent in green tea due to chemical changes which take place during the fermentation stage of the manufacturing process.

Individual thearubigens, the high molecular weight fermentation condensation products were not identified using HPLC. The peaked baseline effect shown in the black tea chromatographic trace is thought to correspond to the presence of thearubigens in the tea sample. Previous work has shown that they do not chromatograph well due to their high molecular weight, and to date have not been identified using mass spectrometry (Lee & Ong, 2000).

5.4.1 Conclusion

Catechins are the most common type of polyphenol found in tea. The consumption of catechins may have a beneficial effect on some aspects of health. The phenolic profile of tea is dependent upon a number of influences including leaf variety and extent of processing. HPLC is by far the most common method of catechin analysis, the improvement of existing technologies and the development of new assays may contribute to an increase in the alleged health benefits of tea compounds.

Chapter 6 General Discussion

This thesis has reported on a series of investigations undertaken to examine the relationship between the vasodilatory activity and antioxidant capacity of polyphenolic compounds, namely extracts of grape and tea and green tea epicatechin derivatives. By determining the effect of such compounds on vascular tension *in vitro* and their related antioxidant activity this provides additional information on the alleged beneficial effects of such beverages. In addition the main components present in samples of green and black tea were determined identifying the main components of each tea type and the chemical differences between green and black tea.

6.1 Vasodilatory & antioxidant activity of grape and tea extracts

The possible beneficial health effects of red wine and tea have mainly focused on their role as potent antioxidants and their ability to scavenge free radicals, but the ability of polyphenols present in these beverages to induce vessel relaxation is also important in their alleged protection of the cardiovascular system. Natural dietary phenolics and polyphenols from grape derived products can induce endothelium-dependent vasodilation in both human and rodent vessels (Andriambeloson *et al.*, 1998; Flesch *et al.*, 1998), with the mechanism of this vasodilation being via the release of nitric oxide (NO) and subsequent increases in cGMP (Fitzpatrick *et al.*, 1993; Andriambeloson *et al.*, 1997; Flesch *et al.*, 1998). Green tea has also been shown in small number of studies to induce vasodilation in isolated vascular vessels. The vasodilator activity of green tea has been attributed to its (-)-epicatechin (EC) content, to the release of NO from vascular endothelial cells, and to the inhibition of calcium influx (Huang *et al.*, 1998, 1999).

Vasodilation induced *in vitro* by grape and tea based extracts were determined and related to their antioxidant activity and phenolic content.

Mechanisms and pathways of action in the vascular system were also determined.

The vasodilatory activity of each individual extract was determined using organ bath pharmacology, a standard, reliable and repeatable experimental pharmacology technique. This study has demonstrated that grape and tea based extracts can induce vasodilation in isolated aortic vessels (Chapter 3). A dried extract of French grape seed was identified to be the most potent extract investigated and the black tea theaflavin extract to be the least, inducing vasorelaxation at the lowest extract concentration and inducing vasorelaxation with the lowest maximum value respectively. Grape based extracts consistently induced greater maximum vasodilation than the tea based extracts. All extracts produced biphasic concentration-dependent vasorelaxation in rabbit aortic isolated vessels.

An extract of red grape skin induced vasodilation by complex mechanisms. These included endothelium-independent mechanisms, via vasodilating prostaglandins, and via prostacyclin and endothelial NO production. Vasodilation induced by a grape powder extract, a dried extract of the whole grape, indicated that the both the flesh and the seeds of the grape have the potential for relaxation. It is evident therefore that not all vaso-active components in grapes are derived from the grape skin. The French grape skin and seed extracts induced vasorelaxation via NO-endothelium-dependent mechanisms. Vasorelaxation induced by green and black tea extracts were due to the combined interactions between vasodilating, endothelium-dependent prostaglandins, and other undetermined mechanisms.

The antioxidant capacity of the extracts consistently showed the tea extracts to be better antioxidants than the grape based extracts. The French grape seed extract was the exception as it was identified a potent vasodilator and a poor antioxidant. This extract was identified to have comparable antioxidant activity to the green and black tea and was consistently higher in all spectral and colorimetric assays than other grape based extracts. Grape

seeds contain flavan-3-ols or catechins (Fitzpatrick *et al.*, 1997), similar to that of green tea. On the other hand, the composition of the grape skin extract is predominately composed of anthocyanins accounting for their deep red colour. Red wine extracts have previously been shown to induce vasorelaxation in rabbit aortic vessels with a potency directly correlated to their antioxidant activity and total phenolic content (Burns *et al.*, 2000). This study on the other hand, has demonstrated a significant inverse correlation between the vasodilatory and antioxidant activity of the extracts, indicating that the higher the antioxidant activity of the extracts, the less active they are as vasodilators. It is also evident that the compounds responsible for inducing antioxidant activity are distinct from those responsible for their vasodilator activity of the extracts *in vitro*.

The opposite nature of the results published by Burns *et al.*, (2000) as compared to the present study may be due to genuine differences in the initial starting products rather than differences in experimental methodology. Red wine extracts were investigated for their antioxidant and vasodilatory activity as compared to various dried grape based extracts in this study. The full wine extracts may therefore have contained a wider range of active compounds other than grape derived products which may have enhanced their vasodilatory and antioxidant capabilities. Red wines will also have a different composition to that of grape extracts because of the chemical changes that occur during extraction, vinification and maturation of the finished wine.

6.2 Vasodilatory & antioxidant activity of green tea epicatechins

Catechins are a group of natural polyphenols found in green teas usually accounting for 30 – 42% of the dry weight of the solids in brewed tea (Yang, 1999). They also are present in black tea but to a much smaller extent. Catechins are oxidised to form the distinctive theaflavins of black tea. Green tea contains the characteristic polyphenolic compounds, (+)-catechin and its

isomer EC, (-)-epicatechin gallate (ECG), epigallocatechin (EGC), (-)-catechin gallate (CG) and (-)-epigallocatechin gallate (EGCG) (Balentine *et al.*, 1997).

Green tea was previously showed in this study (Chapter 3) to be a vasodilator in isolated aortic vessels. To determine the vasoactive components of green tea a selection of the main catechins and epicatechins present were investigated for their vasodilatory activity using standard organ bath pharmacology, and related to their antioxidant activity (Chapter 4). Their mechanisms and pathways of action *in vitro* were also determined.

CG was identified to be the most potent derivative *in vitro* and EC to be the least, inducing vasorelaxation at the lowest derivative concentration added to the organ bath and inducing the lowest maximum relaxation value respectively. Vasorelaxation occurred via the release of NO and was dependent upon the presence of intact functional vascular endothelium. EC and (+)-catechin were devoid of significant vasodilator activity in the rabbit aorta, consistent with studies in the rat aorta where catechin and EC had very low vasodilator activity (Andriambeloson *et al.*, 1997; 1998). The results of this study may account for a degree of the vasodilator activity of green tea but, as discussed previously (Chapter 3), cannot account for all the dilator activity which also involved non-endothelial factors and prostaglandin-mediated effects.

Green tea epicatechins and catechins were also identified to be effective antioxidants, with overall activity ranging in the order magnitude of EGCG>EGC>ECG≥Catechin>CG>EC. Antioxidant activity was positively and highly significantly correlated with vasodilatory activity.

Catechin and epicatechin derivatives are found primarily in the seeds of grapes (Sato *et al.*, 1999). This may contribute to the high antioxidant and vasodilatory capacity of the French grape seed extract investigated in Chapter 3, as compared to the predominately anthocyanin containing grape skin extracts. Grapes also contain gallate esters and gallocatechins but these are

found in only low levels in red wine (Burns *et al.*, 2000) but may, according to the this study account for a small degree of vasodilation induced by red wines. This study has shown CG to be a potent vasodilator *in vitro* but to be a poor antioxidant with low overall phenol content. The red grape based extracts investigated in Chapter 3 had similar vasodilatory and antioxidant responses to that of CG, which is found in low levels in red wine, and is derived primarily from the grape seed (Burns *et al.*, 2000) but which has also been identified in the grape skin. CG may therefore, be in part accountable for the vasodilation induced by grape skin and seed extracts.

Differences in the statistical results presented for the vasodilatory and antioxidant activities of the grape and tea based extracts (Chapter 3) as compared to that of the green tea epicatechin and catechin derivatives (Chapter 4), may be due to genuine differences in the basic chemical composition of the compounds investigated as standard experimental methodology was used in both studies. The grape and tea extracts investigated contained a range of active components in varying concentrations which may have interacted together to produce cumulative antioxidant and vasodilatory effects as compared to the purified single epicatechin standards of a predetermined concentration. Contamination of the grape and tea extracts is another factor which may have had an affect on the results presented and which could not be ruled out.

This study has demonstrated that epicatechins derived from green tea and which are also present to a smaller extent in black tea, can be both vasodilators and antioxidants. The possible health effects of such compounds are likely due to the cumulative effects of these catechins and epicatechins and other components present not yet identified working in synergy.

6.3 Fractionation of green & black tea

Further investigations into the nature of the polyphenols present in tea and their related antioxidant activity was demonstrated. Samples of green and

black tea were separated into fractions on the basis of their polarity using a preparative HPLC system.

This study has demonstrated the pattern of separation and identification using mass spectrometry of the main catechins and polyphenols present in green and black teas following large-scale fractionation (Chapter 5). This study has further determined by the use of complementary methods, the antioxidant activity, total phenolic and catechin content of each tea fraction. Increasing antioxidant capacity was highly correlated with increasing total phenol and catechin content. The effect of each green tea fraction on vascular tension *in vitro* was also determined. A positive association between increasing antioxidant capacity and vasodilator activity was shown.

Mass spectrometry (MS) detection was also been employed for improved catechin identification. The phenolic content of the teas in this study were determined by LC-MS-MS and co-chromatography with authentic standards. The present study identified the presence of 3 of the 4 main catechins present in green tea, EC, EGC and EGCG with additional catechin trimers and large amounts of caffeine also evident. This may account for the poor vasodilator ability of green tea fraction 27 which was identified to have high antioxidant capabilities and a high phenolic and catechin content. The identification of caffeine in green tea may account for the contractile effects of green tea shown in Chapter 3. The theaflavins and thearubigens from black tea were also putatively identified in the fractionated sample.

6.4 Conclusions

The consumption of red wine and tea as part of a healthy diet maybe associated with a reduced incidence of CHD and cancers. This protection is believed to be conferred by the high polyphenol content of both beverages. This study has clearly demonstrated that grape and tea based extracts and green tea epicatechin derivatives can induce vasorelaxation in vascular tissue and has specified the mechanisms of action for each individual extract and

green tea derivative. A number of these compounds are also effective antioxidants, both mechanisms which may contribute to the alleged protective effects of red wine and tea. This study has also demonstrated that the anthocyanins present in red wine account for the vasodilatory activity of grape based extracts, and catechins to be responsible for the antioxidant activity conferred by tea extracts and catechin and epicatechin derivatives. Consumption of red wine and tea may therefore play a role in helping maintain optimal vascular function *in vivo* via antioxidant and vasodilatory mechanisms, contributing to improved cardiovascular health.

6.5 Future work

There has been an explosion of interest in the possible beneficial effects of dietary components on human health over the past few years. The publications arising from this research field appear to be increasing exponentially. Possibilities for continuing research in this field are innumerable.

The isolation and identification of the vaso-active substances present in the extracts examined in this study and their possible effects *in vivo*, continues and are yet to be fully determined. It seems likely that the beneficial effects of red wine and tea are due to the cumulative effects of several polyphenols working in synergy rather than one individual compound present.

Wine and tea contain a huge number of compounds, phenolics, polyphenols and a large number of which at present remain uncharacterised. Work is required to understand the structure of these compounds, particularly the large complex tannins, theaflavins and thearubigens.

Vasodilation induced by the individual phenolics, especially the anthocyanins, present in red wine has not yet been investigated. Although *in vitro* studies have shown the possible beneficial effects of red wine, more *in vivo* studies are required to give a clearer picture of the compounds that are

responsible for maintaining optimal vascular blood flow. Similar studies are also required to investigate the cardiovascular effects of green and black tea *in vivo*.

Extensive trials with human subjects are required to determine the nature and extent of red wine derived phenolic and tea catechin absorption, bioavailability and metabolism. The work in this field to date relies heavily on the use of animal models and the use of pure compounds or plant extracts rather than whole beverages. Such trials may enable the major phenolics and polyphenols that are absorbed and their active metabolites, to be isolated and characterised. This may then allow the production of wines and tea enriched with these active components.

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