The identification and characterisation of quercetin metabolites in humans and rats.

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by

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Abstract.

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Quercetin is a dietary flavonoid, known to possess a range of biological properties in *in vitro* systems. These properties may be of potential therapeutic benefit against certain diseases. Considerable work has focused on its antioxidant capability, which has been shown to be more powerful than that of vitamin E.

Quercetin also has antineoplastic activity, and at 10μM it inhibits the proliferation of malignant cell lines derived from breast, ovarian and gastrointestinal tumours and leukaemias. Therefore, a phase I clinical trial on cancer patients was conducted at the Queen Elizabeth Hospital in Birmingham, UK, in which quercetin was administered intravenously. One patient received quercetin *p.o.* In parallel to this clinical trial, blood and urine, derived from rats, were analysed in order to study the metabolism of quercetin, and to elucidate its bioavailability *in vivo*.

Using human and *in vivo* animal model systems, metabolites were extracted and analysed by on-line liquid chromatography-mass spectrometry (LC-MS). Further characterisation was carried out by on-line tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance spectroscopy (NMR). Enzymatic hydrolysis was also used to corroborate the identity of the metabolites.

The results show that metabolism by F344 rats, which received quercetin *i.v.*, reflected adequately the metabolism seen in humans. Furthermore, analysis of both human and rat samples demonstrated that quercetin is rapidly metabolised *via* phase II biotransformation pathways to methyl, sulphate and glucuronide conjugates, thus eliminating quercetin efficiently from the body. The study shows that quercetin undergoes extensive first pass hepatic metabolism which in addition to the poor absorption, means that quercetin has low bioavailability. Consequently, quercetin might be only of limited therapeutic value, especially when taken orally. Whether or not the constant intake of quercetin *via* the diet may be beneficial to buttress the antioxidant defence system within the body remains to be elucidated.

The existence of GSH-conjugates of quercetin was investigated as a possible explanation for the nephrotoxicity exhibited in rats and the clinical trial. GSH-conjugates of quercetin were not found in vivo, although they could be synthesised in vitro.

Quercetin and isorhamnetin, although not quercetin sulphate nor glycosidic quercetin, exhibited potent COX-2 inhibition as demonstrated by reduced levels of PGE2 in HCA-7 cells.

This thesis is dedicated to my wife Julie, Mum and Dad, my two brothers, Garry and Seán and to the memory of Pat Brown.

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Abbreviations.

AOM - Azoxymethane.

APCI – Atmospheric pressure chemical ionisation.

BDS – Base deactivated octyl

BUN – Blood urea nitrogen.

CAD – Collision activated dissociation.

CE – Capillary electrophoresis.

CHD – Coronary heart disease.

CID – Collision induced dissociation.

Cisplatin - cis-diamminedichloroplatinum [II]

CO – Cyclooxygenase.

COMT – Catechol *O*-methyl transferase.

COX-2 – cyclooxygenase-2

DHPAA - 3,4-Dihydroxyphenylacetic acid.

DMSO - Dimethyl sulphoxide.

DNA – Deoxyribose nucleic acid

DTT - Dithreiotol

ECD – Electrochemical detection.

ER – Estrogen receptor

ESI – Electrospray ionisation.

ESI-MS – Electrospray ionisation mass spectrometry.

EDTA - Disodium ethylenediamine-tetraacetic acid.

FAB – Fast atom bombardment.

FAB-MS – Fast atom bombardment mass spectrometry

γ-GT - γ-glutamyl transferase.

GC – Gas chromatography.

GC-MS – Gas chromatography-mass spectrometry.

GSH – Glutathione.

GST – Glutathione-S transferase.

GSSG - Oxidised glutathione.

h – hour(s)

HK – Hexokinase.

HPLC – High performance liquid chromatography.

I.V. – Intravenous.

I.P. – Intraperitoneal.

mHPAA - *m*-hydroxyphenylacetic acid.

K' – capacity factor.

LC-MS – Liquid chromatography-mass spectrometry.

LC-MS/MS – Liquid chromatography-multistage mass spectrometry.

LDL – Low density lipoprotein.

LO – lipooxygenase.

MDR – Multi-drug resistance

min – minute(s).

MS1 – Mass spectrometer immediately after source.

MS2 – Second mass spectrometer.

MS/MS – Multi-stage mass spectrometry

MRM – multiple reaction monitoring.

NADPH – Nicotinamide adenine dinucleotide phosphate (reduced form).

PDA – Photo diode array.

PGE2 – prostaglandin E2

Pgp – p-glycoprotein

pnESI – Pneumatically assisted electrospray ionisation.

P.O. – per os

Quercetin - 3,5,7,3',4'-pentahydroxyflavone

RP – reversed phase.

TCA – Trichloric acetic acid

TLC – Thin layer chromatography.

Type II EBS – Type II estrogen binding sites.

UGT - UDP-glucuronyl transferase.

UV – Ultraviolet.

Contents:

1.0 Chapter One. Introduction.

1.1 Cancer chemotherapy and chemoprevention.

This thesis describes an investigation into the metabolism of quercetin, a potential cancer chemotherapeutic and chemopreventative agent. The introduction presents the rationale for the potential use of this agent, and its biological properties are outlined. Most importantly, the current state of knowledge of its metabolism and bioavailability is described.

Cancer chemotherapy is a growing area of research. Considerable financial resources and expertise are invested in the search for new or more selective anti-cancer agents. However, it is unlikely that currently available chemotherapeutic agents or new ones to be discovered will cure most cancers. Consequently, there is considerable pressure on cancer pharmacologists and oncologists to develop new strategies not only in the treatment but also in the prevention of the disease.

Cancer describes a wide range of diseases that share certain biochemical or physiological features. Cancer arises from the malignant transformation of normal cells that grow and keep dividing in an uncontrolled manner. Differences between normal cells and transformed cells are often subtle, and tend to be rooted in differential molecular regulatory mechanisms. Our understanding of the cellular and molecular processes involved in carcinogenesis has greatly increased in recent years, with advances in the determination of the role of cell-surface antigens produced by tumour cells, tumour suppressor genes (e.g. p53) and oncogenes (e.g. Bcl-2 and BAX). Such mechanistic insights may eventually be translated into new treatments for patients. It is feasible that strategies such as gene replacement therapy, antisense gene therapy, adoptive immunotherapy and drugs such as growth factor antagonists will be added to standard regimens in cancer treatment(Ciftci 1996).

The clinical effectiveness of many chemotherapeutic agents is limited due to their lack of specificity for malignant cells. In general, conventional cytotoxic anti-cancer drugs are most effective in malignant tumours with a high proportion of dividing cells, and thus cause unwanted toxicity in tissues such as bone marrow and the gastro-intestinal tract, which have an intrinsic high level of proliferation. Consequently, many anti-cancer agents are primarily effective against malignancies such as the leukaemias and lymphomas, which are characterised by high growth fractions. However, the most common malignant tumours are solid ones including those of the colon, rectum, lung and breast. These tissues have a low

proportion of dividing cells and are therefore less susceptible to treatment with drugs.

Future development of cancer therapy will exploit a number of approaches. These include the optimisation of current drug treatment, especially in the area of combination chemotherapy. Also, further research will be carried out into the optimal combination of several treatment modes such as chemotherapy, surgery and radiotherapy. A most important approach seems to be the design and development of new agents that are able to affect proliferation of cancer cells specifically. Alternatively, there are cancer prevention strategies. Chemoprevention is such an approach, and it is defined as the "prevention of cancer by the use of natural or synthetic compounds to inhibit or reverse the process of carcinogenesis"(Tanaka 1997).

Quercetin is one of many naturally occurring compounds that is being tested for its efficacy as an anticancer drug. In addition, quercetin displays biological activities that hint at the possibility that it possesses cancer chemopreventative properties.

1.2 Quercetin – chemical nature and occurrence.

Quercetin is a polyphenolic molecule belonging to the flavonoid family of compounds, which are found ubiquitously in aerial photosynthetic plants. Flavonoids share the diphenylpyran skeleton, which comprises two benzene rings, linked through a heterocyclic pyran or pyrone ring. Within plants, variation of the diphenylpyran structure occurs, both in substitution patterns (mainly hydroxyl and methyl groups) and degree of saturation of bonds within the structure. The most widespread flavonoids are the flavonol or flavone groups. Both comprise the diphenylpyran structure with hydroxyl moieties at the 3' and/or 4' positions. Flavones (e.g. "flavone" or "chrysin") lack the hydroxyl group at C3 that characterises flavonols (e.g. quercetin or kaempferol).

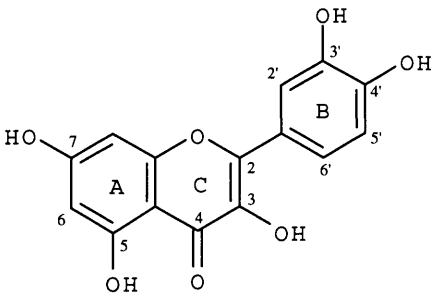


Figure 1.0 *Chemical structure of quercetin.*

Flavonoids are responsive to light and regulate the level of auxins, which are intrinsic to cell growth regulation and differentiation(Moore 1989). Flavonoids occur in a number of colours and consequently attract insects for pollination. Furthermore, flavonoids are organoleptically distasteful to foraging ruminants(Hedin and Waage 1986), thus acting as a natural deterrent to

predators i.e. an anti-feedant. Antifungal activity of isoflavones(Harborne 1986) and bactericidal activity of flavones has also been demonstrated(Tomas-Barberan, Maillard, and Hostellman 1988).

The relative concentration of flavonoids is dependent on the plant species, culture conditions, part of the plant, degree of ripeness and food processing conditions. Flavonoid concentration is greatest in the immature organs of plants, where most cell division takes place, and external or aerial tissues of the plant, due to sunlight stimulating their biosynthesis. Flavonoids appear to be preferentially located in the epidermis, or in the epicuticular zone on the surface of leaves or fruits or they may also be solubilised in the vacuolar sap.

It is important to be able to predict the relative proportion of quercetin and other flavonoids contained within foodstuffs, so that their potential to affect health can be assessed. Hertog *et al* (1992) analysed the edible portion of a number of foodstuffs. The highest concentration of quercetin was found in onions, kale and apples(Hertog, Hollman, and Katan 1992), as shown in table 1.1.

Table 1.1 *Table of quercetin content in commonly consumed fruit and vegetables(Hertog, Hollman, and Katan 1992).*

Foodstuff	**Quercetin (mg/kg)**
onions	284-486
kale	110
french beans	32-45
broccoli	30
tomatoes	8
apples	20-72
white/black grapes	12-15

A number of researchers have carried out similar surveys with values differing significantly. For example, Bilyk and Sapers(Bilyk and Sapers 1985) found quercetin concentrations in kale, between 7-20mg/kg as opposed to Hertog's estimate of 110mg/kg. In addition, the same authors discovered varietal differences in onions(Bilyk, Cooper, and Sapers 1984). Estimates of flavonoid consumption in humans are confounded by similar uncertainties. Among the

variables involved are type of vegetable, food processing, consumption of alcoholic beverages and diet composition. Thus, trying to extrapolate quercetin intake in humans from quercetin food content is difficult, as these variables can confound such estimation. One study has reported that red wine is a poor source of quercetin when compared to onions(de Vries et al. 2001). In this study, human volunteers received either red wine, onions or green tea which each contained equivalent amounts of quercetin. The results suggested that plasma quercetin levels were greater following the consumption of onions than after the consumption of either wine or tea. A widely used figure for the estimated daily consumption in the US of 4-oxo-flavonoids, most of which are presumed to be quercetin, is 170mg(Kuhnau 1976). Another estimate suggests a daily consumption of at least 50mg of quercetin(Brown 1980). A National Toxicology Program (NTP) report gives a more conservative figure of 25mg/day of quercetin, consumed in a normal healthy diet(National Toxicology Program 1992). Hertog (1995) compiled a list of extent of flavonol intake in middle-aged men, in various countries, as part of 'The Seven Countries Study'(Hertog et al. 1995). The results are shown in Table 1.2.

Table 1.2 Quercetin intake for middle-aged men from various countries(Hertog et al. 1995).

Country	**Quercetin intake (mg/day)**
Finland	6
USA	11
Serbia	10
Greece	15
Italy	21
The Netherlands	13
Croatia	30
Japan	31

It is pertinent to note that clear variations exist between different nationalities, which result from different dietary habits. The countries associated with highest quercetin consumption appear to be those in Southern Europe or the Pacific Rim.

The bioavailability of quercetin and its dietary precursors, which is discussed later in this chapter, is considered to be poor and the actual amount of quercetin absorbed by humans has been demonstrated to be less than 1%(Gugler and Leschik 1975). Consequently, if the NTP

report is taken as a benchmark, then less than 250µg of quercetin is actually bioavailable from the 25mg ingested.

1.3 Epidemiological evidence for quercetin-related health benefits.

The link between diet and diseases such as cancer and coronary heart disease (CHD) is well established. A lot of research effort is spent on the exploration of individual components of the diet and their particular role in disease. Data on the effects of agents such as quercetin is obtained in *in vivo* and *in vitro* models as well as in clinical studies. Epidemiological data can help to substantiate conclusions drawn from the mechanistic work.

Quercetin and cancer.

The relationship between cancer and flavonoid intake has been assessed in three epidemiological studies. Two were population based prospective-cohort (the Zutphen Elderly Study(Keli et al. 1996) and the Netherlands Cohort study(Goldbohm et al. 1995)) carried out in Holland, and one was an ecological comparison between cohorts from a variety of countries (the seven countries study(Hertog et al. 1995)).

The Zutphen Elderly Study estimated the flavone intake at baseline in 1985 of approximately 800 men aged 65-85 years. The flavone intake was determined using what is called the cross-check dietary history method. Men were divided into tertiles of flavone and flavonol intake. After 5 years of follow-up, 57 men had developed cancer of the alimentary and respiratory tract. Intake of quercetin alone or quercetin and four other flavonols and flavones did not seem to be associated with these cancers(Hertog et al. 1994). The Netherlands Cohort Study was a study of 120,854 men and women aged 55-69 years, who filled in a semi-quantitative food frequency questionnaire in 1986. Flavonol and flavone intake at baseline in 1986 was calculated and the study population was divided according to quintiles of intake. During 4.3 years of follow-up 200 cases of stomach cancer, 650 cases of colon cancer and 764 cases of lung cancer occurred. Again, intake of flavonol and flavone at baseline was not associated with any of these cancers when considering other risk factors(Goldbohm et al. 1995). In the Seven Countries Study, a cross-cultural study on risk factors for chronic diseases that started around 1960 and involved 16 cohorts and 25 years of follow up, flavonol and flavone intake at baseline was again not related to differences in lung-, colorectal- or stomach cancer mortality rates(Hertog et al. 1995).

Quercetin and cardiovascular diseases.

Flavonoid intake has also been discussed as potentially beneficial in terms of prevention of cardiovascular disease. A conclusion from the Zutphen Elderly Study was that mortality from CHD was inversely associated with flavonoid intake(Hertog et al. 1993). Moreover, a correlation was established between flavonoid intake and incidence of first myocardial infarction(Hertog et al. 1993). The Seven Countries Study demonstrated a similar correlation(Hertog et al. 1995). The Zutphen Study also attempted to correlate long-term flavonol and flavone intake with risk of stroke in a cohort of 552 middle-aged Dutch men, free from a history of stroke at baseline. Men were divided into quartiles of flavonol and flavone intake and followed for 15 years. Flavonoid intake was strongly inversely associated with stroke risk(Keli et al. 1996). Individuals who were in the high intake group (>30mg/d) had about one third the risk of attracting the disease compared to men in the lowest intake category. Quercetin was the major flavonoid in both studies(Hertog and Hollman 1996).

An intriguing link has been made between quercetin intake from drinking red wine and CHD. A number of studies have shown that the consumption of moderate levels of red wine can lower CHD(Paceasciak et al. 1995; Teissedre et al. 1996; Frankel et al. 1993). Mechanisms such as inhibition of lipoprotein oxidation, free-radical scavenging and modulation of eicosanoid metabolism may reduce the likelihood of atherosclerosis and its sequelea, including CHD(Paceasciak et al. 1995). The actual role that quercetin plays in the aforementioned mechanisms will be discussed later.

Tea was the most important source of flavonols in both the Dutch studies(Hertog et al. 1993; Keli et al. 1996). These and a number of other cross-sectional studies imply that there is an inverse association between tea consumption and serum cholesterol. It is feasible that flavonoids play at least in part a role in the protective effect of tea. In contrast, a follow up study evaluating the relationship between tea consumption and cardiovascular disease risk in the UK demonstrated only a weak positive association(Hollman, Tijburg, and Yang 1997).

1.4 Biological properties of quercetin.

Interest in quercetin originates from studies conducted by Szent-Gyorgyi *et al* in the 1930s, who reported that two flavonoids derived from paprika caused decreased capillary fragility

and permeability, associated with scurvy(Bentsath, Rusznyak, and Szent-Gyorgyi 1936; Rusznyak and Szent-Gyorgyi 1936). This property led to the designation of the flavonoids as "bioflavonoids" or vitamin P. Later on, vitamin-like activity could not be substantiated and the vitamin-term was dropped(Roger 1988).

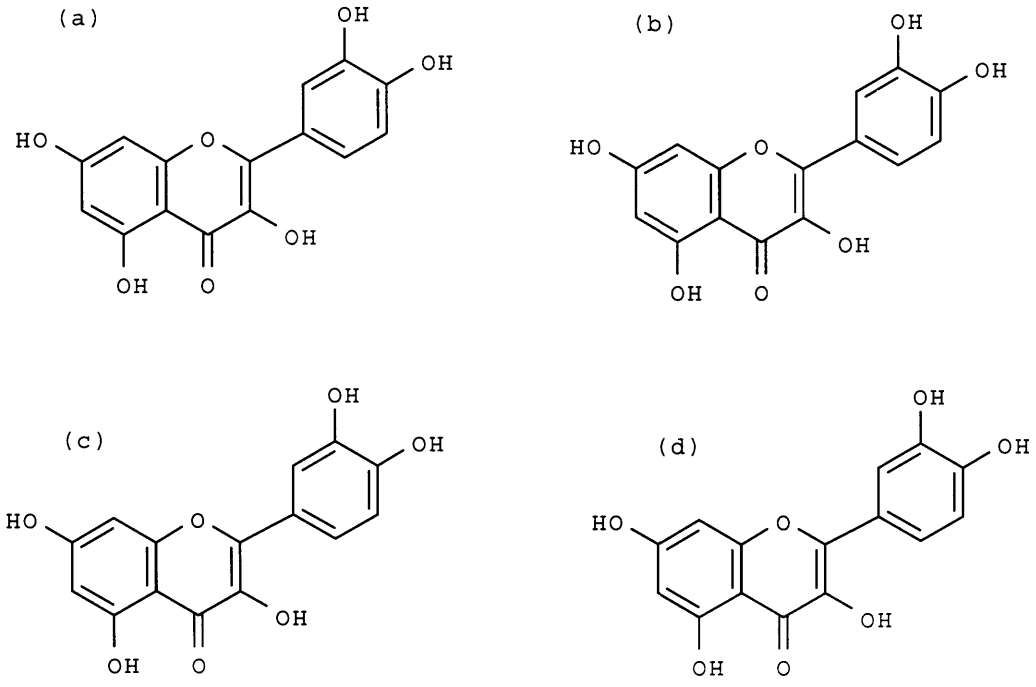
1.4.1 Mutagenicity of quercetin.

The carcinogenic effects of bracken fern were tentatively associated with their flavonoid content(Sumi et al. 1981). In particular, kaempferol and quercetin, two known constituents of bracken fern were suspected to possess mutagenic properties, and consequently a number of assays were used to assess their mutagenicity *in vitro*. It was shown that there are four salient structural features important for mutagenicity of this type of compound(Brown 1980; Macgregor and Jurd 1978):

A free hydroxyl group at the C3 position (Fig. 1.2a), an unsaturated bond between C2 and C3 (Fig.1.2b), a keto group at the 4 position (Fig.1.2c) and the presence of hydroxyl groups at the C3 and C5 positions (Fig.1.2d) which allow tautomerism of the 3-hydroxyl to a 3-keto compound.

Quercetin contains all four features and was subsequently shown to be a potent mutagen(Brown 1980). Conversely, glycosylated quercetin was not mutagenic, thus suggesting that the removal of the glycoside from the aglycone toxifies the flavonoid(Brown 1980).

Figure 1.2 *Structural requirements of quercetin for its proposed mutagenicity.*



The mutagenicity of quercetin has also been demonstrated in *E. coli*(Brown 1980), *S. cerevisiae*(Llagostera et al. 1987) as well as *Drosophila melongaster*(Watson 1982). Quercetin also elicited genetic alterations in mammalian cells, as expressed by chromosomal aberration and sister chromatid exchange, with little effect on point mutations(Vanderhoeven, Bruggeman, and Debets 1984; Meltz and Macgregor 1981).

A study looking at *MLL* chromosomal translocation found that quercetin, as well as other flavonoids, induced chromosomal translocation in this gene. As *MLL* chromosomal translocations are found in 80% of infant leukaemias, it was concluded that dietary bioflavonoids could be partially responsible for certain types of infant leukaemias(Strick et al. 2000).

In order to investigate the mutagenicity further, seventeen feeding studies in mice, rats and hamsters were carried out. Erturk *et al* (1985) reported an increase in bladder tumours. Studies carried out by Pamukcu *et al* (1980) showed that quercetin was a carcinogen, as intestinal and bladder tumours were found in rats(Pamukcu et al. 1980). In contrast, the other studies concluded that carcinogenic activity could not be established(Formica and Regelson 1995).

A study co-ordinated by the NTP showed that renal toxicity occurred in male rats, two years

after consumption of high doses of quercetin(National Toxicology Program 1992). This toxicity study involved feeding F344 rats a diet containing 4% quercetin. While the authors demonstrated some toxicity, quercetin consumption was considerably higher than that expected in a normal diet. In other long-term studies, Stoewsand *et al* (1984) did not find carcinogenicity in rats fed a 0.1% and 0.2% quercetin-supplemented diet for 64 weeks(Stoewsand et al. 1984).

1.4.2 Anticarcinogenic, antimutagenic and antineoplastic properties.

Quercetin is not only mutagenic and potentially carcinogenic, but also anticarcinogenic. It is likely that the anti-carcinogenicity demonstrated by quercetin is not due to one single pathway or mechanism. Instead, the activity of quercetin is probably due to a number of mechanisms that interact to generate the ultimate effect, in either a cumulative or a synergistic fashion. Quercetin displays a variety of properties, as outlined in Table 1.3, which might explain its anti-carcinogenicity.

Quercetin binds to type II oestrogen binding sites (type II EBS), although it does not act as an anti-oestrogen in mice or *in-vitro* systems(Scullion and Mehmood 1999). Type II EBS exhibit a lower affinity, but higher capacity for oestradiol than the classical oestrogen receptor (ER)(Piantelli et al. 1993; Ranelletti et al. 1988). Stimulation of the nuclear type II sites correlates with the proliferation state of mammalian cells. In non-neoplastic tissue, type II EBS are occupied by an endogenous ligand, methyl-p-hydroxyphenyllactate, which inhibits cell growth(Markaverich et al. 1990). It has been postulated that quercetin mediates its effect by mimicking methyl-p-hydroxyphenyllactate, either directly or via a metabolite(Larocca et al. 1990; Markaverich et al. 1990). The fact that quercetin inhibits growth at nanomolar concentrations is consistent with the possibility that this mechanism occurs *in vivo*(Ranelletti et al. 1992). The binding of quercetin to type II EBS is one of the few mechanistic studies, which showed effects at concentrations that could conceivably be reached in human plasma.

Table 1.3 *Effects of quercetin and relative potency. Table adapted from reference(Ferry, Kerr, and Wakelam 1995) with relevant effects from additional sources also referenced.*

Biological activity	EC_{50} dose
Inhibition of Na^+K^+ATPase activity	50μM
Inhibition of pp60src kinase	5μM
Binding to type II EBS	5μM
Inhibition of phosphatidylinositol 3-kinase	2-4μM
Inhibition of HIV reverse transcriptase	0.8μM
Inhibition of DNA polymerase-β	0.25μM
Inhibition of p-glycoprotein expression	100μM
Arrest of cellular proliferation at G2	70μM
Inhibition of 1-phosphatidylinositol kinase activity	6μM (Singhal et al. 1995)
Inhibition of protein kinase C	20μM
Inhibition of growth of ovarian cancer cells	5μM
Inhibition of growth of breast cancer cells	10μM
Down regulation of mutant p53	23μM
Activation of wt p53	60μM(Elangovan et al. 1994)
Induction of apoptosis, role of heat shock proteins	~50μM
Enhancement of TGF-β secretion	10μM
Synergistic effect with cytosine arabinoside	1μM
Synergistic action with genistein	3μM(Shen and Weber 1997)
Sensitisation of human large cell lung cancer xenografts to cisplatin in nude mice	20mg/kg
Inhibition of estrone sulphatase	10μM(Kashfi et al. 1994)
Hyperthermic sensitiser of HeLa cells	100μM

Quercetin has been shown to inhibit cell growth in a number of cell lines including those of the breast, ovaries, gastro-intestinal tract, colon and in leukaemia cells, at concentrations of around 10μM. Additionally, quercetin has been shown to be inhibitory in *in vivo* models of carcinogenicity. For example, quercetin inhibited the initiation of skin tumourigenesis, induced by 7,12-dimethylbenz(a)anthracene (DMBA), benzo(α)pyrene (B(α)P), 3-methylcholanthrene and N-methyl-N-nitrosourea when applied to the skin of mice(Mukhtar et al. 1988). Additionally, quercetin administered via the diet inhibited mammary tumours initiated, by DMBA and azoxymethane (AOM)(Pereira et al. 1996). Both quercetin and its glycosylated precursor, rutin, suppressed hyper-proliferation of colonic epithelial cells, and consequently tumour formation, induced by AOM(Deschner et al. 1991; Deschner et al.

1993).

Quercetin affects key enzymes which regulate cellular signal transduction pathways. The inhibition of protein tyrosine kinases is an example. Quercetin has been shown to be an effective inhibitor by competitively blocking ATP binding(Levy et al. 1984). Tyrosine kinases mediate the activity of several growth factors as well as some oncogene products and consequently, are important in tumourigenesis.

Quercetin inhibits phosphatidylinositol-4-kinase activity, which is important in the maintenance of the phosphatidylinositide pool(Sharoni, Teuerstein, and Levy 1986). Quercetin also inhibits the activity of protein kinase C (PKC)(Srivastava 1985; Ferriola, Cody, and Middleton E 1989; Gescher 1995), an enzyme critical for regulation of growth and differentiation and the growth stimulatory action of transforming ras-oncogenes, the most frequently expressed oncogenes in human tumours.

Quercetin can interact with DNA via intercalation, which may have consequences on its function(Ahmed et al. 1994). It has been suggested that intercalation occurs with the benzopyran-4-one moiety internalised in DNA and the catecholic portion orientated externally. However, studies showed a low level of intercalation occurred, which did not affect the flexibility or hydrodynamic behaviour of the DNA structure(Solimani 1997). The author proposed that when intercalated in DNA, quercetin shields the nucleophilic groups of DNA against attacks by free radicals and reactive electrophiles.

Quenching by quercetin of free radicals found in the vicinity of DNA may prevent the occurrence of mutations. The antioxidant properties of quercetin and other flavonoids have long been proposed to be involved in reducing the overall cellular oxidative stress which can lead to the increased free radicals from endogenous sources e.g. lipid peroxidation(Burcham 1998).

Quercetin has been shown to play a role in various checkpoints of the cell cycle. Quercetin at 10μM can arrest cell development at the G2 stage (as shown in Table 1.3) and decrease p21-ras levels to approximately 50% of those found in untreated cells(Ranelletti et al. 2000). Quercetin has shown inhibitory action against Bcl-2, with expression of the gene being inhibited and apoptosis induced as a result(Zhang, Xu, and Saiki 2001).

1.4.3 Effects of quercetin on drug metabolism.

Quercetin has been shown to be a substrate for a number of enzymes, and can also act as an inhibitor of a number of enzymes. It is conceivable that quercetin acts as a cancer chemopreventative agent at the initiation stage by inhibiting toxifying metabolising enzymes. Quercetin is an inhibitor of cytochromes P450 1A2(Lautraite, Doehmer, and Chipman 1998) and 3A4(Wrighton, VandenBranden, and Ring 1996). The interaction with the family of P450s is particularly germane, as the administration of foodstuffs high in flavonoids has been suggested to increase the efficacy of other drugs by interfering with their metabolism and thus increasing their bioavailability. Accordingly, some therapeutic agents have been administered following the consumption of grapefruit juice(Bailey, Arnold, and Spence 1994; Tassaneeyakul, Guo, and Yamazoe 1998). Quercetin has also been shown to affect in a number of ways the activity of phase II enzymes and factors associated with it.

Hepatic GST can be a modulator of the resistance of cancer cells against cytotoxic drugs. Considerable work has been carried out, assessing the effect of quercetin on cellular levels of GSH and the activity of GST. In isolated rat liver nuclei, quercetin (and other flavonoids) decreased, in a concentration-dependent manner, both the nuclear GSH and GST activity(Sahu and Gray 1996). Moreover, quercetin has been reported to be a potent non-competitive inhibitor of GST activity(Zhang and Wong 1997; Kurata, Suzuki, and Takeda 1992; Zhang and Das 1994). Two different groups have demonstrated that quercetin is a potent inhibitor of a so-called "GSH-efflux pump" for dinitrophenylglutathione (DNP-SG)(Zhang and Wong 1996) and monochloromono-glutathionyl chlorambucil (MG-CMB), respectively(Zhang and Wong 1997). In the case of the DNP-SG, while the efflux of the conjugate is inhibited, glutathione levels were not affected in human adenocarcinoma cells. It is also worth noting that in contrast to these reports, when quercetin was fed to Swiss NMRI mice, hepatic and pulmonary GST activity and GSH levels were both increased(Gandhi and Khanduja 1993). Similarly, in a study carried out by Nijhoff *et al*, the effect of a number of naturally occurring anticarcinogens including quercetin on GST levels, was assessed in the proximal, middle, distal small intestine, large intestine and liver. In essence, an increase in GST activity was noted in all tissues, but especially in the large intestine. A similar pattern of levels was seen for GSH in all tissues, tentatively suggesting that quercetin exerts its effects on GST and GSH with a certain degree of tissue specificity(Nijhoff, Groen, and Peters 1993). In MCF-7 cells, intracellular GSH content increased on incubation with quercetin(Rodgers

and Grant 1998).

In contrast, a study in male SPF Wistar rats in which the effect of flavonoids on drug metabolising enzymes was elucidated, concluded that quercetin, in contrast to other flavonoids, did not alter GST activity(Canivenclavier et al. 1996). Quercetin has also been shown to protect cutaneous tissue-associated cell types including sensory neurones from oxidative stress induced by GSH depletion(Skaper et al. 1997). The mechanism of protection, in this case, is thought to involve quercetins antioxidant properties. Additionally, Duthie *et al* showed in a number of human cell lines, that quercetin (and other flavonoids) caused depletion of reduced GSH, which in the case of quercetin occurred prior to cell death(Duthie, Johnson, and Dobson 1997).

The effect of quercetin on GSH and GST is not easily discernible. The work on the alteration of GSH and GST by quercetin and other flavonoids was carried out in a number of different models, both *in vivo* and *in vitro*. From the literature, it is clear that in the case of rats, there are tissue-specific differences. That is to say, in rat liver, GST and GSH levels are decreased by quercetin, while in the rat intestine GST and GSH levels are increased. The reasons for the tissue-specific differences are unclear. The increase in GST activity and GSH levels by quercetin in the liver and lung of mice may point to inter-species differences between mice and rats.

The goal of the xenobiotic detoxification process is the removal of agents potentially hazardous to the organism. Quercetin has been shown to be a potent and specific inhibitor of p-form phenol sulphotransferase(Walle, Eaton, and Walle 1995; Eaton et al. 1996). This action may be exploited by coadministration, to prolong the half-life of drugs such as paracetamol, which undergo significant detoxification by sulphation.

1.4.4 Sensitisation of tumour cells to cytostatic agents.

Co-administration of quercetin with cytostatic agents can increase their effectiveness. An example is the potential of quercetin to sensitise cells towards cis-platin. Cis-platin (cis-diamminedichloroplatinum [II]) is a commonly used drug in the treatment of ovarian, testicular and head and neck cancers. Approximately 30% of ovarian cancer patient tumours are insensitive to the drug(Gore et al. 1989). Quercetin has been shown *in vitro* to enhance the

anti-proliferative effect of cis-platin, synergistically(Hofmann et al. 1988; Scambia et al. 1992). In addition, quercetin increased the anti-tumour activity of cis-platin in mice bearing human-derived carcinomas(Hofmann et al. 1988). Cross *et al* (1996) showed that the sensitisation to cytotoxicity was not accompanied by an increase in overall genotoxicity(Cross et al. 1996). Synergy was also found in leukaemia cell lines between cytosine-arabinoside and quercetin(Teofili et al. 1992).

Some tumour cells, in which the multidrug resistance (MDR) phenotype is expressed, have the ability to expel intracellular drugs by a ATP-dependent efflux process. The enhanced expression of a transmembrane protein (p-glycoprotein (Pgp)) is responsible for this phenotype(Endicott and Ling 1989). Quercetin has been shown to inhibit the expression of the MDR1 gene, which codes for Pgp, at the transcriptional level(Kioka et al. 1992). The authors implicate blocking by quercetin of the binding of a heat shock factor to the promotor of the MDR1 gene as a potential mechanism.

1.4.5 Protection of coronary heart disease by quercetin.

As pointed out in section 1.3 the palliative effect of red wine may be due, at least in part, to the presence of flavonoids, even though it is conceivable that alcohol is the major protectant(Haskell et al. 1984; Hendriks et al. 1994).

The mechanism by which quercetin might contribute to the protective efficacy of red wine is not completely understood. A mechanism by which quercetin may influence CHD is via regulation of low-density lipoprotein (LDL)(Teissedre et al. 1996). Quercetin has been shown to prevent the oxidation of LDL, thus interfering favourably with the metabolism of cholesterol(Riceevans et al. 1995; Meyer, Heinonen, and Frankel 1998). Oxidation of LDL results in the sequestration by macrophages of oxidised LDL, which accumulate to form 'foam' cells, which are precursors in the formation of atherosclerotic plaques that ultimately can lead to thrombosis.

The link between heart disease and the dietary habits of Mediterranean and French populations has long been noted, and is known as the 'French Paradox'(Constant 1997). In France, the intake of saturated fat has been estimated to be up to three times that in the USA, whilst the incidence of CHD is approximately a third(Constant 1997). It is sagacious to point

out that dietary factors other than wine in the French diet could be involved. The French eat more garlic, cheese, fruit and vegetables than Americans do. Likewise, the French have a higher proportion of lactose intolerance and consequently, the milk intake is proportionally less than that for the Americans. However, the general consensus found in the current literature, is that the most important factor contributing to the French Paradox is the consumption of red wine(Frankel et al. 1993; Waterhouse et al. 1994; Teissedre et al. 1996). Another factor that could contribute to the advantageous role of red wine is that the alcohol contained in wine may beneficially influence the bioavailability of quercetin by acting as a permeation enhancer(Walgren, Walle, and Walle 1998).

Quercetin may protect against CHD, by affecting platelet activation and aggregation. Quercetin blocks platelet aggregation by inhibiting cyclic phosphodiesterases(Lanza et al. 1987). A decrease in cAMP concentration results in enhanced platelet aggregation, and inversely, increased concentrations of cAMP inhibited platelet aggregation(Formica and Regelson 1995). Also, platelet aggregation can be induced by a number of substrates (i.e. ADP, epinephrine), and this can be inhibited by incubation with quercetin(Formica and Regelson 1995).

It is conceivable that quercetin mediates its effect by scavenging oxygen free radicals which are implicated in platelet activation and ultimately hyperaggregability(Xie, Lu, and Gu 1996).

1.4.6 Antioxidant properties of quercetin.

Lipid peroxidation occurs as a consequence of oxidation of polyunsaturated fatty acids, which are present in cell membranes. Oxidation can be mediated by enzymatic and auto-oxidative peroxidation and by free radical chain reactions. A cellular excess of free radicals can lead to a state in which the cell undergoes oxidative stress, whereupon uncontrolled chain reactions and lipid peroxidation can occur. Ultimately, this can result in pathological conditions such as atherosclerosis or cancer. Lipid peroxidation is a very well defined process where, in the initiation phase, free radicals abstract hydrogen from polyunsaturated fatty acids to form a lipid radical. The propagation stage is typified by the lipid radical reacting with molecular oxygen to form the lipid peroxy radical, which begins a cascade generating more free radicals, thus propagating the chain of reactions. The termination stage occurs when the free radical species react together or with 'antioxidants' to form non-reactive species(Burcham 1998).

Antioxidants can act at the termination or initiation stage by scavenging free radicals or singlet oxygen quenchers. Also, the propagation stage can be broken by peroxy-radical scavengers(Cook and Samman 1996).

Flavonoids have two important properties which contribute to their ability to act as antioxidants. Flavonoids chelate metal ions and can interfere with all three stages of the lipid peroxidation cycle. Termination of chain radical reactions occurs by the donation of a single hydrogen atom, which furnishes a flavonoid radical and in turn, reacts with free radicals thus terminating the propagating chain(Afanasev et al. 1989). As a chelator of metal ions, quercetin can inhibit the superoxide-driven Fenton reaction, which results in the generation of active oxygen species(Puppo 1992). *In vitro* studies have shown quercetin to be a more potent antioxidant than α-tocopherol(Riceevans et al. 1995; Ridgway et al. 1996; Riceevans and Miller 1996).

The antioxidant ability of quercetin metabolites has also been assessed. Three separate studies have shown that that quercetin retains its antioxidant ability when conjugated. All studies show that the antioxidant activity of the metabolites is lower than that of the aglycone(Manach et al. 1998; daSilva et al. 1998; Day et al. 2000).

Paradoxically, quercetin has also been suggested to have the potential to act as a prooxidant by existing as a stable radical following abstraction of hydrogen(Sahu and Washington 1992; Sahu and Gray 1993; Sahu and Gray 1996).

1.5 Metabolism of quercetin.

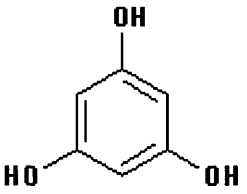
The metabolism of quercetin both in pure form and as a dietary constituent has been reported(Manach et al. 1995; Manach et al. 1996; Manach et al. 1997; Manach et al. 1998). However, some of the methods utilised for the characterisation of metabolites lack specificity, and structural information is scarce. The determination of quercetin metabolites by more sophisticated techniques such as mass spectrometry and tandem mass spectrometry, would allow structural inferences to be drawn. The metabolic fate of quercetin in humans is not completely understood.

The metabolism of quercetin differs depending on its route of administration and the nature of

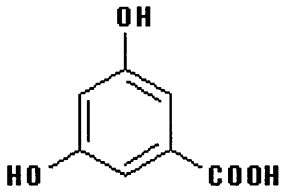
its source, i.e. the form in which it is administered, either as pure chemical or as glycosylated precursors, or as a component of a diet (e.g. within onions).

Booth (1956) administered quercetin and rutin to rabbits (0.5g/kg) via gavage and analysed the urine using TLC. 3,4-dihydroxyphenylacetic acid (DHPAA) was identified as well as *m*-hydroxyphenylacetic acid (mHPAA) and 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid)(Booth et al. 1956). (See Fig. 1.3 for structures).

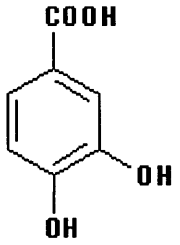
Figure 1.3 *Products of metabolic quercetin degradation(Petrakis et al. 1959; Douglass and Hogan 1958).*



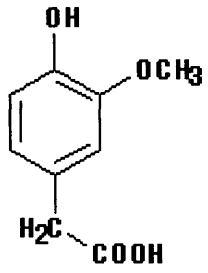
Phloroglucinol



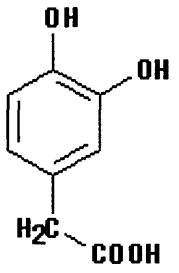
Phloroglucinol carboxylic acid



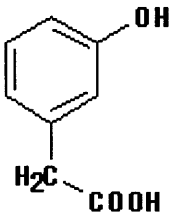
Protocatechuic acid



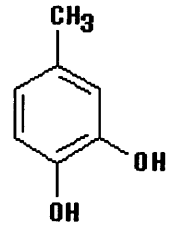
Homovanillic acid



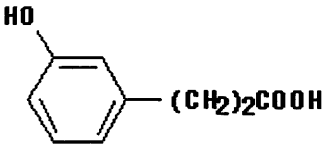
3,4-Dihydroxy-phenylacetic acid



meta-hydroxy-phenylacetic acid



3,4-Dihydroxy toluene



3-(3-hydroxylphenyl] propionic acid

Following ingestion, hydrolysis of flavonoid glycosides occurs. Glycosidases in intestinal bacteria that are not found in mammalian tissues cause the cleavage of the glycosidic flavonoids(Hackett 1986). The resultant aglycone, quercetin in the case of rutin, can be absorbed, excreted or undergo further bacterial metabolism.

Bacterial catabolism also plays an important role in the metabolic destiny of quercetin(Kim et al. 1998). Ring fission occurs, mediated by intestinal bacteria, followed by demethylation and dehydroxylation of the resultant phenolic acids(Kuhnau 1976)(see Fig. 1.3).

Feeding experiments in animals demonstrated that the microbial degradation of flavonoids is strongly influenced by flavonoid hydroxylation pattern. Absence of a free hydroxyl group at C5 and/or C4' lessens the susceptibility towards decomposition by gut bacteria(Griffiths and Smith 1972). It has been suggested that these phenolic acids could act as biomarkers of quercetin consumption(Gross et al. 1996).

Booth *et al* examined the urine of rats, rabbits, guinea pigs and humans that had received pure quercetin orally(Booth et al. 1956). They found that in all four species a striking similarity existed in the metabolic fate of quercetin. In each of the species, DHPAA, mHPAA and homovanillic acid were found in the urine implying that either quercetin is absorbed through the intestine and subsequently metabolised or that the three metabolites are formed in the intestine and subsequently absorbed. A further suggestion from this work is that DHPAA can generate mHPAA by dehydroxylation and homovanillic acid by methylation (see Fig. 3.1). This inference was made following administration of DHPAA, which yielded the same metabolites as quercetin. When quercetin was administered i.p., homovanillic acid was found, but there was no evidence of conjugated metabolites of quercetin in the urine.

In 1958, Douglass and Hogan reported the formation of protocatechuic acid from quercetin in rat kidney homogenates(Douglass and Hogan 1958)(see Fig. 1.3). DHPAA, mHPAA and homovanillic acid were not formed in the rat kidney homogenate, suggesting that they are formed as a consequence of bacterial degradation, or alternatively in some other tissues whilst protocatechuic acid is formed by the kidney. Kallianos *et al* investigated the metabolism of randomly labelled ^{14}C-quercetin force-fed to rats. Phloroglucinol carboxylic acid, phloroglucinol, and protocatechuic acid (see Fig. 1.3) were all identified in the intestinal contents(Kallianos et al. 1959). As a part of the same study Petrakis *et al* described the relative distribution of quercetin following administration of ^{14}C-quercetin. Twelve hours following administration via gavage of pure radio-labelled quercetin, rats were killed and each organ was analysed for radioactivity(Petrakis et al. 1959).

Table 1.4 *Relative distribution of quercetin in rats. Taken from Petrakis et al(Petrakis et al. 1959).*

Organ	Percentage of administered dose.
Gastric contents	0.4
Gastric walls	0.2
Small intestinal contents	10.9
Small intestinal walls	0.3
Large intestinal contents	32.9
Large intestinal walls	2.9
Blood	0.2
Kidney	0.3
Lungs	11.5
Urine	3.9
Respired CO_2	15.1
Total	**78.6**
Total gastrointestinal contents	44.2

Table 1.4 shows the relative distribution of quercetin. Samples of liver, spleen, heart and brain contained less than background radioactivity and were thus not included. A large proportion of the radioactivity was found in the contents of the gastrointestinal tract (44.2%), which constitutes probably un-metabolised quercetin or metabolites of quercetin that have not been absorbed into the systemic circulation. This study also identified ^{14}C-mHPAA and homovanillic acids. After i.p. administration of ^{14}C-quercetin, vanillic acid and homovanillic acid were identified (see Fig.3.1). When the intestinal contents were analysed radioactive phloroglucinol carboxylic acid, phloroglucinol and protocatechuic acids were identified. However, these products were not observed in the urine suggesting they are not readily absorbed from the gut(Petrakis et al. 1959).

A further study of the metabolic fate of pure quercetin using ^{14}C-labelled drug by Ueno *et al* (1983) found that when administered orally, around 20% was absorbed from the digestive tract, more than 30% was metabolised to yield CO_2 and around 30% was excreted unchanged in the faeces(Ueno, Nakano, and Hirono 1983). In this study the absorbed quercetin was excreted within 48h into the bile and urine as the glucuronide and sulphate conjugates of

quercetin, 3'-O-methyl quercetin (isorhamnetin) and 4'-O-methyl quercetin (tamarixetin).

Prior to this study, Gugler *et al* had reported the pharmacokinetics of quercetin in humans(Gugler and Leschik 1975). The authors did not find measurable plasma concentrations of quercetin following oral administration, nor was any quercetin found in urine, either unchanged or in a metabolised form. Recovery of quercetin in the faeces was ~53%, with less than 1% of a 4g dose being absorbed. When deuterated rutin (see Fig. 4.4) was administered p.o. to humans, DHPAA, m-HPAA, homovanillic acid, 3,4-dihydroxytoluene, 3-(*m*-hydroxyphenyl)propionic acid (see Fig. 1.3) were identified by GC-MS as urinary metabolites of rutin(Baba et al. 1983). The urine did not contain measurable levels of rutin or quercetin. Additionally, when deuterated rutin was administered i.p., none of the above metabolites could be detected. The authors also administered labelled rutin orally to neomycin-treated rats and none of the aforementioned metabolites could be detected. This finding, and the fact that all metabolites were observed 14 days after neomycin treatment substantiates the role of intestinal bacteria in the formation of the metabolites of rutin. Finally, when rutin was incubated with rat intestinal content under anaerobic conditions, three of the metabolites (m-HPAA, 3,4-DHPAA and 3-(*m*-hydroxyphenyl)propionic acid) as well as quercetin were recovered. Conjugated derivatives of quercetin or rutin were not detected in this study.

Shali *et al* observed three metabolites of quercetin in the isolated perfused rat liver(Shali et al. 1991). Two double conjugates contained sulphate and glucuronic acid moieties. A third metabolite, which did not undergo hydrolysis by β-glucuronidase, was assumed to be a sulphate conjugate. Shali *et al* also observed that the rate of quercetin sulphation in the male liver was approximately twice that found for the female liver(Shali et al. 1991).

In 1995, Manach *et al* reported that when Wistar rats were fed a diet containing either rutin or quercetin, approximately the same concentration of quercetin was found in the cecum(Manach et al. 1995). When rutin and quercetin when administered in molar equivalents, approximately the same concentration of plasma metabolites were recovered, suggesting that the small intestine is not an effective absorption site, as rutin is predominantly metabolised in the large intestine. Also described within this paper is evidence for albumin binding by quercetin and importantly that the circulating metabolites of quercetin retain the property to bind to albumin.

In 1997, in a follow-up study, Manach *et al* reported the finding that quercetin was more rapidly absorbed in rats than rutin, as rutin underwent bacterial hydrolysis prior to absorption(Manach et al. 1997). In this study, quercetin concentrations were lower in the cecum of animals fed a rutin diet than in those that received a quercetin diet, tentatively suggesting that rutin, in contrast to quercetin, is not absorbed from the small intestine. Ioku *et al* evaluated the metabolism of a number of quercetin glucosides *in vitro* and concluded that dietary flavonoid glucosides are primarily hydrolysed and liberated as aglycones in the jejenum(Ioku et al. 1998). The absorption of rutin in the jejunum was comparatively lower than that of other glucosides, but it has been cited elsewhere that rutin may be metabolised by the microflora in the large-intestine(Baba et al. 1983; Hackett 1986). Recently, the intestinal bacteria responsible for the catabolism of quercetin were isolated(Kim et al. 1998). The conversion of rutin to quercetin was assigned to three bacteria, namely *Bacteroides* J-37, *Eubacterium* A-44 and *Fusobacterium* YK-4. The conversion of quercetin to phenolic acids was assigned to four species, namely *Streptococcus* S-2, *Lactobacillus* L-2, *Bifidobacterium* B-9 and *Bacteroides* JY-6. It is conceivable that since the reaction catalysed by glycosidases proceeds more rapidly then ring fission, free aglycones accumulate in the large bowel, from where they may be absorbed.

Manach *et al* also described the formation of tamarixetin (a 4'-O-methyl derivative of quercetin) as a metabolite of quercetin(Manach et al. 1997). This metabolite was only found in the plasma of rats adapted to a flavonol-free diet before receiving pure quercetin in an experimental diet, whereas it was not found in plasma from rats that did not receive a flavonoid-free diet prior to quercetin administration. The paper described the formation of sulphated, glucuronidated and methylated derivatives of quercetin. This work was corroborated by Piskula and Terao who administered pure quercetin by gavage and identified sulphate and glucuronide conjugates of quercetin and isorhamnetin(Piskula and Terao 1998).

A Canadian double-blind study in humans assessed the effects of supplementing the diet with quercetin-containing tablets on the plasma quercetin levels. The tablets contained quercetin in addition to rutin and other undefined 'bioflavonoids'. The study showed that plasma levels of quercetin were increased, thus indicating absorption. Following the consumption of 1g/day of quercetin for 4 weeks, plasma levels of ~0.5μg/ml of circulating quercetin were observed(Conquer et al. 1998). The plasma levels after 28days were higher than that observed in subjects given a single dose of quercetin (~0.25μg/ml)(Hollman et al. 1997a). This data suggests that an accumulation of quercetin occurs when ingested with the diet.

In a study on the potential antioxidant capabilities of the metabolites of quercetin, Manach *et al* found that isorhamnetin and conjugated derivatives retained antioxidant properties of the aglycone, but were approximately half as effective as quercetin(Manach et al. 1998). The plasma obtained from Wistar rats treated with quercetin by gavage, inhibited copper ion-induced lipid peroxidation(daSilva et al. 1998). As free quercetin or isorhamnetin could not be detected, the metabolites found in the plasma were reasoned to be responsible for this inhibition.

Most work concerned with the metabolism of quercetin has focussed on its role as a dietary constituent. Consequently most studies have not distinguished between quercetin and quercetin glycosides when considering the dietary source of the flavonoids. Additionally, when pure quercetin has been investigated, it has been often mixed in with the diet, an administration mode that may severely effect its distribution and metabolism as quercetin is known to strongly bind to food protein. Only two studies have assessed the metabolism of pure quercetin alone in humans, Gugler *et al* in 1975 (as described earlier) and Ferry *et al* who carried out a pharmacokinetic study in cancer patients giving quercetin by the i.v. route(Gugler and Leschik 1975; Ferry et al. 1996). The latter study will be discussed in more detail later.

1.5.1 Intestinal absorption of quercetin.

As mentioned before quercetin glycosides are thought to undergo intestinal degradation. The aglycone is then available for absorption, as demonstrated in some of the studies described above. However, it has also recently been shown that the glycosides can be absorbed as such *in vivo* in humans(Paganga and Riceevans 1997; Aziz et al. 1998) and *in-vitro* in human Caco-2 cells(Walgren, Walle, and Walle 1998).

These studies have led to investigation of the mechanism of quercetin absorption. Passive diffusion is thought to be the most probable mechanism. However, Hollman *et al* proposed the hypothesis that quercetin glucosides may be actively absorbed in the human intestine by hexose transporters(Hollman et al. 1995). Gee *et al* discovered that quercetin glucosides interact with the intestinal glucose transport pathway in a manner that is at least partially sodium dependent, suggesting that they may be absorbed by the small intestine *in vivo* via this mechanism(Josephy 1997).

Intestinal absorption is crucial to the bioavailability and biological efficacy of quercetin. Drug clearance and intestinal absorption are the principal factors that influence bioavailability. Following administration of 4g of quercetin p.o. in humans, 53±5% was found in the faeces(Gugler and Leschik 1975). It was concluded that approximately 1% of the dose was absorbed, confirming the dominant role of bacterial catabolism. After administration of ^{14}C-labelled quercetin to rats, around 4% of the radioactivity was found in the urine 12 hours after administration, with an estimated 20% having been absorbed(Petrakis et al. 1959). However, two other studies in rats found that 0.3% and 0.5%, respectively, were absorbed and excreted unchanged(Macgregor 1979; Crebelli et al. 1987). Extrapolating from these findings, Formica and Regelson (1995) calculated that, assuming 25-50 mg per diem is ingested and only 1% is absorbed, only 3-12nmol/kg of quercetin ingested in the diet is bioavailable in humans(Formica and Regelson 1995).

A more recent study suggested that approximately 24% of quercetin was absorbed(Hollman et al. 1995). In this experiment, absorption of dietary quercetin was determined in ileostomy patients. In such patients, quercetin is not subject to metabolism by colonic bacteria. Thus, this estimated absorption of quercetin does not reflect the situation in healthy humans. Watson and Oliveira found quercetin to be present in human urine following the consumption of *Ginkgo biloba* tablets which contained ~0.5mg of quercetin per tablet(Watson and Oliveira 1999).

A study of the metabolism of quercetin in pigs, discovered its bioavailability to be 0.54±0.19% when pigs received 50mg/kg (p.o.) of quercetin. Bioavailability was, however, considerably increased to 8.6±3.8% after taking into account conjugated quercetin, and further increased to 17.0±7.1% by including other quercetin metabolites (e.g. isorhamnetin)(Ader, Wessmann, and Wolffram 2000).

Quercetin is highly bound to plasma proteins (upto 99% plasma bound), and specifically to human serum albumin(Manach et al. 1995; Boulton, Walle, and Walle 1998). The binding of quercetin to dietary proteins could explain to some extent the poor absorption and consequently bioavailability of dietary quercetin. The apparent lack of effect on CHD by tea as established in the UK, which is traditionally a high consumer of tea has been postulated to be due to the presence of milk. The binding of quercetin to milk proteins would ultimately limit its availability for absorption(Hertog and Hollman 1996). However, work on the absorption of flavonols from tea in humans, revealed that absorption was not altered by the

addition of milk(Hollman et al. 2001). The binding of quercetin to plasma is not in dispute. McAnlis et al has shown that quercetin has a strong affinity for protein and a s a consequence provides no direct protective effect during LDL oxidation(McAnlis et al. 1999). While the same conclusion was drawn by assessing the antioxidant capacity of α-tocopherol with quercetin, rutin and catechin(Arts et al. 2001). In this study the antioxidant capacity of α-tocopherol was not altered by its interaction with protein. The flavonoids antioxidant capacity was considerably reduced due to their interactions with proteins.

1.5.2 Enterohepatic circulation of quercetin.

Biliary excretion of flavonoids has been shown to be particularly important in the rat(Hackett 1986). However, very little work has been done in other species. An important determinant as to whether compounds such as flavonoids are excreted or not is molecular weight(Smith 1973). Biliary excretion in the rat occurs for compounds with molecular weight higher than about 350, which may explain why flavonoid conjugates are excreted via the bile, but their respective aglycones are not.

Enterohepatic circulation is an important phenomenon in drug metabolism. As a result of biliary excretion, the aglycones and their metabolites are excreted into the duodenum whereupon intestinal bacteria metabolise the glucuronide conjugates to their respective aglycones. The aglycone, once liberated, is absorbed from the intestine. It can then be transported via the hepatic portal vein to the liver, and subjected to conjugative metabolism. The metabolites are excreted into the bile, thus completing enterohepatic circulation. Catechin and genistein have both been shown to undergo such a cycle(Hackett and Griffiths 1977; Sfakianos et al. 1997) and other authors have suggested that this may be the case for quercetin(Paganga and Riceevans 1997; Shali et al. 1991; Hackett 1986), although there is no unambiguous evidence for such a pathway. The human UGT 1A8, an isoenzyme of UGT (uridine diphosphate glucuronyl transferase), found only in the gut and not in other tissues appears to glucuronidate flavonoids(Cheng, RadominskaPandya, and Tephly 1998). Thus, the expression of UGT1A8 in the colon and small intestine suggests that some of the compounds can be reconjugated in the intestinal mucosa, thereby preventing the enterohepatic circulation of the aglycones, at least in part, and promoting elimination of the chemicals by urinary excretion. It has been shown that flavonoids (i.e. quercetin and naringenin) exhibit a very high level of glucuronidation by human UGT 1A3(Green et al. 1998) and UGT1A1(King et al.

1996), but do not display any substrate specificity for UGT 1A4(Green et al. 1998).

1.6 Clinical trials of quercetin.

Two trials on the anti-cancer activity of quercetin have been conducted(Ferry et al. 1996 and unpublished). In the phase I trial the dose limiting toxicity, pharmacokinetics and pharmacodynamics of quercetin were investigated. Inhibition of lymphocytic tyrosine kinase was chosen as pharmacodynamic marker, as quercetin inhibits tyrosine kinases (see section 1.4.4.). In all patients studied, lymphocyte tyrosine kinase activity was inhibited, suggesting that quercetin reaches plasma levels sufficient to cause a biochemical change *in vivo*, indicative of biological activity(Ferry et al. 1996).

Patients' plasma was analysed for quercetin and pharmacokinetics characterised. The dose was escalated up to 1900mg/m^2. The elimination half-life was 26-86 min. The clearance was 0.15-0.49 L/min/m^2, with a median volume of distribution of 3.9 L/m^2. Serum levels achieved immediately after injection of quercetin were in the range 200-400μM at 945 mg/m^2. Four hours later, serum levels of 1μM were found(Ferry et al. 1996).

The dose limiting toxicity was shown to be renal toxicity, which was expressed only at doses exceeding 945mg/m^2.

A phase I/II trial in Birmingham explored the possible beneficial effect of quercetin when given in combination with carboplatin. The results of this trial have apparently not been published yet.

Another clinical trial involved the assessment of bioavailability in patients of a water soluble pro-drug of quercetin (QC12). The drug was not orally bioavailable. When administered i.v., the relative bioavailability of quercetin released from QC12 was determined to be 20-25%. A full scale phase I trial of QC12 in cancer patients was planned to be conducted(Mulholland et al. 2001) but its fate is uncertain.

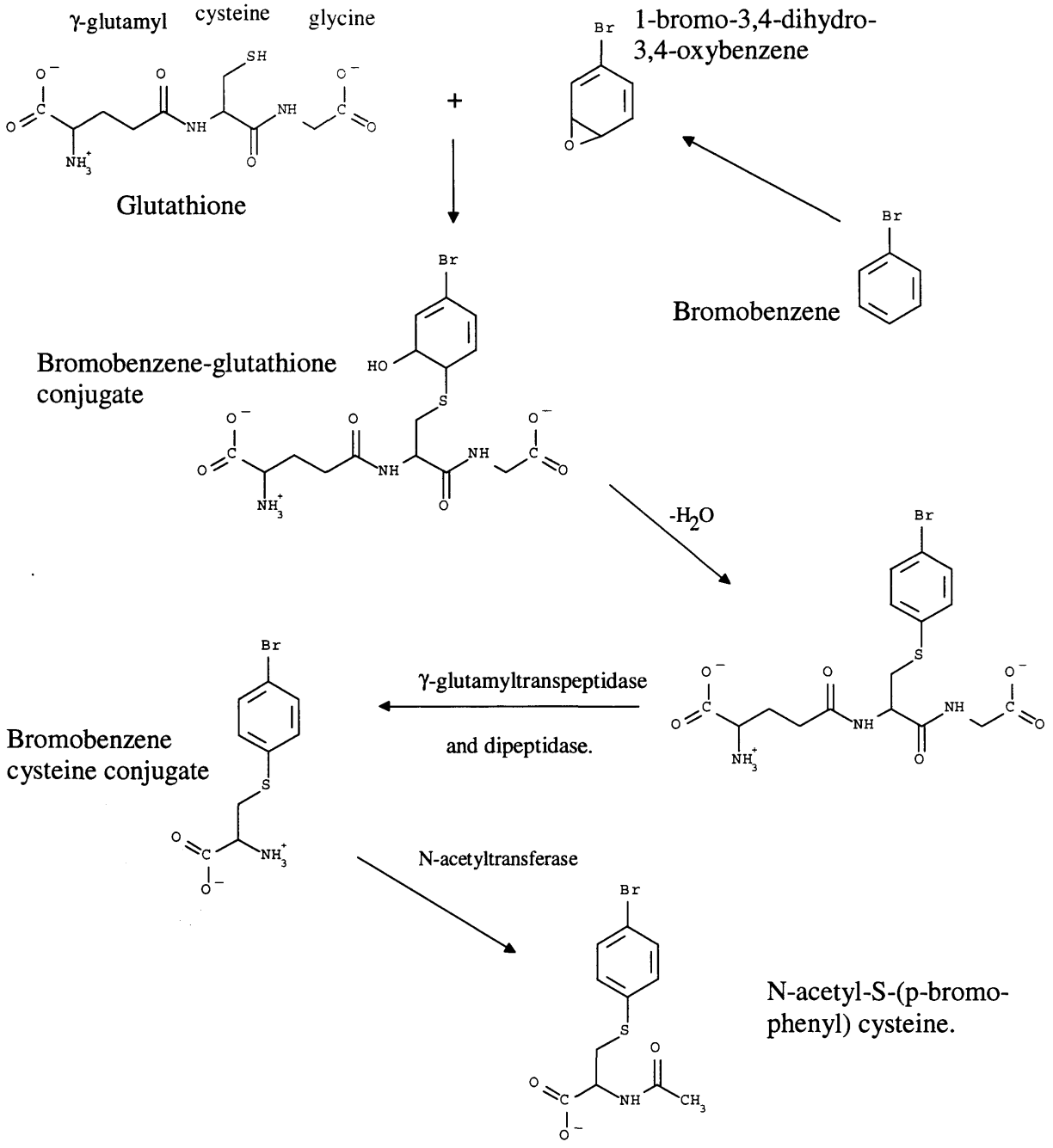
A preliminary study of the HPLC chromatographic conditions that would allow separation of quercetin metabolites was conducted as a prelude to the project described here(Johnstone 1994).

1.7 Role of glutathione in quercetin toxicity.

Part of the work described here is concerned with the potential generation of glutathione (GSH) and cysteine conjugates of quercetin, as a possible explanation of its kidney toxicity in humans(Ferry et al. 1996). Glutathione is present in high concentrations in most living cells and participates in a variety of vital cellular reactions. Among the earliest evidence for the involvement of GSH conjugation in the disposition of xenobiotics is the observation in 1879, according to which N-acetyl-S-p-bromophenyl-cysteine was isolated from the urine of dogs treated with bromobenzene(Meister 1982). This metabolite, a mercapturic acid (N-acetyl-S-(substituted)-cysteine), is the ultimate product of the metabolism of the bromobenzene-GSH conjugate.

GSH protects cells against potentially toxic electrophiles. However, reactions between reactive electrophiles and GSH can also give rise to conjugates that are toxic. For example, the nephrocarcinogen hydroquinone is transformed to GSH conjugates including 2,3,5-tris-(glutathio-S-nyl)hydroquinone, which has been demonstrated to be nephrotoxic(Lau et al. 1988; Lau, Kleiner, and Monks 1995).

Scheme 1.1 *Glutathione conjugation of bromobenzene and subsequent metabolism to mercapturic acid derivative.*



Scheme 1.1 presents the salient features of the pathway involving conjugation with GSH. In the scheme bromobenzene is used as an example of an electrophilic species which is conjugated, metabolised and excreted. The conjugation with GSH occurs predominantly in the liver, and conjugates are transported to tissues such as the proximal tubule of the kidney, which contains abundant amounts of gamma-glutamyl transpeptidase (γ-GT). There, the GSH conjugates are metabolised further to mercapturic acids. Bromobenzene is initially conjugated to glutathione catalysed by a glutathione transferase (GST)(Scheme 1.1). Removal of the γ-

glutamyl moiety is catalysed by γ-GT followed by the action of cysteinylglycine dipeptidase which cleaves the glycine peptide, leaving the cysteine conjugate. The final step involves acetylation of the amino group of cysteine, which requires N-acetyltransferase and acetyl-coenzyme A as co-factor. This pathway is specific to glutathione conjugates, but involves many enzymes with broad substrate specificity(Zhu et al. 1994; Josephy 1997).

In recent years, many authors have shown that in some instances, GSH can react to generate toxic metabolites, and an example, hydroquinone, has been mentioned above. Compounds such as hydroquinone, bromohydroquinone, 17β-oestradiol and haloalkenes are converted *via* conjugation with GSH into cytotoxic, genotoxic or mutagenic metabolites(Iverson et al. 1996). These metabolites appear to exhibit their toxic potential selectively in the kidney. The mechanisms by which the nephrotoxicity is expressed differ depending on the molecule. The nephrotoxicity may in part, be due to the formation of reactive quinone species, such as those displayed by 17β-oestradiol. Nephrotoxicity associated with some GSH conjugates seems to require the participation of γ-GT, for example in the cases of hydroquinone(Peters et al. 1997) and 17β-oestradiol(Butterworth, Lau, and Monks 1997). Haloalkanes such as trichloroethylene are converted to GSH conjugates which after mercapurate generation, are metabolised via the β-lyase pathway, which furnishes the ultimate toxicants of haloalkanes(Green et al. 1997). Details of these mechanisms are described on the following pages.

In the light of the fact that quercetin is a catechol and thus potentially subject to similar metabolic processes as those operative for hydroquinone, one might hypothesise that it may undergo hepatic GSH conjugation followed by toxification analogous to hydroquinone, ultimately causing damage in the renal proximal tubules.

1.7.1 Hydroquinone.

Figure 1.4 *Structure of hydroquinone.*

Hydroquinone (Fig. 1.4) is used as an antioxidant in the rubber industry, as a developing agent in photography and as an intermediate in the manufacture of rubber and food antioxidants and monomer inhibitors. It has also been identified in the smoke of non-filtered cigarettes (up to 155µg /cigarette). A 2-year National Toxicology Program bioassay in male F344 rats concluded that hydroquinone was a nephrocarcinogen(National Toxicology Program. 1989). Peters *et al* have associated this toxicity with GSH conjugates of hydroquinone(Peters et al. 1996; Peters et al. 1997).

Hydroquinone itself is not mutagenic in short-term bacterial assays or in the Ames test. Mutagenic activity was not been found in mouse cells *in vivo*. However, it caused base-pair changes in the TA 1535 *Salmonella* tester strain and was mutagenic in the oxidant-sensitive (TA104) salmonella tester strain. In addition to the mutagenicity renal tubular cell degeneration was observed in the renal cortex of male and female F344/N rats which received hydroquinone for 13 weeks(Monks and Lau 1997).

Hydroquinone is primarily metabolised via conjugation with sulphate and glucuronic acid. In addition 2-(N-acetylcystein-S-yl)–hydroquinone, 2-(glutathion-S-yl) hydroquinone, 2,5-bis (glutathion-S-yl) hydroquinone, 2,6-bis (glutathion-S-yl) hydroquinone and 2,3,5-tris (glutathion-S-yl) hydroquinone (TGHQ) have been characterised as metabolites of hydroquinone *in vivo*(Lau, Kleiner, and Monks 1995). TGHQ caused severe renal proximal tubule necrosis in male Sprague-Dawley rats(Lau, Kleiner, and Monks 1995). Immunohistochemical analysis revealed distinct patterns of staining for TGHQ-protein adducts in the rat kidney, and this phenomenon was not demonstrated in mouse kidney(Kleiner et al. 1998a). Since oxidation of a quinol conjugate to a quinone is a prerequisite for reactivity resulting in covalent adduction, differences in the ability to oxidise quinols and reduce quinones may contribute to the difference in susceptibility between the rat and mouse(Kleiner et al. 1998b; Kleiner et al. 1998a).

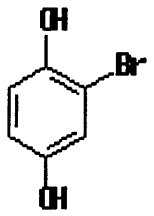
Mechanistically, chemical-induced carcinogenesis may proceed via an initial cytotoxic insult followed by regenerative hyperplasia then leading to tumour formation. This may be the case

for TGHQ, which has been shown to induce cell proliferation in proximal tubular cells of the S_3M region probably as the corollary of a cytotoxic insult, with proliferation being proportional to the degree of toxicity(Peters et al. 1997). TGHQ is about 600-fold more potent than hydroquinone as an inducer of nephrotoxicity, and consequently only a small proportion of hydroquinone is required to be metabolised to TGHQ to express nephrotoxicity.

Pretreatment of rats with acivicin, an inhibitor of γ-GT, prevented hydroquinone nephrotoxicity, thus suggesting that the toxicity is dependent on the formation of metabolites that require processing by γ-GT(Peters et al. 1997). Therefore hydroquinone toxicity may be caused by cysteine conjugates generated from GSH conjugates. The exact mechanism of nephrotoxicity is unclear, but it is conceivable that formation of a semiquinone of the hydroquinone-cysteine conjugate that binds covalently to macromolecules is responsible for toxicity. In terms of structural explanation it is possible that the presence of cysteine on the hydroquinone ring promotes electron destabilisation, which might favour redox cycling of the quinone. This mechanism may lead to oxidative stress in the cellular environment.

1.7.2 2-Bromohydroquinone.

Figure 1.5 *Structure of bromohydroquinone.*

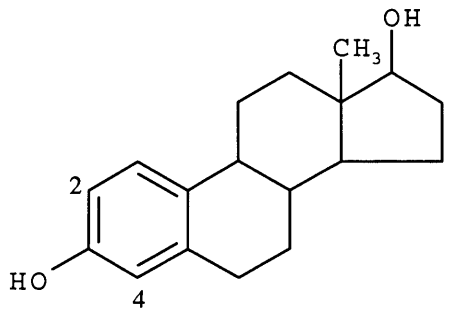


In the case of 2-bromohydroquinone the relative toxicity of quinol-GSH conjugates increased with the addition of GSH molecules(Monks et al. 1985). The initial conjugation of 2-bromohydroquinone with GSH is thought to be a detoxification step as it has been shown that the GSH conjugates themselves are more difficult to oxidise than 2-bromohydroquinone(Monks and Lau 1997). 2-Bromo-bis (glutathion-S-yl) hydroquinone, like TGHQ, appears to target its toxic potential to the renal proximal tubule cells, involving brush border γ-GT(Monks et al. 1985). Acivicin protected against 2-bromo-bis (glutathion-S-yl) hydroquinone mediated nephrotoxicity. Aminooxyacetic acid (AOAA), an inhibitor of β-lyase, decreased 2-bromo-bis (glutathion-S-yl) hydroquinone nephrotoxicity only to a minor extent, suggesting that toxicity does not involve β-lyase, which is described in more detail in section 1.7.4. These results suggest that metabolism of the GSH conjugates by γ-GT furnishes reactive intermediates.

The toxicity of 2-bromo-bis(glutathion-S-yl)hydroquinone seems to be related to its ability to generate reactive oxygen species which cause DNA fragmentation(Monks and Lau 1997). Scavengers of hydrogen peroxidase and Fe^{3+} inhibited the formation of single DNA strand breaks induced by this conjugate in renal proximal tubular epithelial cells(Monks and Lau 1997).

1.7.3 17β-Oestradiol.

Figure 1.6 *Structure of 17β-oestradiol.*



Exposure to oestrogens has been associated with neoplastic changes in humans and experimental animals(Zhu and Liehr 1994). In an established model of oestrogen-mediated carcinogenesis, male Syrian golden hamsters develop renal carcinoma following prolonged exposure to oestrogens. The mechanism of this carcinogenicity has been proposed to be related to the metabolism of oestrogens to reactive catechols or quinones(Butterworth, Lau, and Monks 1997). Hydroxylation of 17β-oestradiol, whilst lowering the affinity for estrogen receptors, generates catechol oestrogens which are readily oxidised to reactive semiquinones, *o*-quinones and quinone thioethers(Iverson et al. 1996). The major site of catechol oestrogen formation is the liver but the kidneys are the target organ for oestrogen-mediated carcinogenesis in male Syrian hamsters. A number of mechanisms have been proposed to explain this species-selective nephrocarcinogenicity of oestrogens, one of which implicates GSH. Firstly Liehr *et al* showed that hamsters unlike rats have high renal levels of the P450 responsible for catalysing the 4-hydroxylation (see Fig. 1.6) of 17β-oestradiol(Hammond et al. 1997), forming a catechol oestrogen. This agent, as outlined above, may be the proximate or ultimate toxicant in hamsters(Hammond et al. 1997; Butterworth, Lau, and Monks 1997). Thus, the tissue specificity demonstrated by 17β-oestradiol could be due to the *in situ* synthesis of 4-hydroxy-17β-oestradiol. Secondly, the difference between hamsters and rats in susceptibility to oestrogen induced nephrotoxicity, may in part be due to the role of catechol *O*-methyl transferase (COMT), and the variation in levels of COMT between hamsters and rats. The methylation of catechol oestrogens by COMT is a major route of metabolism(Butterworth, Lau, and Monks 1996). Hamsters exhibit much lower levels of hepatic and blood COMT than rats(Li, Purdy, and Li 1989). COMT

effectively quenches the ability of catechol oestrogens to redox cycle, while GSH conjugation does not eliminate such a mechanism(Monks and Lau 1992). A greater amount of free catechol present in the hamster compared to the rat may be responsible for 17β-oestradiol mediated nephrotoxicity specifically in the hamster.

A third proposed mechanism of estrogen-mediated renal carcinogenicity involves GSH conjugates of the catechol oestrogens(Monks and Lau 1992). Catechol oestrogen quinone-thioethers are thought to be generated in the liver and exert their toxicity in the proximal tubule of hamster kidneys.

Hamsters were administered a dose of 17β-oestradiol at a concentration high enough to induce very mild renal toxicity. Of this dose, 11.7% was recovered as GSH conjugates of the catechol estrogens of either the 17β-oestradiol or estrone(Butterworth, Lau, and Monks 1997). A problem with identifying this mechanism in humans, is the absence of mercapturic acids derivatives of oestrogens in human urine(Monks and Lau 1997). However, it is conceivable that they are not readily excreted or that they bind to macromolecules. When 2-hydroxy-1-(glutathion-S-yl)-17β-oestradiol was administered to rats, 15% of drug related species was found in the urine and only 5% in the faeces. Only the N-acetylcysteine metabolite was identified in the urine(Elce 1972). Acetylation and deacetylation in the kidney are further factors that may contribute to the overall nephrotoxicity. Thus, high activities of renal deacetylase may cause the regeneration of reactive quinone species from quinone-mercapturic acids. The release of the *N*-acetyl group may provide a quinone that can undergo redox cycling(Lau, Kleiner, and Monks 1995).

1.7.4 Haloalkenes.

The hypothesis concerning the GSH conjugation of quercetin being a precursor to nephrotoxicity, relies ultimately on the formation of quinones. However, other mechanisms of GSH-mediated nephrotoxicity exist. It has been revealed by many different approaches that a multi-step, GSH-dependent pathway, the so-called cysteine β-lyase (β-lyase) is involved in the expression of nephrotoxicity of haloalkenes(Anders and Dekant 1998). Haloalkenes undergo hepatic GSH conjugate formation, the conjugates are transported to the kidney and bioactivation occurs via renal β-lyase.

The formation of reactive intermediates by cysteine conjugate β-lyase is thought to play a role in the target-organ toxicity and in the possible renal tumourigenicity of several chlorinated olefins widely used in chemical processes(Anders and Dekant 1998).

1.7.5 Quercetin associated nephrotoxicity and GSH conjugation.

Quercetin contains a catechol moiety, which in analogy to hydroquinone, bromo-hydroquinone and the catechol estrogens might be capable of forming a quinone. Reactivity of quinones depends on their ability to undergo redox cycling, generating an oxidative stress and/or to interact with cellular nucleophiles such as proteins, DNA or GSH. This nucleophile may, as in the case of hydroquinone and catechol estrogens, generate metabolites of quercetin that cause toxicity.

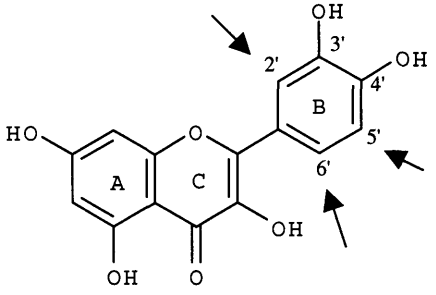
As part of the National Toxicology Program (NTP), the NIH carried out a toxicology and carcinogenesis study on F344 rats fed quercetin(National Toxicology Program 1992). In this assay, rats were fed quercetin in their drinking water for two years at different doses. After 2 years, toxic and neoplastic lesions were seen in the kidney of male rats, including increased severity of chronic nephropathy, hyperplasia, and neoplasia of the renal tubular epithelium. The report concludes that 'quercetin showed carcinogenic activity in the kidney of the male rat, causing primarily benign tumours of the renal tubular epithelium'. Further, 'quercetin did not cause tumours at any other sites. Quercetin is a genotoxic chemical (at high concentration), but the neoplastic response may be due in part to non-genotoxic and genotoxic events.'

During the phase I clinical trial of quercetin carried out by Ferry *et al*(Ferry et al. 1996), dose-limiting human nephrotoxicity was encountered at high quercetin concentrations. All three patients treated at 1700mg/m^2, exhibited renal toxicity as demonstrated by elevation of serum creatinine. At 1400 mg/m^2 only 25% of patients exhibited renal toxicity, while at 945 mg/m^2 and 630 mg/m^2, 37.5 and 20% of patients respectively, encountered renal toxicity. At the same time there was no cumulative fall in creatinine clearance with multiple weekly treatments, indicating that any nephrotoxicity was transient and reversible within 7 days(Ferry et al. 1996).

Quercetin increases the severity of oestradiol-induced tumourogenesis in the hamster

kidney(Zhu and Liehr 1994). The reasons for the potentiation of the oestradiol effect on the kidney by quercetin, could be linked to the inhibition of COMT that decreases in activity by 22-34%. The COMT-catalysed *O*-methylation of catechol metabolites of oestradiol is also potently inhibited by quercetin. The kinetic data suggest a mixed-type (competitive and non-competitive) of inhibition. It is likely that the methylation of quercetin is related to the competitive inhibition. It is conceivable, that from the evidence cited here that the nephrotoxicity exhibited in the long term feeding study and the clinical trial is induced via conjugation of GSH to quercetin and subsequent metabolism.

Figure 1.7. *Quercetin and potential sites of GSH adduction (explained in text).*

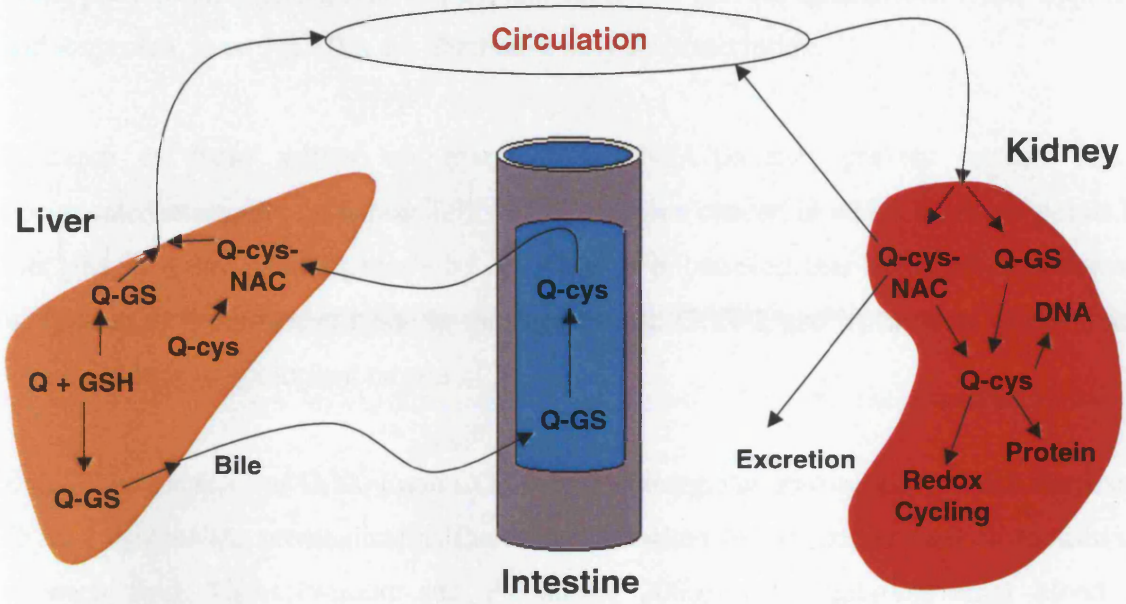


The hypothesis for a role of quercetin-GSH conjugates in the nephrotoxicity of quercetin is outlined in scheme 1.2, and implicates GSH conjugation in the liver. Quercetin undergoes a 2-electron oxidation via a semiquinone to form a ortho-quinone(Metodiewa et al. 1999). This reactive intermediate could react with GSH, forming a monosubstituted GSH conjugate, conceivably at the 2', 5' or 6' sites of the B-ring as shown in Fig. 1.7. The quercetin mono-GSH conjugate may then undergo a second round of oxidation/GSH conjugation in a manner analogous to hydroquinone and 2-hydroxyoestradiol(Monks et al. 1985; Butterworth, Lau, and Monks 1997) to form a bis-GSH conjugate. Three potential conjugates are possible involving positions 2' and 5', 2' and 6' or 5' and 6' of the B-ring, as seen in Fig. 1.7. The possibility of further conjugation to form a tris conjugate is also viable at positions 2', 5' and 6' (seen in Fig. 1.7.).

The resultant conjugates may be excreted into the bile, which is rich in γ-GT, and metabolised to cysteine derivatives, which could be reabsorbed into the liver, whereupon they may undergo conversion via acetylation, to form *N*-acetylcysteine conjugates. *N*-Acetylcysteine conjugates could then be taken up into the general circulation whereupon they might reach the kidneys. The *N*-acetylcysteine conjugates might be deacetylated in the kidney to form cysteine conjugates in a manner analogous to the GSH conjugate of hydroquinone(Lau,

Kleiner, and Monks 1995), (refer to scheme 1.2). Alternatively it is conceivable that quercetin undergoes conjugation with GSH, and the conjugate(s) reach the general circulation and is transformed in the kidney to quercetin-cysteine conjugates. The proximal tubule of the kidney is especially rich in γ-GT and there glutathione conjugates of quercetin could be metabolised to cysteine derivatives (see scheme 1.2).

Scheme 1.2 *Hypothetical metabolism of quercetin-glutathione conjugates.*



Part of the work described in this thesis was designed to test the hypothesis that quercetin undergoes metabolism to GSH conjugates as outlined above, that cause toxic lesions to the kidney. It is conceivable that on formation of a GSH-conjugate, a quinone could form that could subsequently generate a semiquinone, which in turn could cause damage directly. In addition, the quinone could undergo redox cycling to return to its catechol precursor, a process that would probably necessitate NADPH or NADH, thus depleting the cell of reducing power. Ultimately a decrease in reducing power would lead to the cell undergoing oxidative stress, and consequently, cytotoxicity.

1.8 COX-2 as potential target of quercetin.

A recent development in cancer research is the recognition that some drugs that share the property of inhibiting the cyclooxygenase (COX) enzyme can delay or prevent cancer(Prescott and Fitzpatrick 2000). These agents, known as non-steroidal anti-inflammatory drugs (NSAIDs) play an important role in clinical medicine. NSAIDs are used to suppress inflammation, relieve pain and fever and prevent thrombosis. Other than aspirin and ibuprofen, most NSAIDs are obtainable only by prescription.

Research on these agents has revealed that NSAIDs may prevent cancer. The best documented example of this possibility involves colon cancer, in which evidence points to the blocking of colon carcinogenesis by NSAIDs. It is believed that the COX-2 isoenzyme is implicated as the target enzyme in the mechanism. COX-2 and its isoform COX-1, are the established pharmacological targets of NSAIDs.

Essentially the roles of COX-1 and COX-2 can be integrated into a model which suggests that COX-1 synthesises prostaglandins that serve housekeeping functions such as protecting the stomach from ulcers(Prescott and Fitzpatrick 2000) and regulating renal blood flow. However, COX-2 synthesises prostaglandins associated with pain and fever(Prescott and Fitzpatrick 2000). COX-1 is constitutively expressed to fulfil its housekeeping role, while COX-2 is not constitutively expressed in appreciable amounts by most normal tissues, but is rapidly induced by certain inflammatory cytokines, tumour promoters, growth factors and oncogenes. Conventional NSAIDs inhibit both COX-1 and COX-2 non-selectively at concentrations encountered in the gastrointestinal tract. Agents have been developed that specifically inhibit COX-2. One such agent, celecoxib, has received much attention and has showed promise in clinic trials(Schnitzer 2001).

The evidence for the discrete role of COX-2 in colon carcinogenesis is three fold. Epidemiology, whole-animal pharmacology and *in vitro* pharmacology all indicate that COX-2 plays a role in colon carcinogenesis. In essence, epidemiological studies have shown that individuals that take NSAIDs have a reduced risk of developing colon cancer and its non-malignant precursor, the adenomatous polyp(Muir and Logan 2000). In whole animal studies, the dosing of rats with NSAIDs prevented, or markedly reduced, the formation of colon tumours(Reddy, Maruyama, and Kelloff 1987). Knockout mice that lacked the COX-2 gene

were bred with mice that harboured a mutated allele of the gene encoding adenomatous polyposis coli (APC), a tumour suppressor, germ lime mutations of which are associated with elevated risk for colon cancer(Oshima et al. 1996). The results from this experiment showed that selective genetic elimination of COX-2 protected mice with a defective APC tumour suppressor from the development of intestinal tumours(Oshima et al. 1996). Additionally, analyses of human colon and normal colon tissue indicate that COX-2 is consistently absent in normal tissue and increased in tumours, while COX-1 is present at low levels in both (Sano et al. 1995).

The COX isoenzymes catalyse the oxidation of arachidonic acid to various prostaglandins, including PGE-2. PGE-2 can inhibit programmed cell death by inducing expression of the Bcl-2 proto-oncogene(Sheng et al. 1998). PGE2 and other prostaglandins often elevate intracellular cyclic AMP concentrations which can suppress apoptosis(Orlov et al. 1999). It has been reported that PGE2 promotes colon carcinogenesis. The long term administration of PGE2 and its effect on colon carcinogenesis was investigated in rats(Inaba, Uchiyama, and Oka 1999). The authors concluded that endogenous PGE2 in colon mucosa may be adequate to promote colon carcinoma. Conversely, to block colon carcinogenesis, PGE2 levels in colonic mucosa must be decreased to less than endogenous levels.

The relationship between flavonoids, quercetin in particular, and key steps in the arachidonic acid pathway are well documented. In macrophages cyclooxygenase activity and PGE2 secretion were both inhibited by the flavonoid catechin(Formica and Regelson 1995). The methoxy flavone, wogonin inhibited inducible-PGE2 production in macrophages at levels as low as 0.5μM, while the protein expression of COX-2 was depressed by wogonin at 10μM(Chi, Cheon, and Kim 2001). Nobiletin, a flavonoid extracted from citrus fruit reduced production of PGE2 in both synovial cells and rabbit articular chondrocytes(Ishiwa et al. 2000). Quercetin inhibited cyclooxygenase enzyme activity with a IC_{50} of 16 μM(Formica and Regelson 1995). Six different flavonoids (including quercetin) were shown to markedly decrease PGE2 and COX-2 expression in a concentration dependent manner(Raso et al. 2001). In human platelets, a methoxycarbonylflavonol inhibited the release of PGE2(Silvan et al. 2000). Moreover, quercetin, kaempferol, genistein, resveratrol and resorcinol were found to effectively suppress the COX-2 promotor activity in DLD-1 cells(Mutoh et al. 2000a). In this study, it was suggested that the common link between all these compounds was the resorcinol moiety in the structure, which may play an active role in the inhibition of COX-2 expression in colon cancer cells. An assessment of 12 flavonoids was carried out and

quercetin was found to be the most potent suppressor of COX-2 transcription(Mutoh et al. 2000b). Quercetin can inhibit phospholipase A2, 5- and 12- lipoxygenases and cycloxygenase(Fawzy, Vishwanath, and Franson 1988; Kato et al. 1983; Gschwendt et al. 1984; Welton et al. 1986).

Another polyphenol, curcumin, has also been shown to effect COX-2 expression(Ireson et al. 2000). In cells where PGE-2 levels were induced by phorbol-ester, curcumin reduced PGE-2 levels to pre-induction levels(Ireson et al. 2000). It has been shown previously that curcumin does inhibit prostaglandin synthesis by inhibition of COX-2 induction(Ireson et al. 2000). The reduced and conjugated metabolites of curcumin showed either week inhibition or were ineffective.

1.9 Aims of project.

The introduction above illustrates that a considerable amount of knowledge is available on the biochemical and physiological effects of quercetin. Furthermore, since this work commenced, an appreciable amount of information has been published on the absorption and metabolism of dietary quercetin glycosides. In contrast, little data exists on the metabolism of pure quercetin, especially in humans. The biological activities displayed by quercetin, such as the regulation of signal transduction pathways, its antiproliferative effects and its ability to bind to type II EBS (refer to section 1.4), have led to clinical trials of quercetin. The trials provided some information on the pharmacokinetics of quercetin, but little on its metabolism. In order to interpret the reported effects of the drug and assess its potential viability as a therapeutic or preventative agent, more knowledge of its metabolism is desirable. The detection of methylated, sulphated and glucuronidated derivatives of quercetin has been shown in rats. However, the metabolic fate of quercetin in humans, when administered as a pure agent is still unknown. Extent of conjugation and sites of conjugation on the quercetin molecule are not known. The principal aim of the work described in this thesis is to shed light on these particular gaps in the knowledge of quercetin metabolism.

The work described here made use of human plasma samples obtained from the phase I trial, conducted in Birmingham, which were analysed for the presence of metabolites. In order to explore quercetin metabolism further, its biotransformation was studied in rats, and the hypothesis was tested that the rat is a suitable model for quercetin metabolism in humans.

One rationale for using animals was to generate sufficient quantities of quercetin metabolites to aid identification and characterisation. The study of quercetin in rodents *in vivo* was thought to allow more insight into the absorption, distribution and bioavailability of quercetin. The production of quercetin metabolites was also explored in *in vitro* systems. By improving the understanding of the metabolism of quercetin the results might help to contribute to the development of quercetin as a potential therapeutic and chemopreventative agent.

To meet these objectives, a sensitive and selective method for the separation and analysis of quercetin and quercetin metabolites in various biological matrices was developed and validated. High-performance liquid chromatography (HPLC) with UV and ECD detection was used. On-line HPLC-mass spectrometry (LC-MS) and on-line HPLC-multistage mass spectrometry (LC-MS/MS) allowed subsequent characterisation. ^{1}H-NMR was employed for the characterisation of certain synthesised metabolite standards.

In order to explore the potential generation of thioether metabolites of quercetin, which might explain its renal toxicity, special care was taken to identify conjugates of quercetin with glutathione and cysteine.

Finally, the effect of quercetin and some of its metabolites on COX-2 was investigated. The overall aim of this part of the work was to elucidate the potential contribution that quercetin metabolites might make to an important biological effect, exerted by the parent compound *in vivo*. In order to explore this aim, the effect of quercetin and its metabolites on the activity and/or expression of COX-2, by way of assessment of PGE2 levels, was compared.

Overall, the work was designed to improve the understanding of the metabolism of quercetin in order to aid interpretation of its pharmacological effects and to improve potential future studies of its therapeutic value in humans.

2.0 Chapter Two. Analytical techniques and method development.

2.1 Introduction.

The detection, identification and characterisation of molecules can be carried out using a variety of analytical techniques. The work described in this thesis encompasses high-performance liquid chromatography (HPLC), which utilises reverse-phase (rp) chromatography for separation, and UV, electrochemical and mass spectrometric (MS) techniques for detection. Thin layer chromatography (TLC) was also used for separation. Mass spectrometry, tandem mass spectrometry (MS/MS) and NMR were used for characterisation of individual molecules.

The aims of this chapter are four-fold. Firstly, a description and explanation of the techniques used in the work outlined in the thesis are presented. Secondly, the development and optimisation of a rp-HPLC system for the separation and sensitive detection of quercetin and its metabolites in plasma, urine, bile, and tissue is outlined. Thirdly, a mass spectrometric system is described which has been optimised for LC-MS and on-line LC-MS/MS experiments, thus allowing versatile molecular characterisation. Finally, the validation of the HPLC system for the quantitation of quercetin is described.

2.2 Experimental.

2.2.1 Materials and reagents.

Quercetin, fisetin, myricetin, morin, kaempferol, naringenin, mushroom tyrosinase, horse-radish peroxidase, hydrogen peroxide, HPLC grade acetone, HPLC grade dimethyl sulphoxide (DMSO), ammonium acetate, were all obtained from Sigma (Poole, UK). Disodium ethylenediamine-tetraacetic acid (EDTA), AnalaR grade was obtained from BDH (Poole, Dorset, UK). Methanol (HPLC grade) and glycerol formal from Fisher (Loughborough, Leics., UK). Isorhamnetin and quercetin 3-O-sulphate were obtained from Extrasynthese (Genay, France).

2.3 High performance liquid chromatography of quercetin.

2.3.1 HPLC of quercetin.

The analysis of quercetin requires a sensitive, selective and robust system that can detect and distinguish between quercetin, metabolites and other flavonoids.

Early work had used randomly-labelled ^{14}C-quercetin and measured total radioactivity following administration(Petrakis et al. 1959). This type of approach is obviously not feasible in humans and is limited to animal studies. Further studies used ^{14}C-labelled quercetin and analysed extracts of urine, faeces and bile by TLC(Ueno, Nakano, and Hirono 1983). TLC has poor resolving power in comparison with HPLC, and is unable to yield a much data on the characterisation of the molecule. Gugler *et al* carried out a human study in which quercetin was derivatised to produce a fluorescent quercetin-tetraphenyldiboroxide complex allowing a limit of detection of 100ng/mL(Gugler and Leschik 1975). A spectrophotometric method has also been utilised, but can be criticised because it lacks sensitivity and specificity(Nikolovskacoleska et al. 1996). Contemporary methods of analysis that are used in flavonoid analysis include gas chromatography (GC), gas chromatography coupled with MS (GC-MS), and capillary electrophoresis (CE)(Baba et al. 1983; Payares et al. 1997; Watson and Pitt 1998; Berahia et al. 1993; Liang et al. 1996). GC and GC-MS require derivatisation before analysis, while CE often lacks the necessary sensitivity due to the very small volume of sample injected onto the capillary. The most widely used method for the routine analysis of quercetin is HPLC with UV, fluorescence or electrochemical detection. Table 2.1 is a summary of the HPLC systems, which have been used for the analysis of quercetin, and shows the various separation and detection systems. The majority of HPLC systems used for the detection of quercetin have been optimised for the detection of quercetin as a constituent of a mixture of naturally occurring flavonoids. The published methods have not been optimised for resolution of quercetin metabolites, especially those that elute close to the solvent front. Less then half of the systems are compatible with mass spectrometry due to the use of involatile buffers in the mobile phase, while the remaining mobile phases consist of water and organic modifier, which can lead to poor resolution and unsymmetrical peak shape.

The best method for the analysis of quercetin and related compounds is HPLC-MS, which has sensitivity, specificity and potentially provides characterisation of unknown compounds.

Table 2.1 *Different HPLC systems used for the separation and detection of quercetin and related compounds.*

Column	Mobile phase	Detection	Compounds separated	Ref.
Hypersil ODS C_{18}, 150x4.6mm, 5μm	73% water/H_3PO_4 (99.5/0.5) in acetonitrile.	UV (370nm and PDA)	Quercetin and rutin.	(Manach et al. 1997)
Nova-Pak C_{18}, 250x4.6mm, 4μm	20% methanol in 0.1% HCl: Acetonitrile. (Gradient).	UV (PDA)	Quercetin glycosides	(Coward et al. 1996)
Nova-Pak C_{18}, 300x3.9mm, 4μm.	Water:acetonitrile:0.5% TFA (pH3.0). (Gradient).	UV (PDA)	Quercetin glycosides and 31 related phenolic compounds from Norway Spruce.	(Slimestad and Hostettmann 1996)
Nova-Pak C_{18}, 150x3.9mm, 4μm	i) acetonitrile: 0.025M KH_2PO_4,(25:75) ii) acetonitrile 0.025M KH_2PO_4, (45:55)	UV (370nm and PDA)	Quercetin, and flavonoid glucuronides, glycosides and glucosides.	(Barnes et al. 1998)
μBondapak C_{18},	Water:acetic acid: methanol (80:6:65)	UV (370nm)	Quercetin, rutin and taxofolin and metabolites.	(Brown and Griffiths 1983)
Vydak C_{18}	0.1% TFA: methanol (Gradient).	UV (PDA)	Quercetin metabolites.	(Gross et al. 1996)
Inertsil ODS-2, 150x4.6mm, 5μm	31% acetonitrile: 0.025M phosphate buffer.	Fluorescent (490nm) following derivatisation	Quercetin and quercetin glycosides	(Hollman et al. 1995)
ODS-HC-SIL-X1	Water:methanol:acetic acid (60:75:5)	UV (370nm)	Propolis	(Daigle and Conkerton 1983)
Micropack MCH-10 300x4.0mm,	Methanol:0.1M Citrate buffer (1:1)	UV (360nm)	Rutin	(Romero et al. 1983)
μBondapak RP-18 300x3.9mm, 10μm	Water:tert-butanol (86:14)	UV (355nm)	Flavonoid glycosides	(Debernardi et al. 1984)
Lichrosorb Si60, 300x3.0mm, 5μm	isooctane:diethyl ether:acetonitrile (50:30:10)	UV (230nm)	Quercetin, kaempferol, myricetin and glycosidic derivatives	(Siewek and Galensa 1984)
Ultrasorb ODS, 2500x4.6mm, 5μm	1% Acetic acid: acetonitrile. (gradient)	UV (360nm)	Quercetin and related glycosides	(Siewek, Galensa, and Herrmann 1984)
μBondapak C_{18}, 300x3.9mm, 10μm	Methanol:water:acetic acid (50:42:8)	UV (280nm)	Quercetin and kaempferol aglycones and gylcosides.	(Elce 1972; Bilyk and Sapers 1985)
Spherisorb S5 ODS-2, 250x 4.0mm,	Methanol: water:acetic acid: 0.1M tetrabutyl-ammonium phosphate. (Gradient)	UV (365nm)	Flavonol glycosides	(Harborne and Boardley 1984)
Partisil 5 CCS/C_8, 250x5.0mm	Water:methanol:acetic acid. (Gradient).	UV (365nm)	Flavonol sulphates	(Harborne and Boardley 1984)
μBondapak C_{18}, 300x4.1mm, 10μm	Methanol:water	UV (254nm)	Flavonol glycosides	(Lehoang et al. 1985)
μBondapak C_{18}, 300x3.9mm, 10μm	Acetic acid:water: acetonitrile(or MeOH). (Gradient).	UV (340nm)	Flavonoids and coumarins	(Tamma, Miller, and Everett 1985)

Various	Methanol:acetic acid:water. (Gradient).	UV (365nm)	Flavonoids and their glycosides	(Harborne, Boardley, and Linder 1985)
RP-C18, 250x4.6mm, 5µm	Acetonitrile:phosphate buffer (26:74), with SDS and EDTA.	ECD (+0.72V)	Quercetin and quercetin glucoside.	(Hopkins and Ahmad 1991)
Nucleosil, 250x4.6mm, 5µm	Methanol:acetic acid:water. (Gradient).	UV (340nm) & PDA	Flavonoid aglycones	(Voirin and Bayet 1992)
LiChrocart RP-18, 125x4.0mm, 5µm	Water:formic acid: methanol. (Gradient).	PDA	Flavonoid aglycones and glycosides	(Gil et al. 1993)
LiChrospher RP-18, 250x4.0mm, 5µm	Methanol:water: acetic acid (30:70:3)	UV (330)	Flavone glycosides	(Krauzeba ranowska and Cisowski 1994)
Hypersil ODS C_{18}, 100x4.0mm, 5µm	Methanol: orthophosphoric acid. (Gradient).	UV (370 and 260nm)	Flavonol glycosides.	(Rehwald, Meier, and Sticher 1994)
Cosmosil 5C_{18}-AR, 250x4.6mm, 5µm	tetrabutylammonium bromide: $K_2H_2PO_4$: methanol:acetonitrile. (Gradient).	UV (254nm)	Flavones	(Sheu and Chen 1995)
µBondapak C_{18}, 150x3.9mm,10µm	45% acetone: sodium dihydrogen sulphate.	UV (375nm)	Quercetin and unidentified metabolites	(Liu et al. 1995)
LiChrospher 100 RP-18, 250x4.0mm, 5µm	0.01M Phosphoric acid : methanol. (Gradient).	UV (PDA)	Quercetin and 24 other flavonoids found in *citrus*.	(Nogata et al. 1994)
Novapak C18, 250x4.6mm, 4µm.	Methanol:HCl: acetonitrile. (Gradient).	UV (320 and 520nm)	Flavonoids and hydroxy-cinnamates	(Bourne and Riceevans 1998)
Hypersil ODS C_{18}, 60x4.6mm, 3µm	Aq. THF :ortho-phosphoric acid: methanol. (Gradient).	UV (220, 280, 320 and 360nm)	Flavonoids	(Keinanen and JulkunenTi itto 1998)
Hypersil ODS C_{18} 150x3.0mm 5µm	i) Sodium acetate, and MeOH (80:20). ii) Sodium acetate :MeOH:AcCN (40:40:20).	Coulometric array	Quercetin, and 12 other phytoestrogens and polyphenols	(Gamache and Acworth 1998)
Hypersil ODS C_{18} 250x4.0mm, 5µm	Acetic acid: methanol : water. (Gradient).	UV (PDA)	Quercetin, Cis- + trans- resveratrol, rutin, catechin, epicatechin and trans resveratrol glycoside.	(Goldberg et al. 1996)
Inertsil, 150x4.6mm,	30mM NaH_2PO_4 : methanol.	ECD	Quercetin + unidentified metabolites.	(Manach et al. 1998)
Hypersil ODS C_{18}, 150x4.6mm, 5µm	73% water/H_3PO_4 (99.5/0.5) in acetonitrile.	UV (370nm and PDA)	Quercetin and rutin.	(Manach et al. 1997)

2.3.2 HPLC system for the detection of quercetin.

2.3.2.1 Pumps and detectors.

For the majority of HPLC-UV detection, two HPLC systems were employed, both of which were manufactured by Varian (Walton-on-Thames, Surrey, UK). The first system was the Varian Prostar system, which comprised of a UV detector (model 310), solvent delivery system (model 230) and a autosampler (model 410). The autosampler contained a 100μL loop. The second system for UV analysis used a Varian model 9012 liquid chromatograph with a Linear UVIS-204 detector set at 375nm. Injection of sample was *via* a Rheodyne 7125 injector fitted with a 100μL loop.

For electrochemical detection (ECD), a Gynkotek model 300 solvent delivery system with an Antec electrochemical detector (Presearch, Letchworth Garden City, Herts., UK) set at oxidation mode (+0.6V) was used. Injection of sample was *via* a Rheodyne 7125 injector fitted with a 100μL loop.

On a few occasions at the start of the project, a Pye-Unicam HPLC system, which comprised a Pye-Unicam Pump and UV detector was used. This HPLC system was attached to a chart recorder for data collection.

2.3.2.2 Column

The flavonoids were separated on a 250×4.6mm I.D. BDS-Hypersil C_{18} column (5μm particle size) from Hypersil, Runcorn, Cheshire, UK. The flow rate was 1.0 mL/min using ammonium acetate and methanol as mobile phase components. The development of the mobile phase is described below. For ECD, the mobile phase was continuously purged with a slow stream of helium during the separation.

2.3.2.3 Mobile phase

The choice of mobile phase is paramount to optimal separation. For efficacious separation, it is desirable to have a silanol-masking reagent within the mobile phase. Triethylamine can be used to eliminate silanol effects, but it is more suitable to use a buffer containing a silanol

masking agent, such as ammonium acetate, which has been shown to mask residual silanol groups(Lim and Peters 1984). Ammonium acetate is now routinely used in mobile phases, especially as it is also compatible with electrospray ionisation mass spectrometry.

Another common problem found with silica columns, is the presence of metal ions, which are found as an impurity within the packing. The presence of metal ions can result in broader peaks and peak tailing. Polyhydroxylated compounds, such as flavonoids are particularly susceptible to chelation by metal ions, and as a consequence, the separation can result in poor resolution and peak symmetry. The addition of a metal ion chelator into the mobile phase allows the performance of the separation to be improved drastically. EDTA is a proven metal ion chelator and was used in the mobile phase and assessed for its effect on resolution.

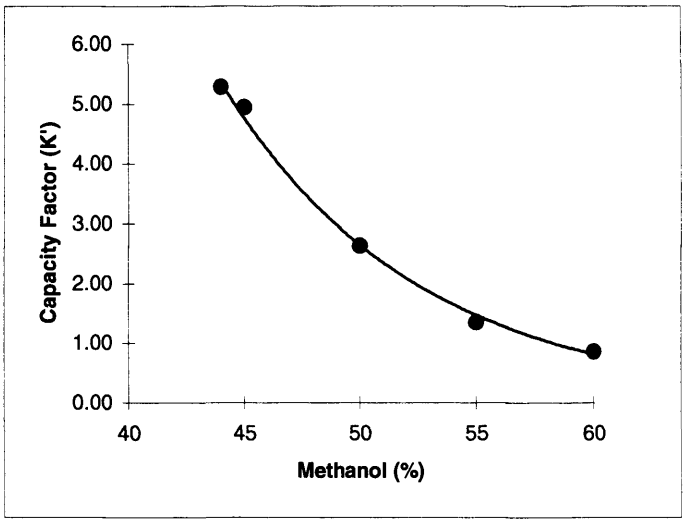
A mobile phase of ammonium acetate containing EDTA in methanol was chosen for optimisation of the separating system. Following the injection of a quercetin standard, peak areas were assessed and the variation in capacity factor (k′) was calculated for each of the parameters using equation one. In this equation R_o represents the time of the solvent front and R_t represents the retention time of the compound of interest.

Equation 1. $$k' = \frac{R_t - R_o}{R_o}$$

Methanol content.

In reversed-phase liquid chromatography, the organic part of the mobile phase effectively causes the elution of the analytes, by competing for the hydrophobic interactions formed between the stationary phase and the analyte. The capacity factor (k′) of a column is a direct measure of the strength of the interaction of the sample with the packing material. The higher the capacity factor of the column, the greater is its ability to retain solutes. Typically, a k′ value should lie between 2 and 5 for suitable balance between retention time and resolution. An experiment was carried out where the proportion of methanol was varied and the k′ calculated. Fig. 2.1 shows that k′ decreases with increasing methanol, which is expected with reversed-phase HPLC. As can be seen for the chosen isocratic system of 44% methanol, the capacity factor is approximately 5.

Figure 2.1 *The relationship between methanol proportion and the capacity factor (k'). The experiment compared between five different proportions of methanol i.e. 45, 46,50, 55 and 60%. The capacity factor was assessed by injecting a quercetin standard and determining the solvent front time and the retention time for quercetin. These figures were incorporated into Equation 1.*

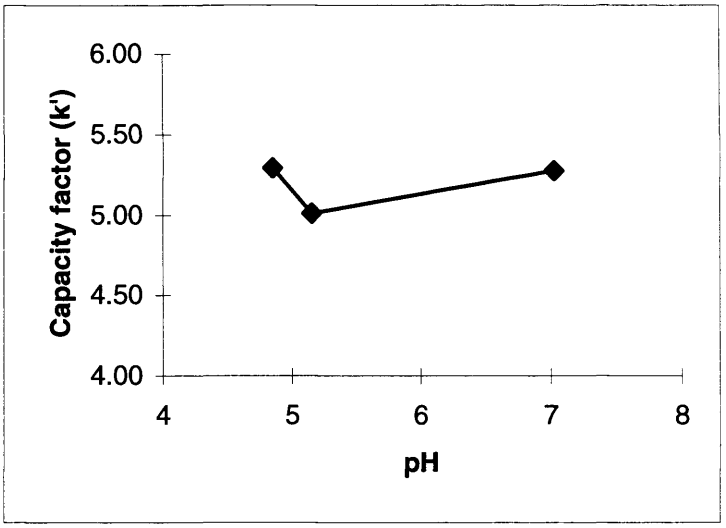


Buffer pH.

A similar experiment was carried out to assess the effect of pH on k′. Using a 56% 0.1M ammonium acetate buffer in methanol, the pH was altered and the k′ calculated. Fig. 2.2 shows that a clear relationship between the pH of the ammonium acetate buffer used for quercetin analysis and the capacity factor does not exist. The effect of pH on reversed-phase liquid chromatography is a complex one and depends on the analyte, the stationary phase and the mobile phase chosen. For most stationary phases, it is important that the pH lies between 2.0 and 8.0, as pH values above 8.0 causes dissolution of the silica and pH values below 2.0 elicit hydrolysis of the bonded functional groups in the stationary phase, resulting in loss of retention - and inevitably column failure. The stability of the analyte at different pH is also a consideration.

For the retention of quercetin, varying the pH between 5 and 7 had little impact as the capacity factor varies between 5 and 5.5. A pH of 5.15 was chosen as the most suitable for the ammonium acetate buffer in the mobile phase, as the k′ remained below 5.0. An additional factor that influenced the choice was the need to maintain an alkaline buffer for negative-ion electrospray mass spectrometry.

Figure 2.2 *The relationship between changes in pH and the capacity factor, k'. The capacity factor was assessed by injecting a quercetin standard and determining the solvent front time and the retention time for quercetin. These figures were incorporated into Equation 1.*



Buffer strength.

A decrease in the ammonium acetate concentration from 0.25 to 0.1M, had little effect on either Rt, with a change of approximately one minute observed, or resolution. In order to avoid suppression of ionisation that ammonium acetate has been reported to cause(Schaefer and Dixon 1996), the molarity was kept to a minimum, and thus 0.1M was chosen.

Buffer additives.

In order to optimise peak shape, EDTA (100mg/L) was added to the buffer. Reversed-phase columns often contain within the packing, metal ions which are contaminants of the silica. Metal ions are trapped by residual silanol groups, which can then form complexes with the analytes. Fig. 2.3 shows the chelation of analytes (represented by the letter Q) by metal ions.

Figure 2.3 *Chelation of analytes by metal ions. Structure (a) shows the C_{18} bonded phase with residual silanol groups. Structure (b) shows residual silanol-groups trapping metal ions, which are able to capture analytes in the column by complexation.*

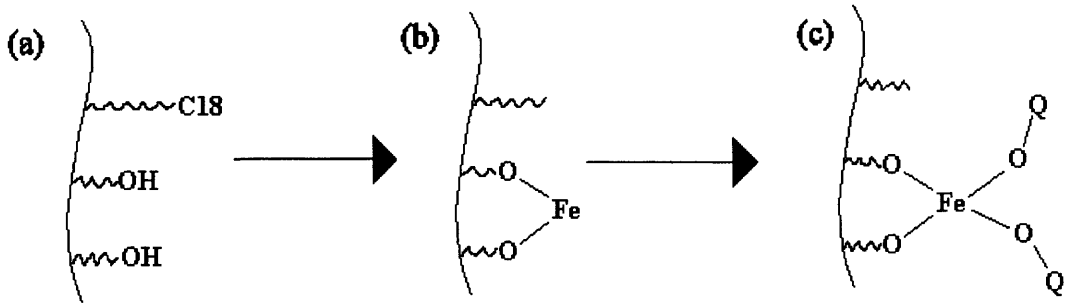
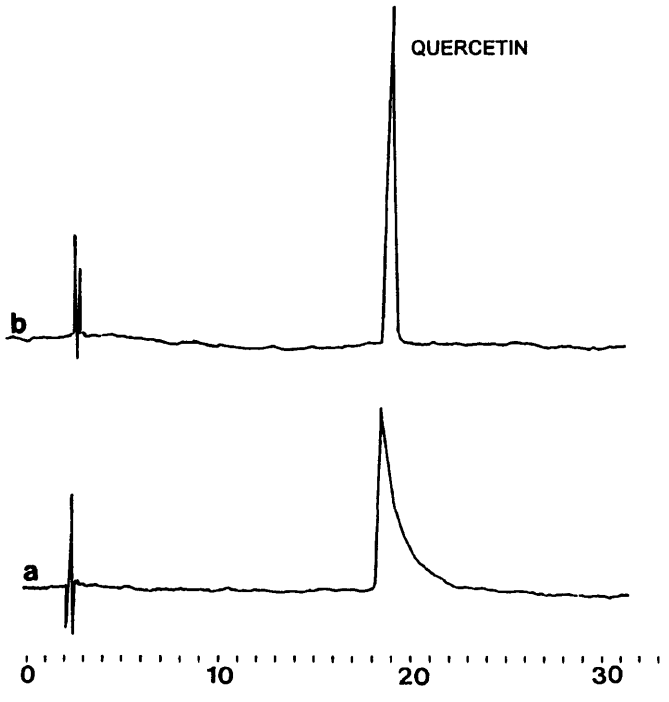


Fig. 2.4 shows the effect of the presence of EDTA in 0.1M ammonium acetate. Chromatogram (a) shows the elution of quercetin when EDTA is not present in the mobile phase, while chromatogram (b) shows the same injection when EDTA is added to the buffer.

Essentially, the presence of EDTA improved the resolution and peak tailing was not observed.

Figure 2.4 *Effect of EDTA on peak shape. Isocratic chromatography was employed as described in section 2.3.2.4.*



The isocratic system of 56% 0.1M ammonium acetate, pH 5.15 in methanol is suitable for demonstration of the effects of EDTA on the resolution of quercetin chromatographic peaks. However, it soon became apparent that a gradient system would have to be incorporated to separate structurally related quercetin metabolites. A suitable gradient began with 75% 0.1M ammonium acetate in methanol, dropped linearly to 55% for 10min, and then to 45% for a further 20 minutes. The mobile phase remained isocratic at this stage for a further 5 minutes.

2.3.2.4 Chromatographic systems.

Two chromatographic systems are used in this thesis:

Chromatographic system A - Isocratic: 56% 0.1M ammonium acetate, pH5.15 in methanol.
Chromatographic system B - Gradient: 75% 0.1M ammonium acetate in methanol, dropped linearly to 55% for 10min, and then to 45% for a further 20min. The mobile phase remained isocratic at this stage for a further 5min.

Chromatographic system B was tested for a number of parameters in order to validate the method.

2.3.3 Validation

The HPLC system was validated according to the Good Research Practice (GRP) requirements of the MRC Toxicology Unit. Kaempferol was used as an internal standard (IS), due to its structural similarity to quercetin as shown in Fig. 2.8. Additionally, it was known that kaempferol eluted at a time that did not interfere with metabolites of quercetin.
Validation is clearly very important when carrying out studies where the imperative issue is quantitation. Key components of validation include good linearity of response across different concentrations, the accuracy and precision of the method, the efficiency of the recovery of the drug in question, robustness of the assay and the stability of the drug in storage prior to analysis(Swartz and Krull 1997).

2.3.3.1 Extraction efficiency (recovery)

The recovery of a drug from a biological matrix is important to assess, in order to determine the true accuracy of the overall assay. The extraction efficiency was determined by measuring

the amount of quercetin in spiked plasma following extraction. Using a calibration line containing known amounts of quercetin, the percentage recovery could be assessed by comparing equivalent concentrations of quercetin in mobile phase (assumed to be 100% recovery) and that recovered in plasma.

The protocol for testing the extraction efficiency of the extraction solution involved spiking 980μL of drug free human plasma with 20μL of different concentrations of quercetin (10, 5, 1, 0.1 and 0.05mg/mL). Plasma was then measured into 200μL aliquots and 400μL of DMSO/methanol (1:4) was added. Following rigorous vortexing, 200μL of the supernatant was removed and added to 200μL of deionised water. This solution was mixed thoroughly and 50μL injected onto the column. For each concentration, three replicates were generated. Two injections of each replicate were analysed.

Table 2.2 shows the data for the recovery of quercetin for each concentration from plasma. The mean percentage recovery for all concentrations is 90% and there is no clear evidence for any concentration dependent differences in percentage recovery.

Table 2.2 *The percentage recovery from plasma of quercetin. The mean recovery from plasma for all concentrations is 90.08%. The recovery was calculated by comparing between the known amounts of quercetin in mobile phase and the recovered amount of quercetin in plasma.*

Quercetin concentration (μg/mL)	Quercetin in plasma		Quercetin in mobile phase.		Recovery (%)
	Peak area	s.d.(n=6)	Peak area	s.d.(n=6)	
1	19450	1594	21980	1703	88.5
2	39640	2097	42810	3163	92.5
10	406600	30200	449200	31860	90.5
100	2052000	76660	2360000	142400	86.9
200	3551000	235300	3835000	301700	92.6

2.3.3.2 Precision and accuracy.

The accuracy is determined by the percent recovery. The mean percent recovery over the concentration range is 90.2% with a standard deviation of ±2.5%. The precision was assessed by comparing the standard deviation of the Rt and peak areas of quercetin and kaempferol over the 5 concentrations of quercetin that were used. For each concentration used, three separate samples were prepared and each sample analysed twice to give six results for each concentration.

Table 2.3 shows the results obtained for the collated repeat injections. The upper part of the table shows the mean averages (and s.d.) in retention time (Rt) and peak area obtained for quercetin. The lower half of the table shows the mean averages (and s.d.) in retention time (Rt) and peak area obtained for kaempferol.

Table 2.3 *Variations in retention time and peak area when different concentrations of quercetin are spiked in plasma. Each sample is spiked with internal standard (kaempferol) and the retention time (Rt), standard deviation and COV are taken from duplicate analysis of three individual samples (hence n=6).*

Quercetin

Conc. (μg/mL)	Rt	s.d	COV	peak area	s.d.(n=6)	COV
1	21.4	0.19	0.9	19450	1594	8.2
2	22.4	0.50	2.2	39640	2097	5.3
20	22.6	0.08	0.4	406600	30200	7.4
100	22.4	0.22	1.0	2052000	76660	3.7
200	21.6	0.28	1.3	3551000	235300	6.6

Kaempferol

Conc. (μg/mL)	Rt	s.d.	COV	peak area	s.d. (n=30)	COV
200	28.3	0.57	2.0	4096000	235461	5.7

Individually, both flavonoids are detected with good precision. Mean COV values, over all concentrations, of 1.2% (Rt) and 6.3% (peak area) were obtained for quercetin. Kaempferol displays a COV of 2.0% (Rt) and 5.7% (peak area). However, determination of quercetin is carried out by assessing the ratio of peak areas of quercetin to the internal standard, kaempferol. The COV for the ratio of drug to internal standard was 6.0%, which is well in accepted guidelines(Swartz and Krull 1997).

Interday variation was not assessed. However, the good precision of the Rt is probably due to contemporary chromatographic systems which employ temperature regulation of the column and automated injection of the samples.

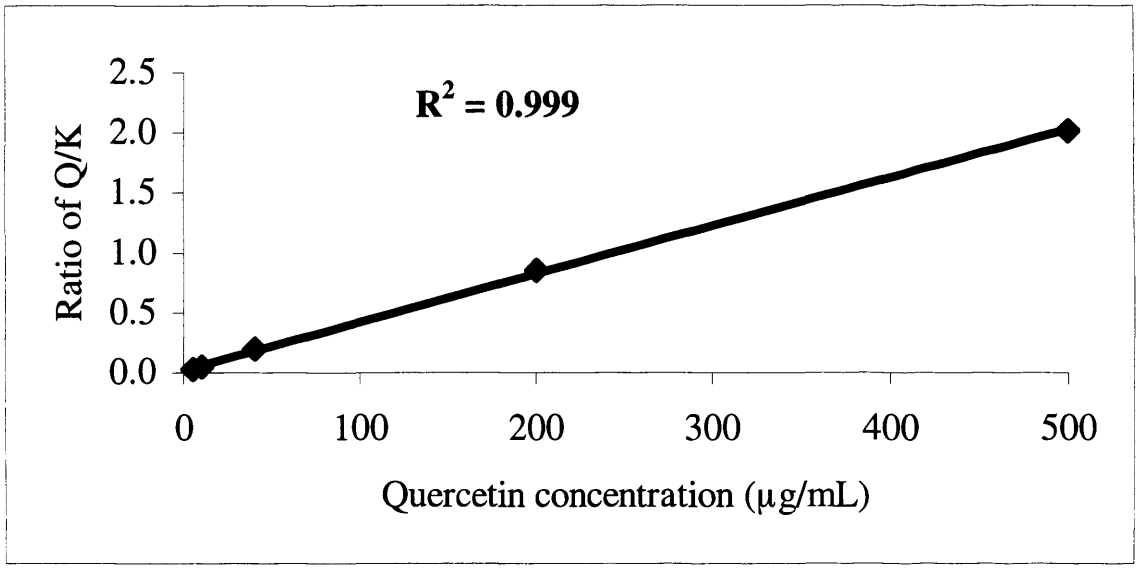
2.3.3.3 Linearity of response.

Linearity was assessed by the creation of a calibration graph using kaempferol as an internal standard (10mg/mL). Kaempferol (4μL of 10mg/mL) was added to 196μL of plasma containing different concentrations of quercetin. Five concentrations of quercetin (500, 200, 40, 10, 5 μg/mL) were assayed against kaempferol. Quercetin and kaempferol were extracted as described in section 2.3.3.1.

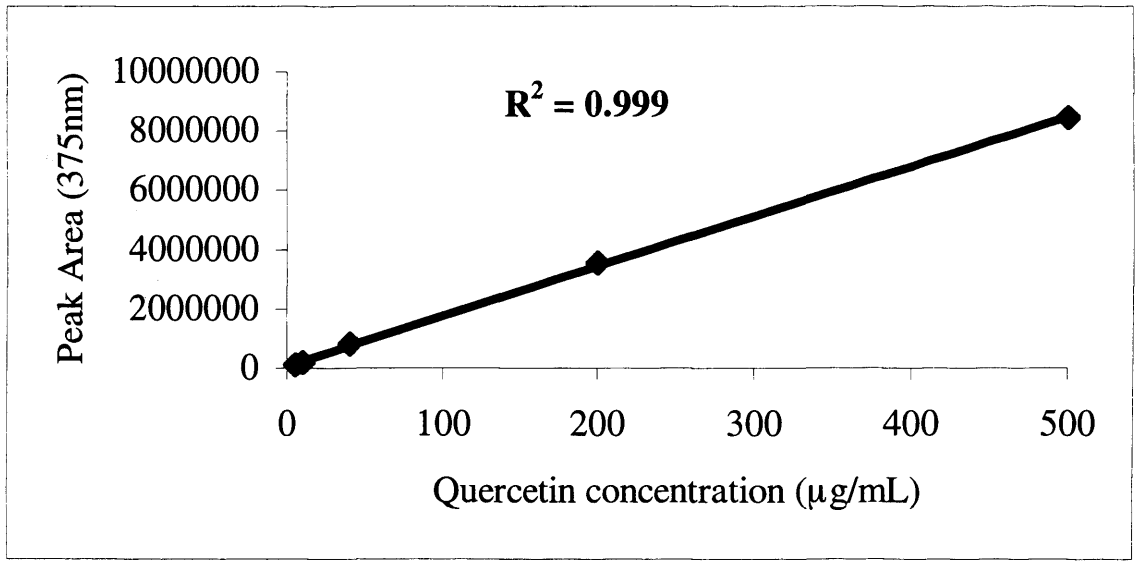
Table 2.4 *The analysis of plasma containing different concentrations of quercetin assayed against an internal standard, kaempferol. The peak area (P.A.) of quercetin is divided by the P.A. of kaempferol for two sets of samples. The mean for each concentration is then evaluated and the average ratio plotted against injected quercetin concentration (shown in Fig. 2.5). Q=quercetin and K=kaempferol.*

Quer conc. (μg/mL)	Quer peak area	Kaemp peak area	Q/K	Quer peak area	Kaemp peak area	Q/K	Mean Q peak area	Mean Q/K
5	107800	4279000	0.025	127500	4345000	0.029	117600	0.027
10	195000	4434000	0.044	205200	4341000	0.047	200000	0.046
40	828600	4163000	0.199	817000	4236000	0.193	823000	0.196
200	3597000	4197000	0.857	3500000	4104000	0.853	3548000	0.855
500	8276000	4096000	2.020	8604000	4280000	2.010	8440000	2.015

Figure 2.5 *Calibration curves for quercetin in plasma with (A) or without (B) the use of an internal standard. In both cases, the linearity is seen to be excellent from 5μg/mL to 500μg/mL with r^2 values in excess of 0.999 are obtained.*



(A)



(B)

Figure 2.5 demonstrates that the method has good linearity from 5.0μg/mL to 500μg/mL with r^2 values in excess of 0.999. Thus demonstrating, that samples elicit a UV-absorbance response that is proportional to the concentration of the analyte.

Additionally, linearity is displayed in the absence of an internal standard (Fig. 2.5 (B)). Using the averaged peak area from five different concentrations of quercetin an r^2 of 0.999 was obtained.

2.3.3.4 Stability of quercetin.

Samples obtained *in vivo* were inevitably going to be stored prior to analysis, thus the stability of quercetin at -20°C and -80°C was assessed. The estimation of the stability of a compound in the chosen storage media and temperature, is an important factor when establishing a reliable analytical method. Stability was assessed by spiking plasma with known concentrations of quercetin. Each concentration of quercetin was prepared in triplicate and each of the triplicates was aliquoted into a number of eppendorfs, to allow repeated analysis over various time points. The peak area in Fig. 2.6 is derived from the mean of the triplicates.

Figure 2.6 *Stability of quercetin in plasma over two weeks at -20°C.*

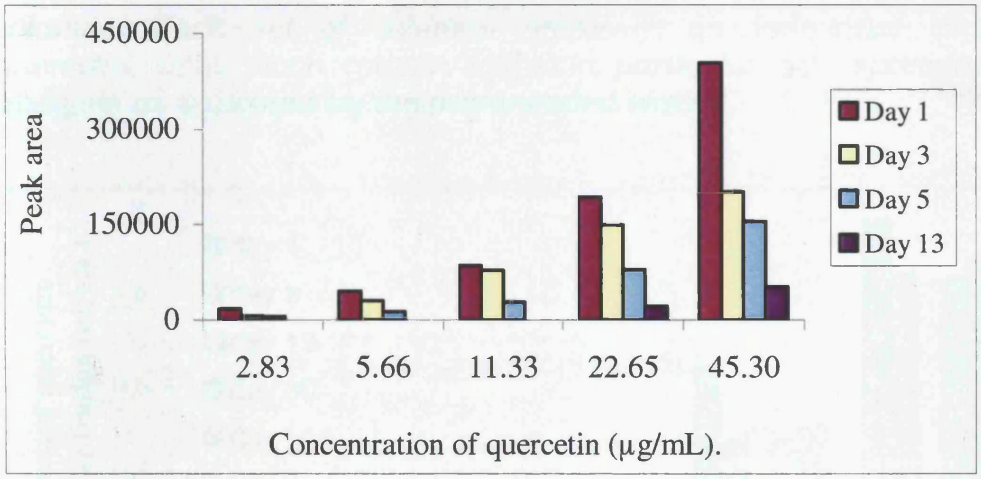


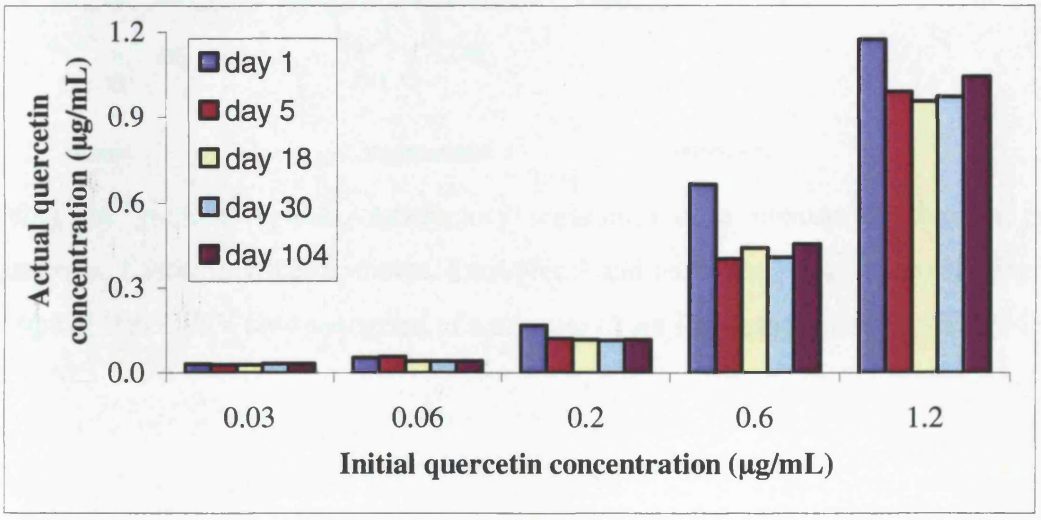
Fig. 2.6 shows that during 13 days, quercetin rapidly degrades in plasma. More specifically, the experiment showed that by two weeks three plasma samples originally containing approximately 11.3µg/mL of quercetin (37.5µM) had deteriorated such that, quercetin was undetectable.

A repeat study was carried out to assess the effect of storage at -80°C. The study was carried out in the presence of the internal standard, kaempferol. Five concentrations of quercetin were made up in plasma giving final plasma concentrations of 1.2, 0.6, 0.2, 0.06 and 0.03 mg/ml. One mL of plasma for each concentration was aliquoted into 10 separate eppendorfs each containing 98µL of spiked plasma. At discrete time points, an aliquot of each concentration was removed from the -80°C freezer and allowed to thaw at room temperature. Two microlitres of 10mg/ml of kaempferol in DMSO was added to each aliquot. The sample was vortexed and 200µl of 1:4 DMSO/MeOH was then added. The extraction solutions were vigorously vortexed, centrifuged at 17060g for 15min, and the supernatant transferred to a

fresh Eppendorf tube. Prior to injection the extractant was diluted 1:1 with deionised water. Fifty microlitres of this aqueous extraction solution was injected onto the column. Shown in Fig. 2.7 is the degradation of quercetin over time.

On day one, fresh plasma was analysed and then stored at -80°C. Subsequent analyses show that quercetin is relatively stable over time when stored at -80°C, with a loss of quercetin after day one, presumably due to the initial effects of freezing. The average loss of quercetin between day one (i.e. prior to freezing) and the mean average of the other days analysis was calculated to be 20%. The difference in levels of quercetin for subsequent analyses showed no particular pattern, with little obvious degradation of quercetin up to day 104, other than the initial loss of 20%. Subsequently, all samples were stored at -80°C.

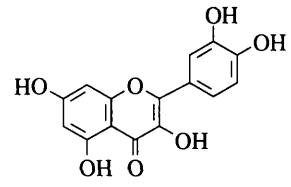
Figure 2.7 *Stability of quercetin stored at -80°C. Shown are 5 sets of columns. Each set of columns represent an individual concentration of quercetin, while each column within a particular set represents the day of analysis as indicated by the colour-coded legend.*



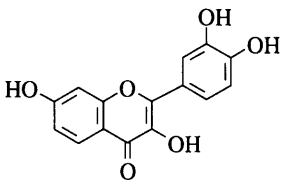
2.3.3.5 Separation of other related compounds.

The method described is suitable for the separation of other flavonoids. The six compounds, seen in Fig. 2.8, are all structurally related flavonoids, with differences existing between the number and position of hydroxyl groups and the absence and presence of the 2,3 double bond.

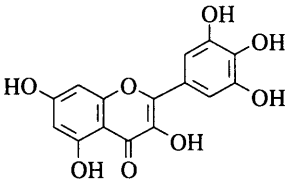
Figure 2.8 *Structures of quercetin and related flavonoids.*



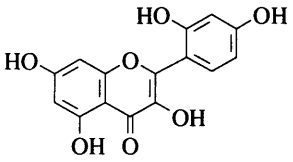
QUERCETIN



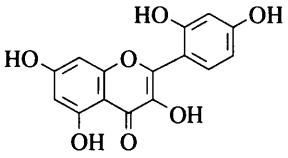
FISETIN



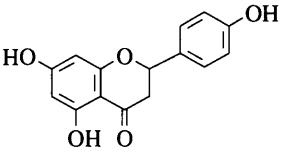
MYRICETIN



MORIN



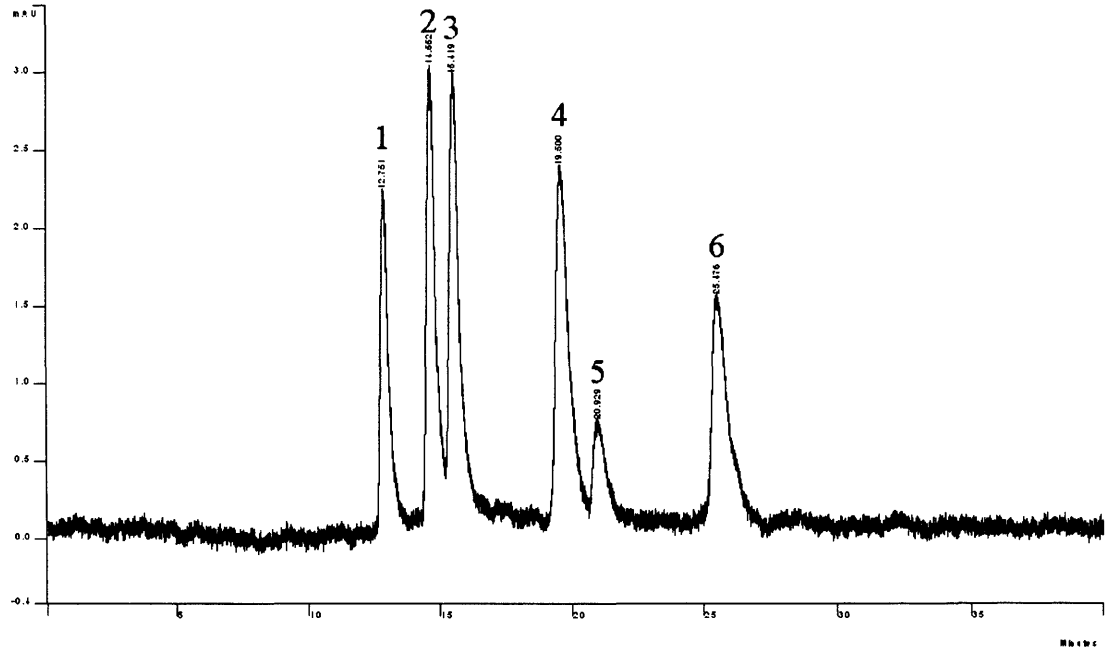
KAEMPFEROL



NARINGENIN

Using the gradient system, satisfactory separation of a mixture of the six polyphenols quercetin, fisetin, myricetin, morin, kaempferol and naringenin was achieved. Fig. 2.9 shows a typical HPLC-UV chromatogram of a mixture of six flavonoids.

Figure 2.9 *HPLC separation of a mixture of flavonoids. Using chromatographic system B. Peaks correspond to 1=morin, 2=myricetin, 3=fisetin, 4=quercetin, 5=naringenin and 6=kaempferol. A Varian HPLC was used as described in section 2.3.2.1 using gradient chromatography as described in section 2.3.2.4.*



2.3.3.5 Changes in retention time.

The project was carried out over 5 years and most of the analyses were carried out on 3-4 HPLC columns. Many factors such as laboratory temperature, mobile phase temperature and protein accumulation on column top affect retention time. Such factors may have contributed to changes in retention time seen in some of the data presented in the next four chapters. Where the same chromatographic conditions were used, differences of upto 4 min were observed. Where temperature regulation was employed, differences in retention time appeared to be minimised suggesting a pivotal role of temperature in the elution of quercetin. The careful choice of controls and using mass spectrometry confirmed the identity of the species detected by UV.

2.4 Mass spectrometry of quercetin.

A mass spectrometer is a device that allows the separation of ions according to their masses. Mass spectrometers have the following elements. Firstly, an inlet through which samples can be introduced. Introduction can be via an injection valve such as a Rheodyne injector, or a syringe pump. Alternatively, a HPLC or GC can be coupled to the mass spectrometer and samples are introduced via capillary tubing. The second element of a mass spectrometer is the source, which is responsible for the ionisation of compounds, which ultimately generates charged species. There are many different forms of ionisation techniques currently in use, including electrospray (ESI), fast atom bombardment (FAB), atmospheric pressure chemical ionisation (APCI), electron ionisation (EI), chemical ionisation (CI), laser desorption, thermospray and plasma desorption. For this project ESI, FAB and APCI were used with the majority of data being collected using electrospray. ESI, FAB and APCI ionisation are considered to be less destructive ionisation techniques (consequently, referred to as 'soft' ionisation techniques) and thus they are considered to be more applicable to characterisation.

The next element of a mass spectrometer is one or a number of analysers which are responsible for the separation of charged species according to their mass. Sector instruments incorporate an electro-magnetic field while quadrupole mass spectrometers incorporate a combination of radio-frequency and direct current fields.

Further analysers are used if characterisation of specific molecules is required. Ions can be selected from the first analyser and repelled into a collision cell, where they are fragmented using a neutral gas such as helium, argon or xenon. Product ions are then selected and repelled towards a second analyser, where the individual product ions are separated. The ions are then sequentially focused at a single point - the detector. In the case of the Quattro Bio-Q mass spectrometer, the second analyser is a quadrupole and the collision cell is a radio-frequency only hexapole.

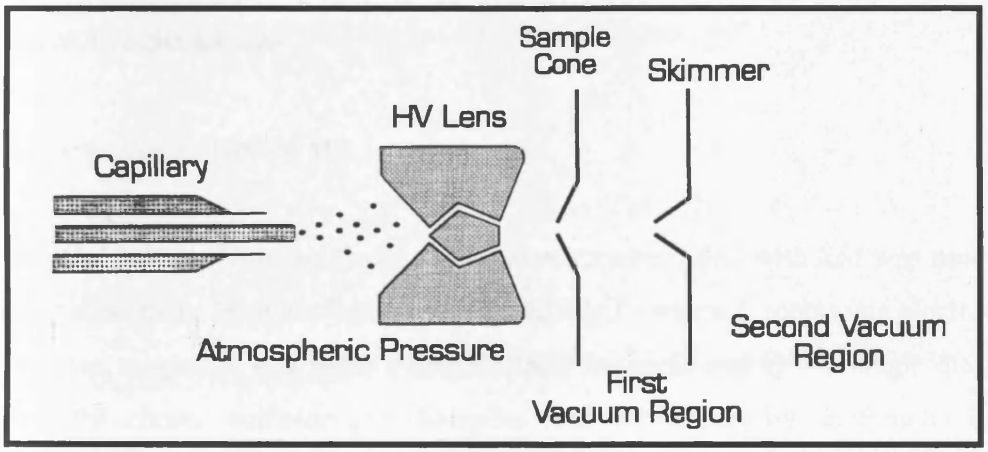
2.4.1 Electrospray ionisation.

The process of electrospray is simple in theory. An electrospray is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a low flow rate. The Quattro Bio-Q instrument has pneumatically-assisted electrospray

(pn-ESI) incorporated into it, which is when the capillary is surrounded by a concentric sheath arrangement, that allows a flow of nitrogen gas, hence, aiding nebulisation (see Fig. 2.10). The electric field is obtained by applying a potential difference of 3-6kV between the capillary and the counter electrode, separated by 0.3-2.0 cm. This field induces a charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets.

The electric field and the sheath of gas combine to create a mist of highly charged droplets, which pass down a potential and pressure gradient towards the analyser portion of the mass spectrometer. During this passage, the solvent contained within the droplets evaporates, causing them to shrink to the point where the repelling coulombic forces come close to their cohesion forces, thereby resulting in their explosion.

Figure 2.10 *The typical configuration for pneumatically assisted electrospray.*



The droplets then undergo a cascade of ruptures, yielding smaller and smaller droplets until the electric field on their surface becomes large enough to produce the desorption of the ions(Gaskell 1997a). The ions that are obtained carry a great number of charges if several ionisable sites are present on the molecule(Gaskell 1997a).

The electrospray technique has made an enormous contribution to modern MS methodology, because it provides a logical coupling of solution introduction of compounds of analysis; and secondly, it allows ionisation of highly polar and involatile compounds(Gaskell 1997b).

2.4.2 Mass spectrometry of quercetin and metabolites in extracts.

Four mass spectrometric systems were utilised:

Routine LC-MS and LC-MS/MS.

VG Quattro Bio-Q tandem quadrupole mass spectrometer fitted with a pneumatically assisted electrospray (pnESI). The configuration of the mass spectrometer utilised a hexapole collision cell in between two quadrupoles in 'hQ1hQ2' geometry; where Q1 describes the first quadrupole, h describes the hexapole gas collision cell and Q2 is the second quadrupole analyser. Following an upgrade of the mass spectrometer, a hexapole was fitted to the front-end which allows greater targeting of the ions into the first quadrupole. Argon was used as the collision gas. On-line LC-MS was performed, using the Varian system (described above in 2.3.2.1.) and a capillary flow splitter (1:7) which allowed approximately 142μL of column eluant into the source.

Electrospray ionisation MS.

A VG Autospec Ultima-Q hybrid mass-spectrometer fitted with ESI was used in the negative ionisation mode. The configuration was 'EBEhQ'; where E represents electrostatic analysers, B is the magnet, h is a radio frequency-only hexapole and Q is a single quadrupole. Argon, was the chosen collision gas. Samples were introduced by continuous infusion using a Harvard syringe pump.

High-resolution tandem FAB-MS.

VG 70 SEQ hybrid mass-spectrometer using Fast Atom Bombardment (FAB) ionisation. The FAB matrix used was glycerol. Tandem mass spectrometry was obtained using the 'EBqQ_2' configuration; where E is an electrostatic analyser, B is the magnet and q is a radio frequency-only quadrupole that acts as a collision cell. Q_2 acts as a second quadrupole analyser for the detection of product ions.

LC-MS with ESI and APCI.

Micromass Platform single quadrupole mass spectrometer with ESI or APCI, both in the negative mode, were used for LC-MS analysis. The geometry of this mass spectrometer was the simplest having 'Q' geometry; where Q is the single quadrupole. Samples were introduced into the source via direct injection, continuous infusion or via on-line liquid chromatography using a Hewlett Packard Series 1100 HPLC system.

2.4.3 Optimisation of mass spectrometric conditions.

In respect to all mass spectrometric devices used, at the start of each experimental session, the instrument was fine tuned using a quercetin standard, typically containing 10pg/μl quercetin in 50/50 (v/v) methanol and water.

Optimisation of specific mass spectrometric conditions was mainly carried out on the Quattro Bio-Q mass spectrometer. Modes of ionisation, polarity of ionisation, source temperature, cone voltage were all varied to assess ultimate improvements in sensitivity. Mass spectrometric parameters of the two analysers (i.e. MS1 and MS2) were also altered to improve sensitivity. These conditions formed a 'tunepage-spine', around which the mass spectrometric conditions could be altered on a daily basis, to effectively fine-tune the mass spectrometer.

Tandem mass spectrometry experiments.

The majority of tandem mass spectrometry (MS/MS) experiments were carried out on the VG Quattro Bio-Q instrument. In general, three types of experiment were carried out using tandem mass spectrometry.

(a) The first consisted of selecting an ion from the first quadrupole. The selected ion was then dissociated in the hexapole collision cell, whereupon a scan was carried out in the second quadrupole. This type of experiment is termed a product ion scan.

(b) A second type of experiment was carried out where an ion was selected in the first quadrupole, and subsequently introduced into the collision cell. The second quadrupole was

then set to transmit one product ion. This type of experiment is termed multiple reaction monitoring (MRM).

(c) The third experiment, mainly used in the elucidation of the dissociation mechanism, involved subjecting the precursor ions to 'high' cone voltage in the source, thus promoting fragmentation, a process termed in-source collision induced (or activated) dissociation (CID (or CAD)). The resultant fragment ions were then introduced into the hexapole collision cell via selection from the first mass spectrometer, and subjected to further dissociation. The resultant product ions were then analysed in the second mass spectrometer (MS2). Typically, a product ion scan was carried out in MS2.

2.4.4 Development and optimisation of mass spectrometric techniques for the detection of quercetin.

Initial work was carried out using the separating power of the previously described HPLC method, collecting fractions and analysing the concentrated fractions via direct injection- or direction infusion- mass spectrometry. For these instances, the Autospec instrument was used and all optimisation was carried out using a quercetin standard.

However, the majority of analyses were carried out on the Quattro Bio-Q mass spectrometer, with some samples being analysed on the VG Platform.

2.4.4.1 Optimisation of Quattro Bio-Q mass spectrometric conditions.

On the Quattro Bio-Q mass spectrometer, electrospray was the chosen method of ionisation.

Atmospheric pressure chemical ionisation (APCI) was also assessed when using the Platform mass spectrometer. APCI is a useful ionisation technique that theoretically demonstrates greater sensitivity as it can inherently deal with higher flux. The technique differs from ESI in that contained within the source is a corona pin, where primary ions are produced by corona discharges on a solvent spray.

Evaluation of a quercetin standard showed that sensitivity was approximately 20 times greater using APCI instead of ESI when attached to LC. APCI achieved a sensitivity limit of

approximately 1.5ng injected onto the column. However, APCI appeared to have reduced sensitivity as a consequence of interference derived from biological samples, which could be due to the particular geometry of the Platform mass spectrometer.

The Quattro Bio-Q mass spectrometer was not equipped with APCI capability; thus, APCI could not be assessed for its tandem mass spectrometric capability. The polarity of the ionisation mode had to be established. Direct infusion was used to assess whether or not quercetin would be ionised in ESI negative or positive modes. Typically, positive ion ionisation can increase sensitivity compared to negative ion analysis due to negative ion ionisation inherently producing fewer ions. Quercetin has five hydroxyl groups, which can lose protons. The molecule has few sites for the addition of protons and as a result, it is likely that quercetin would preferably ionise in the negative mode. When a quercetin standard was placed into the source and analysed for comparison between electrospray positive and negative modes, it was quite clear that negative ion ionisation gave a much greater signal than when using positive mode. Consequently, negative ion electrospray ionisation was used as the preferred mode of ionisation.

Tune page settings initially gave an optimised quercetin molecular ions at m/z 301 $[M-H]^-$ using the conditions shown in Table 2.5. These conditions were always used at the start of any analysis. Fine-tuning was centred on these core values.

Table 2.5 *Tune page settings.*

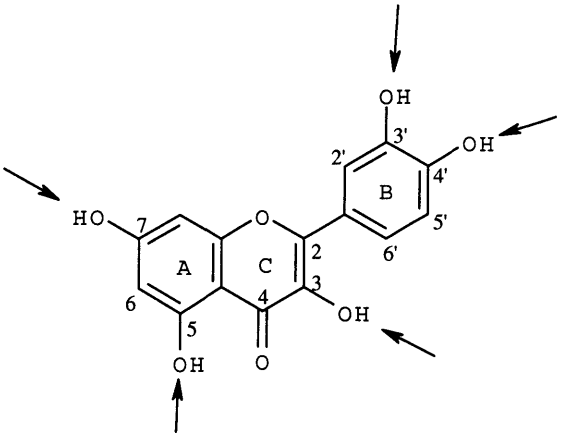
Parameter	Unit	Value
Capillary	kV	3.78
HV lens	kV	0.20
Cone		46.0
Skimmer O/S		5.0
Skimmer		0.9
RF lens		0.4
Source temperature	°C	110

2.4.5 Development of tandem mass spectrometry for characterisation of quercetin metabolites.

The characterisation of a molecular species, such as quercetin, in a mass spectrometer after negative-ion electrospray ionisation is often difficult to ascertain. In the following work, the fragmentation patterns were obtained by using a quercetin standard that is not isotopically labelled. In the absence of such a labelled compound, it is difficult in the case of quercetin to ascertain the exact position of ionisation. The quercetin molecule is polyhydroxylated and polyphenolic. The five hydroxyl groups are all candidates for the removal of a proton, which would facilitate negative ion ionisation (see Fig. 2.11).

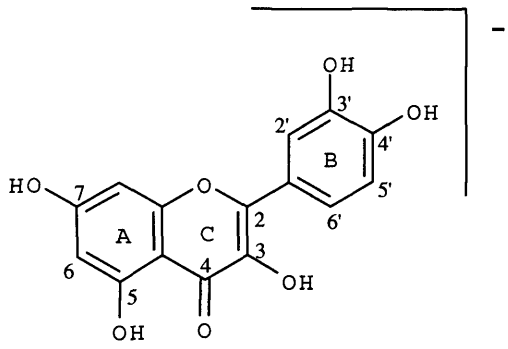
All five hydroxyl groups, as indicated by the arrows in Fig. 2.11, have the potential to be ionised. The hydroxyl groups on both the catechol and resorcinol rings (ring B and A respectively, in Fig. 2.11) could become ionised. Stabilisation could occur through the benzene rings and the other hydroxyl group. The remaining hydroxyl group (i.e. 2-C position) could also conceivably form a stable ion through the electron stabilisation of the pyran ring and the ketone group. When these sets of experiments were initiated, it was thought that the fragment ions would allow the characterisation of the quercetin molecule, and indicate the site of ionisation.

Figure 2.11 *Potential sites of ionisation on the quercetin molecule.*



The exact site of ionisation could not be deduced in the absence of isotopically labelled quercetin. Consequently, in the following section, the quercetin molecule will be designated with an overall negative charge (Fig. 2.12).

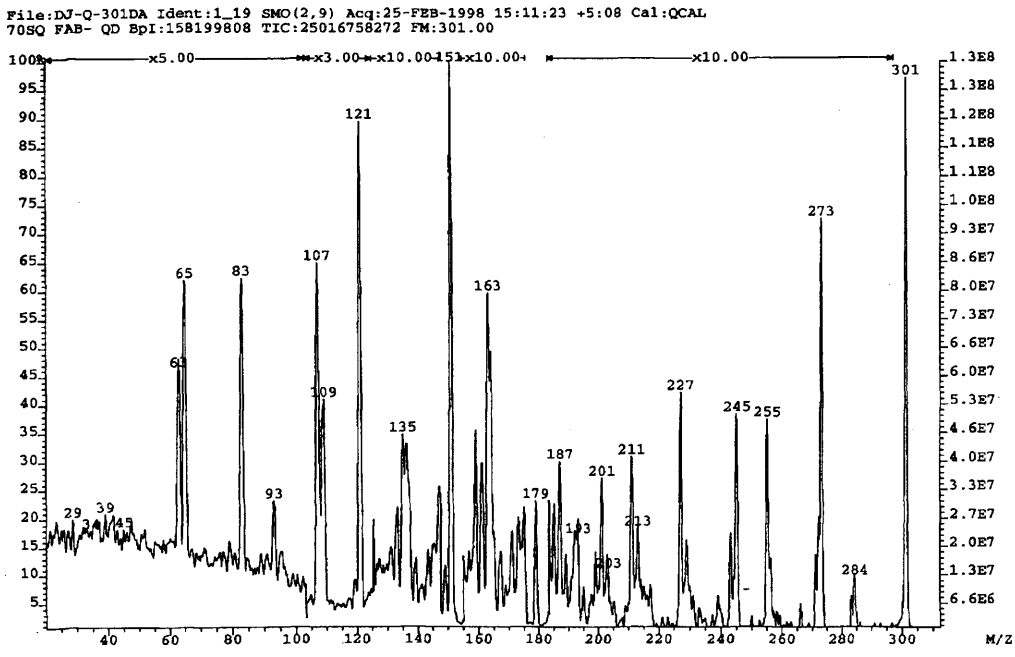
Figure 2.12 *Ionised forms of the quercetin molecule*



In order to facilitate structural characterisation of quercetin metabolites, it was important to establish the fragmentation pathway of a quercetin standard. MS/MS of quercetin was executed to yield structural data that would act as a template for characterisation of quercetin metabolites, via comparison of the fragments obtained, as is standard procedure when deriving structural information of unknown compounds.

For comparative purposes, initial MS/MS analysis was carried out on quercetin using the VG 70-SEQ. Fast Atom bombardment (FAB) ionisation was employed to derive alternative source product ions. FAB is considered a harsher ionisation technique due to the increased energy input into the source. The FAB spectrum obtained is shown in Fig. 2.13.

Figure 2.13 *Mass spectrum of quercetin product ions obtained via negative ion fast atom bombardment (FAB). Collision energy set at 45eV, collision gas=air, and attenuation set at approx. 60%. Product ion scan from m/z 20-310. Data obtained from a VG 70 SEQ hybrid mass-spectrometer as described in section 2.4.2.*

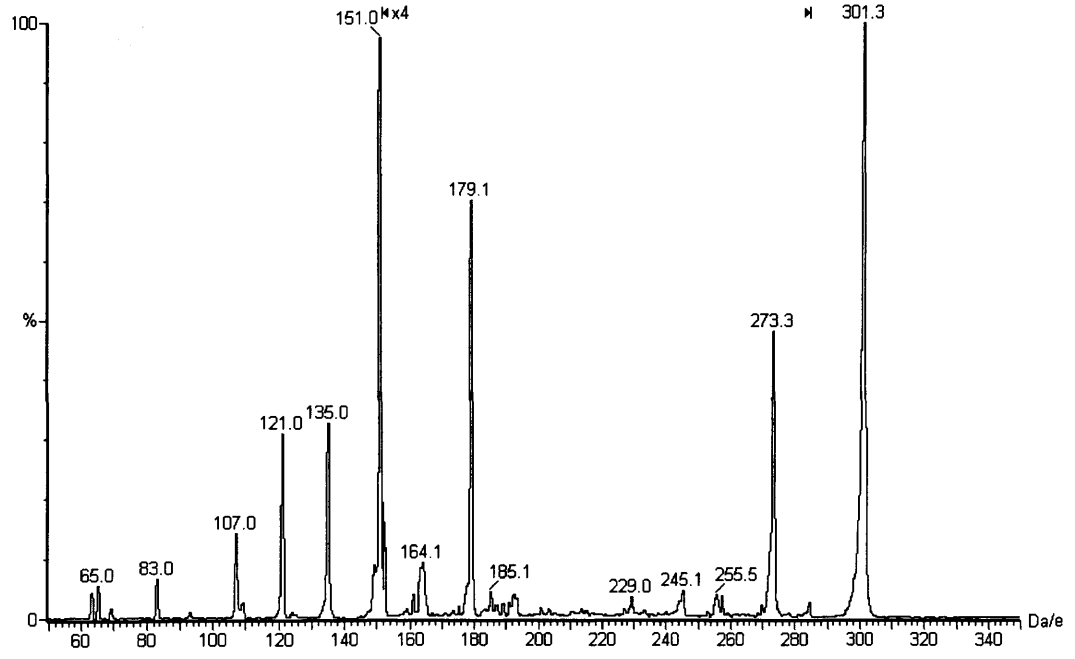


Shown in Fig. 2.13 are a number of highly resolved ions attributable to the quercetin molecule when ionised under FAB conditions.

A typical MS/MS spectra of quercetin obtained on the Quattro Bio-Q instrument using negative-ion electrospray ionisation is shown in Fig. 2.14. The FAB product ion spectrum shows a larger number of fragments that are probably produced as a result of FAB being a higher energy, or 'harder', ionisation technique than ESI, thus resulting in increased dissociation. However, in both cases there are product ions with $[M-H]^-$ at m/z 273, 255, 245, 227/229,179, 163/164, 151, 135, 121, 109, 107, 83, 65, and 63. An interesting feature is the close homology between the product-ion spectra produced for both ESI and FAB. Considering the different mechanisms involved in ionisation, the products ions arrived at are remarkably similar.

Deriving the structural nature of each of the product ions can be difficult to assess, especially considering that the exact source of each product ions is not alluded to solely by its m/z value. Thus, it is important to assess whether product ions are derived from a single dissociation pathway, or as a consequence of a number of dissociation pathways.

Figure 2.14 *Mass spectrum of quercetin product ions obtained via negative ion electrospray ionisation. Collision energy 42eV using argon as collision gas. Product ion scan from m/z 50-350. Experiment executed on a Micromass Quattro-BioQ instrument as described in 2.4.2.*



Essential to the characterisation of any putative quercetin metabolite was the complete structural characterisation of the quercetin molecule. Quercetin underwent in-source collision-

induced dissociation (CID) (or collision activated dissociation (CAD)), and then individual product ions underwent further fragmentation via introduction to a hexapole collision cell and the product ions generated were analysed in the second mass spectrometer. This set of experiments allowed direct assessment of secondary dissociation.

Table 2.6 shows the secondary product ions generated when quercetin product ions from CID underwent further fragmentation via a hexapole collision cell. This data allows greater scope for interpretation and consequently confidence in the mechanisms of fragmentation.

Table 2.6 *Secondary products ions obtained for primary product ions subjected to MS/MS in a second mass spectrometer. These data were obtained on the Quattro Bio-Q mass spectrometer.*

Primary fragment ion (m/z) produced 'in source'.	**Product ions (m/z) produced in a hexapole collision cell.**
301	273, 255, 245, 227/229,179, 163/164, 151, 135, 121, 109, 107, 83, 65, 63
273	245,163,151,121,95,83 and 65
179	151,65,41
151	65,41
135	69
121	41

Table 2.6 shows that quercetin (m/z 301) fragments to all the ions mentioned previously. As m/z 245 is found in both the product ion spectra for m/z 301 and m/z 273, it is likely that m/z 245 arises as a consequence of secondary fragmentation of m/z 273. m/z 163 is also derived from m/z 273, while m/z 255 is unlikely to be a secondary product of $[M-H]^-$ ion at m/z 273 due to its absence in the m/z 273 product ion spectra. m/z 255 is probably derived directly from $[M-H]^-$ at m/z 301.

Scheme 2.1 Dissociation pathway of quercetin to m/z 83, 164, 245 and 273. *The structures contained within brackets indicate the formation of an unstable intermediate. Also, when two routes of fragmentation are proposed, the colour of bond breaks indicates the route of fragmentation.*

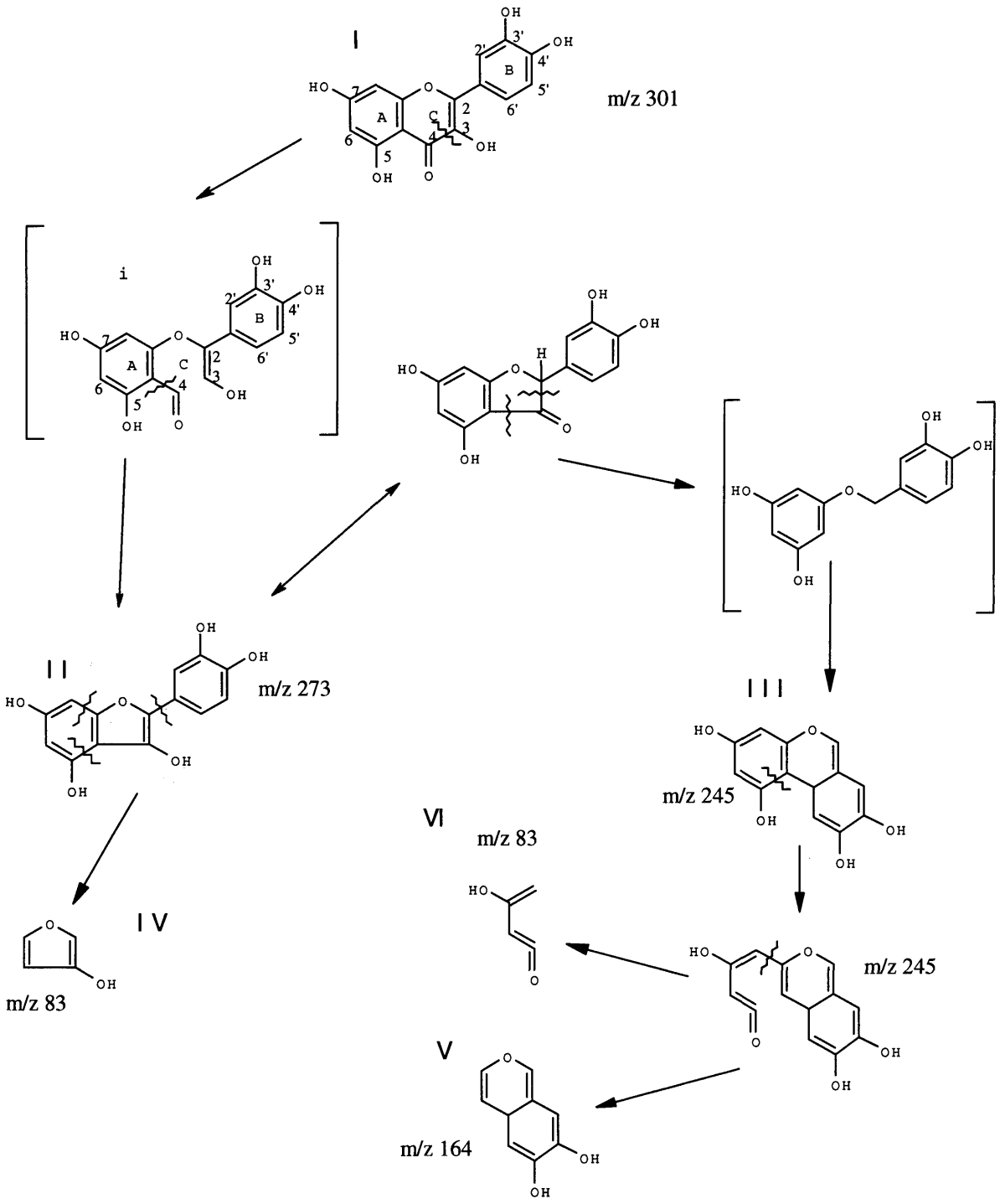
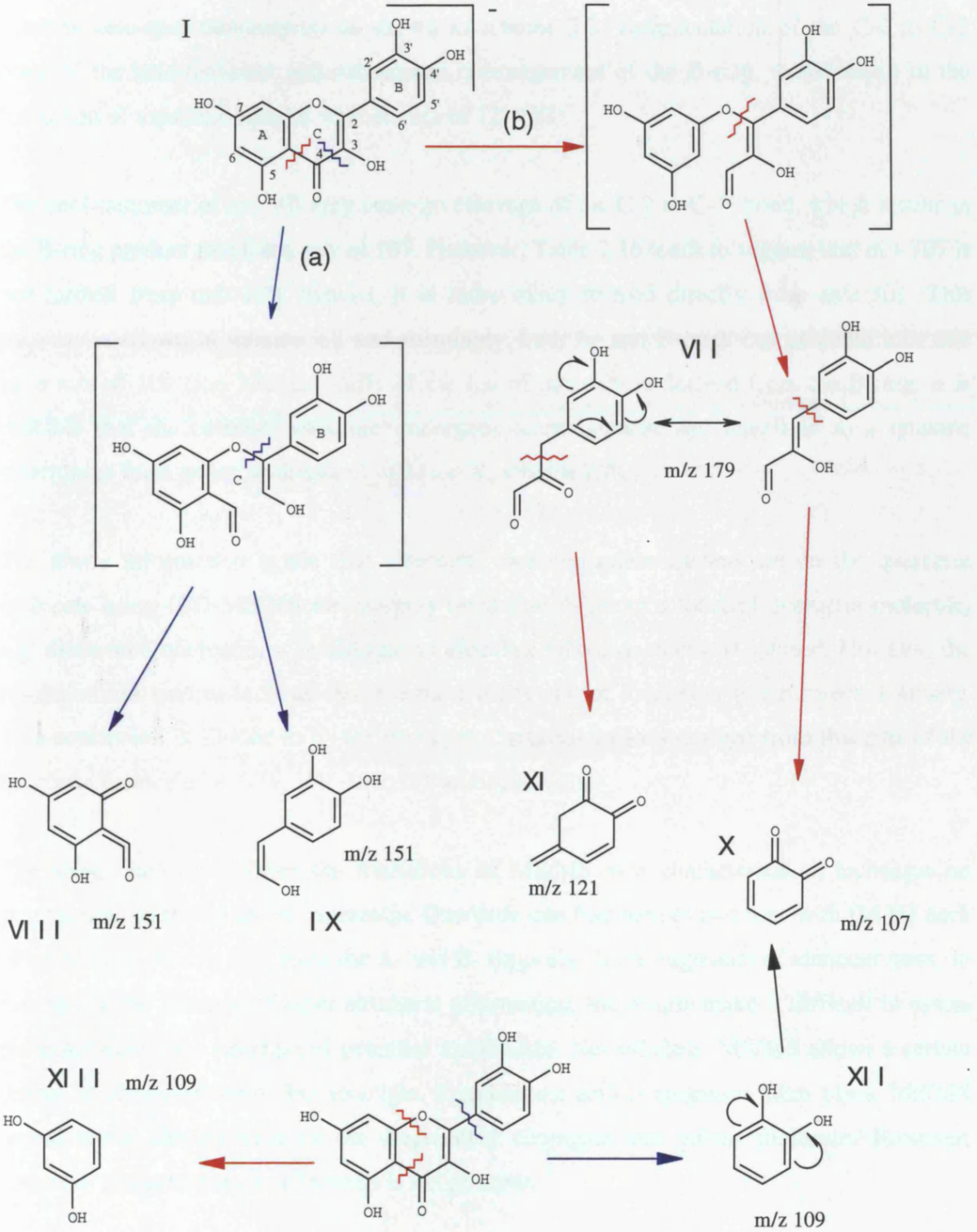


Fig. 2.14 shows a number of ions derived from the quercetin molecular ion (m/z 301). Using ESI-, m/z 273 is an abundant ion which is likely to be formed by an initial bond cleavage between C-3 and C-4 followed by cleavage between C-4 and C-10 giving rise to the loss of a CO moiety. This mechanism is shown in scheme 2.1. Ion I (scheme 2.1; m/z 301) leads to the formation of ion II (scheme 2.1; m/z 273) which would necessitate the formation of an intermediate with an m/z 303 (I in scheme 2.1). This proposed intermediate ion was not found in the fragmentation pattern of quercetin when using either FAB or ESI. However, this may be an indication of the inherent instability of the proposed ion.

Ion II (m/z 273) may also exhibit keto-enol tautomerism which through cleavage of the C-2 to C-3 and C-3 to C-10 bonds might furnish an intermediate which undergoes cyclisation to ultimately form ion III, which has an m/z of 245. Furthermore, ion III through cleavage of the bond lying between C-10 and C-5 and then the bond between C-8 and C-9 would produce two ion species. Ion VI (scheme 2.1) has a m/z of 83 while ion V has a m/z of 164. It is also quite conceivable that the m/z 83 ion is derived more directly from the m/z 273. This pathway as shown in scheme 2.1, requires the cleavage of three single bonds to form ion IV.

The most abundant product ion derived from quercetin is that of m/z 151 which is the predominant ion in both FAB and ESI. Quercetin, when fragmented, may cleave either side of the C-4 ketone group. In either case it is likely that an unstable intermediate is formed. Route (a), shown in scheme 2.2 would lead to the formation of an intermediate ion that was postulated to be formed prior to the formation of ion II (m/z 273) in scheme 2.1. This intermediate could also fragment following cleavage of the pyran-O and C-2 bond as shown in scheme 2.2. This fragmentation would result in one of two theoretically possible ions (VIII or IX). Cleavage of the pyran-O-C2 bond would yield the B-ring product attached to a CHCHOH moiety giving a ion with an m/z of 151 (ion IX). Alternatively, an A-ring product containing a carbonyl group and a ketone group (ion VIII) could also be formed as a consequence of such a bond breaking, which would also give an m/z of 151.

Scheme 2.2 Dissociation pathway of quercetin to m/z 179, 151, 121, 109 and 107. *The structures contained within brackets indicate the formation of unstable intermediates. When two routes of fragmentation are proposed, the colour of bond breaks indicates the route of fragmentation.*



Ion VII in scheme 2.2 (m/z 179) is derived straight from m/z 301. It is not obtained as an intermediate from m/z 273 or 245. Ion VII is formed by the cleavage of the C-4 and C-10 bond. The unstable intermediate formed undergoes a further round of cleavage between the pyran-O and C-2 bond, which yields the m/z 179 product ion. It is conceivable that ion VII exhibits keto-enol tautomerism as shown in scheme 2.2. Fragmentation of the C-2 to C-3 bond of the keto-tautomer and subsequent rearrangement of the B-ring, would result in the formation of a quinoid species with an m/z of 121 (XI).

The enol-tautomer of ion VII may undergo cleavage of the C-2 to C-1' bond, which results in the B-ring product that has a m/z of 107. However, Table 2.16 tends to suggest that m/z 107 is not formed from m/z 179. Instead, it is more likely formed directly from m/z 301. This pathway is shown in scheme 2.2 and seemingly, both A- and B-rings can generate ions that have m/z of 109 (ion XIII and XII). If the ion of m/z 109 is derived from the B-ring, it is possible that the catechol structure undergoes rearrangement and oxidation to a quinone structure to form an ion with m/z of 107 (ion X, scheme 2.2).

The above information is the first structural characterisation carried out on the quercetin molecule using CID-MS/MS electrospray ionisation. Without a labelled quercetin molecule, e.g. deuterated quercetin, it is difficult to elucidate where quercetin is ionised. However the results would tend to indicate that the most likely site of ionisation is the catechol moiety. This conclusion is alluded to by the prevalent formation of ions derived from this part of the quercetin molecule i.e. 273, 179, 151, 135 and 109.

The above data exemplifies the limitations of MS/MS as a characterisation technique on polyphenolic compounds like quercetin. Quercetin can fragment to two ions with $[M-H]^-$ each of equal mass, at m/z 151. Both the A- and B- rings also form fragments of identical mass, In essence, in the absence of other structural information, the results make it difficult to assess the exact molecular structure of potential metabolites. Nevertheless, MS/MS allows a certain degree of characterisation. For example, if glucuronic acid conjugation takes place, MS/MS would allow identification of the fragmented conjugate and parent molecule. However, structural characterisation of isomers is not possible.

2.5 Nuclear magnetic resonance spectroscopy (NMR).

2.5.1 Principles of NMR.

Nuclear Magnetic Resonance spectroscopy (NMR) is a technique used to elucidate molecular structures that utilises the fact that certain nuclei possess a magnetic moment. When placed in a magnetic field these nuclei align either with or against the applied field.

Nuclei are composed of protons and neutrons, the former carrying a positive charge and the latter being electrically neutral. If there is mechanical spin and electric charge a nucleus will behave like any spinning charged object and produce a magnetic field.
Magnetic properties occur when the nuclei have a) odd atomic number and odd mass number, b) even atomic number and odd mass number and c) odd atomic number and even mass number. For nuclei that have both mass and atomic number even it is assumed that the particles in these atoms are paired, i.e. spinning in the opposite directions. This results in no overall spin and giving no magnetic moment.

The nuclei that possess spin behave as spinning magnets, and will orient themselves in a magnetic field. The simplest example is the proton, here I =1/2, and due to quantum restrictions it can only align itself with (low energy) or against (high energy) an applied field.

As a result of the proton behaving as a spinning magnet it can not only align with or opposed to the field but it will also move in characteristic way under the influence of an external magnet. This movement is called precessional motion and is proportional to the strength of the applied field. If a proton is precessing in an aligned orientation it can absorb energy to achieve the higher energy state. The energy is provided by the application of a radio-frequency source.

When the frequency of the precessing proton and the radio-frequency beam are the same the energy is absorbed and this is described as resonance. This energy change can be monitored by the NMR and converted into spectra.

NMR produces spectra from which structural information can be gleaned. The scale used is chemical shift and measured in parts per million (ppm). There are regions of these spectra

characteristic of certain types of proton. For example primary alkyl groups are generally found at a chemical shift of between 0 and 3ppm, secondary groups at 3 to 5 ppm etc. Different substituents can either shield the proton from the effects of the field or deshield. This has an effect on the shift of the protons, moving the peaks either upfield or downfield.

More information is available, as the integration of the peaks is proportional to the number of protons in the group. The most useful data is gathered as a result of spin-spin splitting. Effectively the groups surrounding a proton have an effect on its magnetic environment, leading to the peak being split into doublets, triplets etc.

Splitting patterns are given by the n=1 rule, if a peak is assigned to a particular proton it can be split by an adjacent system in this manner. For example, a CH_2 group (n=2) will result in the neighbouring group being seen as a triplet. The size of the split, the J value is again a useful guide to the structure.

Many more types of experiment are available; correlation spectroscopy (COSY) is a two-dimensional method that shows through-bond connections, Nuclear Overhauser Effect Spectroscopy (NOESY) shows effects through space.

2.5.2 Application of NMR.

NMR was used for the structural determination of sulphate conjugates of quercetin synthesised by the addition of quercetin to triethylamine-sulphate (TEA) which in the absence of air is a strong sulphate-donating group. The protocol for the synthesis and the subsequent NMR analysis is found in chapter three.

2.5.3 NMR instrumentation.

Bruker ARX 250 (Bruker, UK) 250MHz.

2.5 Discussion.

The HPLC method for the detection of quercetin in human plasma has been demonstrated to be sensitive and specific. The assay was found suitable for monitoring drug and metabolites in patient plasma.

The HPLC separation system provided satisfactory resolution and robustness for detection by UV, ECD and MS. UV was the detection mode of choice for quick analysis, however, in cases where sensitivity was an issue, electrochemical detection was used, as it provided a forty-fold increase in sensitivity.

Mass spectrometry is a useful tool for the analysis of quercetin metabolites, with electrospray negative-ion ionisation proving to be satisfactory for the identification of metabolites. Sensitivity, in terms of mass spectrometric detection, was limited due to quercetin ionising only in negative-ion mode.

This chapter has set out a number of approaches to analysing quercetin which allow sensitive, selective and robust detection. Also described are a series of MS/MS experiments designed to allow characterisation of quercetin. The complete structural characterisation of quercetin was not achieved by MS due, in part, to the structural similarities exhibited by different parts of the molecule.

The theory behind NMR is explained in order to allow characterisation of the sulphate conjugates synthesised in chapter 3.

3.0 Chapter Three. Metabolite identification and characterisation.

3.1 Introduction.

Previous investigations into the identification of quercetin metabolites have used hydrolysis of the conjugates by enzymatic means (see section 1.5)(Hollman and Katan 1997; Piskula and Terao 1998). Full characterisation of individual intact metabolites has not been carried out. In some studies, the position on the quercetin molecule at which it undergoes glucuronide conjugation, using *in vitro* methods, has been assessed(Day et al. 2000; Oliveira and Watson 2000).

The aim of this chapter is to contribute to the identification and characterisation of metabolites generated from quercetin in hepatic fractions *in vitro* and intact rodents.

3.2 Experimental.

3.2.1 Materials and reagents.

Quercetin, HPLC grade dimethyl sulphoxide (DMSO), ammonium acetate, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dithiothreitol (DTT), magnesium chloride, dimethyl sulphide (DMS), S-adenosyl methionine (SAM), pyridine, dioxane and porcine liver catechol O-methyl transferase (COMT), bovine serum albumin (BSA), sodium carbonate, sodium hydroxide, sodium-potassium tartrate solution, copper sulphate solution, phenol reagent, HEPES buffer were all obtained from Sigma (Poole, UK). Disodium ethylenediamine-tetraacetic acid (EDTA), AnalaR grade was obtained from BDH (Poole, Dorset, UK). Triethylamine sulphate (SO_3-TEA), methanol (HPLC grade) and glycerol formal from Fisher (Loughborough, Leics., UK). Isorhamnetin and quercetin 3-O-sulphate was obtained from Extrasynthese (Genay, France).

3.2.2. Metabolites of quercetin in hepatic fractions.

Preparation of hepatic fractions.

F344 rats were killed by cervical dislocation, the livers excised and transferred to ice-cold

1.15% potassium chloride (KCl). The liver (3g) was placed in buffered sucrose (27mL) (0.25M sucrose, 10mM TRIS, 1mM EDTA with pH adjusted to 7.4 with 1M HCl), and homogenised with 10 slow passes at 1100rpm using a Potter homogeniser. The homogenate was centrifuged at 10,000g for 20min at 4°C and the lipid layer was removed. The supernatant, which represents the S9 fraction, was decanted to polycarbonate tubes. The S9 fraction was then centrifuged at 100,000g for 1h. The supernatant (cytosolic fraction) was removed. KCl, 1.15% (5mL) was added to the pellet, which was resuspended using a Teflon homogeniser. The resuspended homogenate underwent a further 100,000g centrifugation step, after which the supernatant was decanted and the pelleted microsomes were resuspended in glycerol and 0.25M phosphate buffer, pH 7.4 (30/70, v/v).

Incubations.

The protein content was determined in fractions by Lowry's method(Lowry et al. 1951).

Lowry's protein determination:

Solutions:

- Bovine serum albumin (BSA), 1mg/mL in distilled water (dH_2O).
- Lowry solution (Na_2CO_3 (60g) and 10M NaOH (20mL) and made up to 2L with dH_2O).
- Solution A- 4% Sodium potassium tartrate solution.
- Solution B- 2% Copper sulphate solution.
- Phenol reagent (1:1 phenol with dH_2O).
- Solution C (Lowry solution (60mL) + solution A (0.6mL) + solution B (0.6mL)).

The standard curve was created with 5 tubes containing zero, 0.02, 0.05, 0.1 and 0.2mL BSA, made up to 0.5mL with dH_2O. Samples (0.5mL) were typically diluted 1/20 in distilled water. To each tube 5mL of solution C was added, vortexed and left to incubate for 10min. Phenol reagent (0.5mL) was added, tubes vortexed and allowed to incubate for 10min. For all the tubes, the absorbances were then read at 660nm.

Protein concentration of hepatic cellular fractions in all experiments was 1mg/mL of incubation mixture.

Incubation solutions:

- Incubation buffer – HEPES (20mM) in 1.15% KCl at pH 7.4.
- $MgCl_2$ (1mM in final incubation mixture).
- NADPH generating system sufficient for 20 tubes – NADP (8.16mg) and glucose-6-phosphate (48.7mg) and glucose-6-phosphate dehydrogenase (240µL of 2500units/mL) in 1.76mL of incubation buffer.
- Quercetin (1mM) in DMSO.

Incubations were carried out by adding quercetin (final concentration, 10µM), $MgCl_2$ (final concentration, 1mM) and the NADPH-generating system (100µL) to the cellular fraction (1mg of protein per mL). The final volume of the incubation mixture was 1mL. The three types of fraction (S9, cytosolic and microsomal) were incubated with or without quercetin. Control incubations contained fractions that had been boiled for 10min prior to incubation with quercetin. All tubes were incubated for 1h at 37°C. Following incubation, protein was precipitated using 1:4 (v/v) DMSO/methanol (400µL) and 20% TCA (100µL) solutions. The extraction mixture was then vortexed and centrifuged for 15min at 17060g, following which the supernatant was removed and analysed by HPLC (for conditions see 2.3.2.4.).

3.2.3 Synthesis of quercetin metabolites.

Methylated quercetin.

Catechol *O*-methyl transferase (COMT) was incubated with quercetin and S-adenosyl methionine (SAM), which served as a methyl donor. Magnesium chloride ($MgCl_2$) was used as a cofactor, and dithiothreitol (DTT) was added as a reducing agent.

Solutions: (final incubation concentrations),

- Tris-HCl buffer 0.01M, pH7.4.
- $MgCl_2$ (1mM).
- SAM (23µM).
- Quercetin (123µM).
- DTT (95µM).

- COMT (50u/mL).

Apart from COMT, the components of the assay were added together and allowed to equilibrate at 37°C. After 5min, COMT was added and the incubation was left overnight. Placing the incubation tubes at -80°C for 10min stopped the reaction. Following thawing, samples were extracted and analysed as described for plasma (see chapter 2.3.3.1.).

For the chemical synthesis, of dimethyl sulphide (DMS, 10μL) was added to quercetin to give a 100μM equimolar solution. Methanol (980μl) was added and the mixture was incubated for 40min at 37°C. The incubation mixture was then diluted 1:1 with distilled water and analysed using HPLC.

Alternatively, quercetin (31.43mg, to give 100μM), DMS (11.80μL, to give 120μM) and K_2CO_3 (18.97mg, to give 140μM) in acetone (10mL) were refluxed for various time periods. The incubation mixture was then diluted 1:1 with distilled water and analysed using HPLC.

Quercetin sulphate.

A method for sulphating quercetin was adapted from a previously published method for sulphating curcumin(Ireson et al. 2000). Sulphated derivatives of quercetin could be generated via an organic synthetic route utilising triethylamine sulphate (SO_3-TEA). The procedure required absolute water free quercetin, which was obtained by dissolving quercetin (0.5126g, 1.69mM) in pyridine and the solution mixed. Using a rotor evaporator, the pyridine was totally removed and the dehydration step repeated a further two times. SO_3-TEA was weighed out (3.06g, 16.9mM) under an atmosphere of argon to avoid contact with air. The dried quercetin was dissolved in dioxane and added to SO_3-TEA in a 0.5L bottle under argon. The bottle was then placed in a water bath for 1.5h at 40°C. The resultant mixture was then analysed for the presence of quercetin sulphate conjugates.

3.2.4 Metabolism of quercetin *in vivo.*

Fischer (F344) rats are commonly chosen for toxicological studies and have been used in the long-term feeding studies of quercetin executed by the US National Institute of Health(National Toxicology Program 1992). Male F344 rats weighing approximately 200-250g were used in the study described here.

Quercetin metabolites in the bile.

Six F344 rats were anaesthetised by injecting 100µl of pentobarbitol (60mg/mL) i.p. following which the body cavity was opened and the bile duct located. Using micro-scissors, the bile duct was cut to allow the insertion of a polythene cannulae (i.d 0.28mm; o.d. 0.61mm) which was cut to approximately 30cm in length. The body temperature was maintained using a heat lamp. Bile was collected for 20mins following which, quercetin (12.5mg/kg) in glycerol formal was injected i.v. via the lateral vein in the tail. Bile was collected in 20min intervals for 2h. Quercetin and its metabolites were extracted using the same procedure as for plasma (see section 3.2.5).

Quercetin metabolites in plasma.

Six F344 rats were initially anaesthetised using 100µL pentobarbital (60mg/mL). Quercetin (62.5mg/kg) was then injected via the lateral tail vein. After 5min, a cardiac exsanguination procedure was carried out with throacotomy and blood collected. Four repeat test rats were injected with quercetin in 50:50 (v/v) glycerol formal in water, while two rats that acted as controls were injected with injection vehicle only.

Quercetin metabolites in urine.

Sixteen F344 rats in four groups of four rats were used. The first group received 0.5mL/kg of DMSO. The three remaining groups received, by gavage, 1900, 2300 or 2500 mg/kg of quercetin in DMSO. Rats were transferred to metabolism cages and urine collected at 4, 12 and 24h.

3.2.5 Sample preparation.

To each aliquot of sample (plasma, bile or urine) twice the volume of DMSO/methanol (1:4, v/v) was added. The extract was vortexed and centrifuged at 17060g for 15min. The supernatant was removed and diluted 1:1 with water prior to HPLC analysis. Typically, 50µL were injected onto the HPLC column.

Sample concentration for off-line LC-MS.

Eluant attributed to peaks detected at 375nm was collected and concentrated for off-line mass spectrometric analysis. This was done in either of two ways:

1) Individual peaks were collected as eluant fractions, and the solvent was vacuum-evaporated. The samples were reconstituted in 250µL of methanol prior to MS analysis.

2) Individual peaks were collected as eluant fractions, and the solvent was vacuum-evaporated. The dried fractions were reconstituted in water and methanol (4:1, v/v, 200µL) and subjected to solid phase extraction (SPE). The protocol for the SPE of quercetin and metabolites was as follows:

   a) Wash Varian C18 Bond-elut SPE cartridge with methanol (2mL).
   b) Wash cartridge with water (5mL).
   c) Transfer reconstituted fraction to cartridge and collect eluant (Eluant A).
   d) Wash cartridge with water (5mL) and collect wash (Eluant B).
   e) Elute analytes of interest with methanol (2mL) and collect eluant (Eluant C).
   f) Vacuum-evaporate to dryness and reconstitute in 200µL of methanol.

The eluant collected in step (e) represents the cleaned-up fraction and was used for mass spectrometric analysis. Eluants A and B were routinely analysed for potential hydrophilic metabolites of quercetin and served as negative controls for mass spectrometry.

3.2.6 Enzymatic hydrolysis of conjugates.

Five hundred units of β-glucuronidase solution (5000units/mL in 0.1M ammonium acetate, pH 6.8) were added to 100µl of plasma acidified with 20µl of 0.5M acetic acid. Incubations were carried out for 30min at 37°C.

Following incubation, 196µL of the incubate was removed and kaempferol (4µl of 10mg/mL in DMSO) was added and the mixture vortexed. To this mixture 400µL of 1:4 (v/v) DMSO/MeOH was then added, the resultant solution vortexed vigorously, and centrifuged at

17060g for 15min, and the supernatant transferred to a fresh eppendorf tube. Prior to injection the extractant was diluted 1:1 with deionised water.

3.2.7 Identification of metabolites.

Identification of metabolites was predominantly achieved using HPLC coupled to UV detection. Unless otherwise stated the chromatographic system used was the gradient system described in section 2.3.2.4. In a small number of cases the isocratic system described in section 2.3.2.4. was used. Comparison of Rt with authentic standards was carried out in all cases where possible.

The LC-MS of samples involved the monitoring of a range of molecular ions typically between 200 and 1300 m/z. After analysis had taken place individual ions were selected and shown as stand-alone ion chromatograms. These chromatograms are referred to from this point on as extracted chromatograms.

3.2.8 Characterisation of metabolites.

Individual metabolites were characterised by either HPLC-UV, LC-MS, mass spectrometry, tandem mass spectrometry or NMR, or a combination of methods.

3.3 Results.

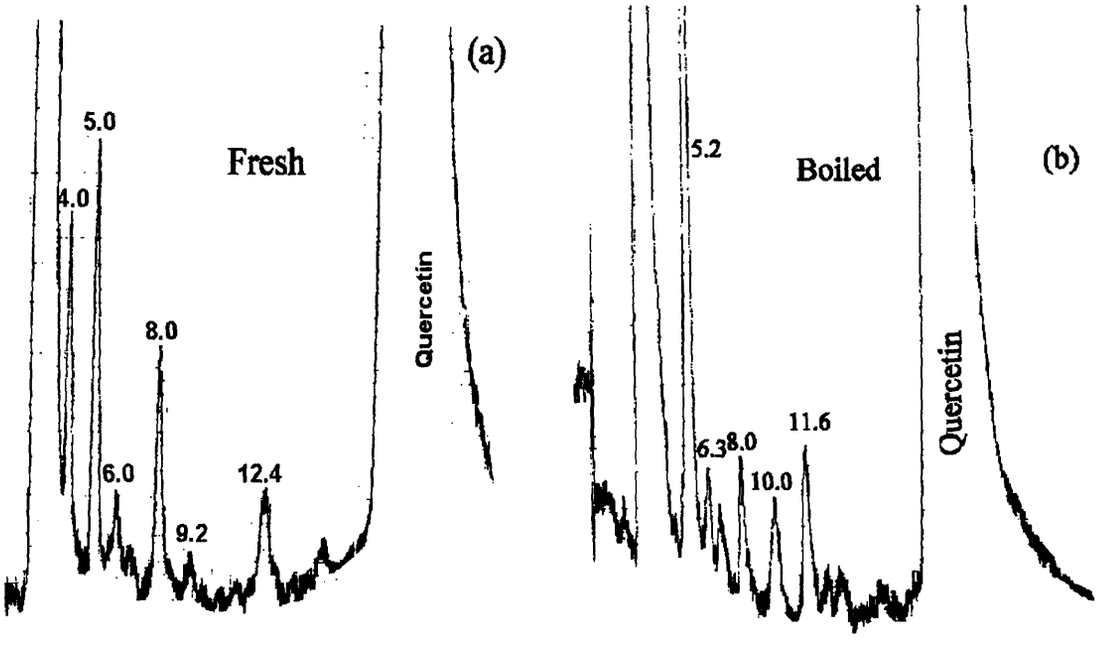
3.3.1 Quercetin metabolism in hepatic fractions.

In order to characterise quercetin metabolites, it was necessary to generate sufficient amounts of its metabolites *in vitro* to enable analysis by HPLC and MS. The first approach attempted the incubation of quercetin with cellular fractions i.e. liver microsomal, cytosolic or S9 fractions.

The HPLC chromatograms revealed several peaks in incubates with quercetin but none in those without quercetin and control incubations i.e. those with boiled cellular fractions. Peaks were seen in the extracts of S9, cytosolic and microsomal fractions, tentatively suggesting that quercetin metabolites were generated. However, when this experiment was repeated, HPLC analysis of the control incubations contained peaks similar to those found in the test incubations. Fig. 3.1 shows chromatograms of an extract of a S9 fraction incubated with quercetin, and of an extract obtained from an incubation containing inactivated S9. Inactivated S9, cytosolic and microsomal fractions all contained peaks that were found in the test incubations. It was concluded that quercetin is not metabolised, or at such a low rate which confounds analytical detection.

These results are consistent with the results of previous attempts to synthesise quercetin metabolites in microsomes, which failed to generate metabolites in either Aroclor 1254-induced or uninduced rat liver microsomes(Nielsen et al. 1998). Additionally, unpublished data has also shown that microsomes did not lead to the biosynthesis of quercetin metabolites (pers. comm. Peter Mackenzie).

Figure 3.1 *Quercetin was incubated with S9 fraction (a) or boiled S9 (b). Numbers indicate the Rt (min) of each peak. This particular analysis was carried out on a Pye-Unicam HPLC system, linked up to a chart recorder. Chromatographic system A as described in 2.3.2.4 was used.*



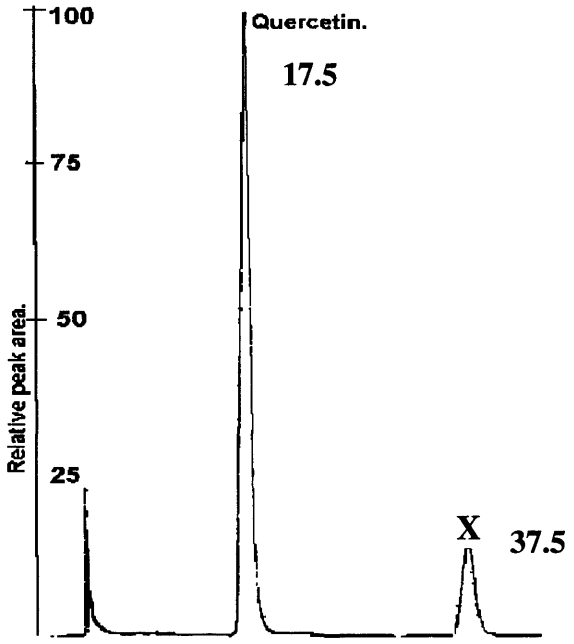
3.3.2 Synthesis of potential metabolites as reference compounds.

Isorhamnetin.

Several methods of synthesis of authentic isorhamnetin were attempted. Initially, enzymatic synthesis was carried out(Zhu, Ezell, and Liehr 1994). Following an overnight incubation of quercetin with COMT and SAM, products were extracted. HPLC analysis of the extract revealed two peaks. The first peak co-eluted with authentic quercetin (see Fig. 3.2).

The second peak, probably isorhamnetin (annotated with X), eluted after quercetin with a peak height approximately 15% of that of quercetin. Assuming that the extinction coefficients of quercetin and isorhamnetin are similar, the chromatogram suggests that approximately 15% of quercetin is converted to isorhamnetin using this biomimetic procedure.

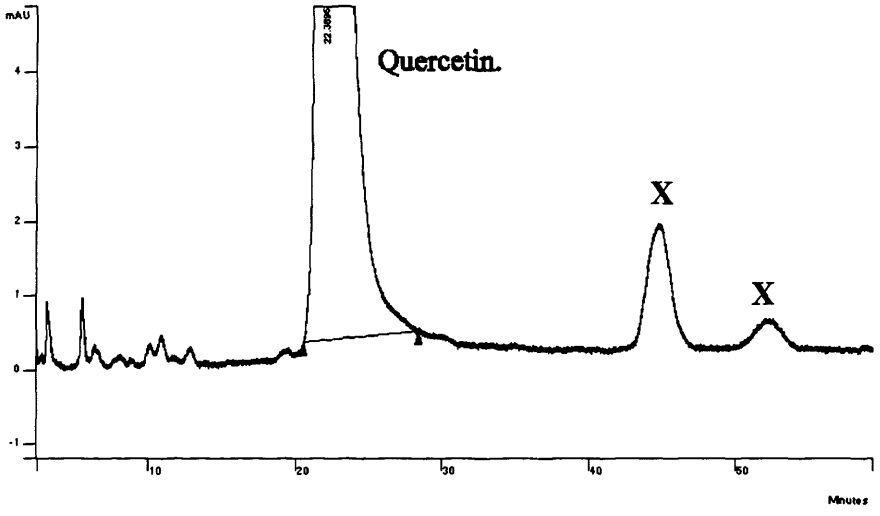
Figure 3.2 *HPLC analysis of an incubation of catechol O-methyl transferase, S-adenosylmethionine and quercetin. This analysis was carried out on a Pye-Unicam HPLC system, linked up to a chart recorder. Chromatographic system A as described in 2.3.2.4 was used. Retention time is shown for each peak.*



In order to allow mass spectrometric analysis, sample preparation involved removal of Tris-HCl, which interferes with MS, via SPE as described in section 3.2.5. Mass spectrometric analysis proved inconclusive, in that an ion corresponding to a mono-methylated derivative of quercetin was not detected. It is possible that the product was lost during desalting. A 15% conversion level was considered low, consequently, chemical synthetic methods were attempted.

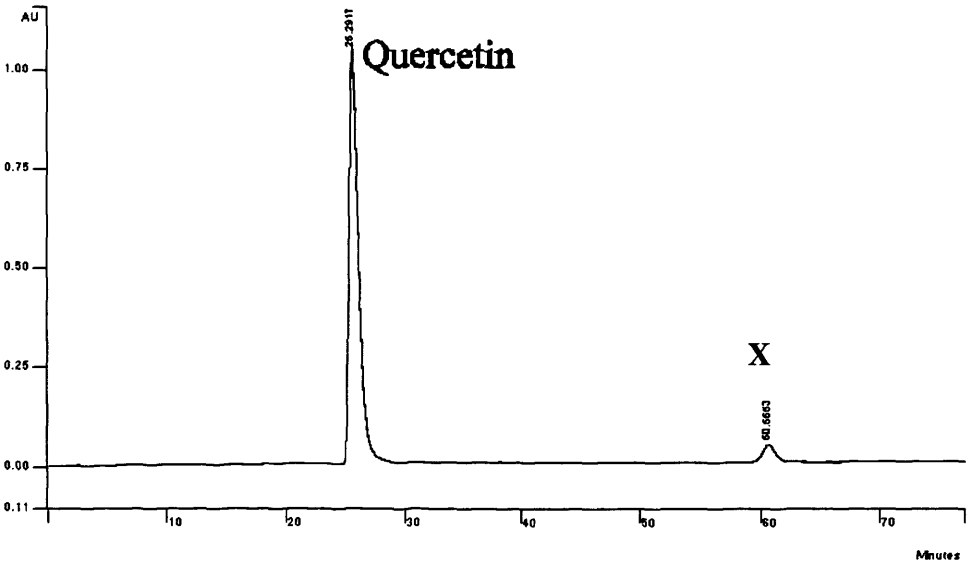
Two chemical synthetic methods were used to generate isorhamnetin, as described in 3.2.3. The first method involved incubating quercetin and DMS, a methylating agent, in methanol. HPLC analysis of the incubation extract revealed a number of peaks as shown in Fig. 3.3. The large peak with a Rt of 22.4 in Fig. 3.3 is consistent with authentic quercetin. Two peaks that eluted after quercetin are probably methylated derivatives of quercetin (annotated with X), the first might be isorhamnetin and the second dimethylated quercetin. Mass spectrometric analysis failed to confirm this assumption.

Figure 3.3 *Products generated by the incubation of quercetin and DMS in methanol. HPLC was carried out on a Varian HPLC system as described in section 2.3.2.1 using chromatographic system A as described in section 2.3.2.4.*



Quantitatively, the synthetic conversion was very poor. Consequently, the method was modified to include the base K_2CO_3, and refluxing in acetone instead of methanol. Quercetin was reacted with DMS in the presence of K_2CO_3, and HPLC analysis revealed a few peaks as shown in Fig. 3.4. Again mass spectrometry was unsuccessful in identifying the methylated species (which is annotated with X), which may have been due to the low conversion rate, which was less than 5%.

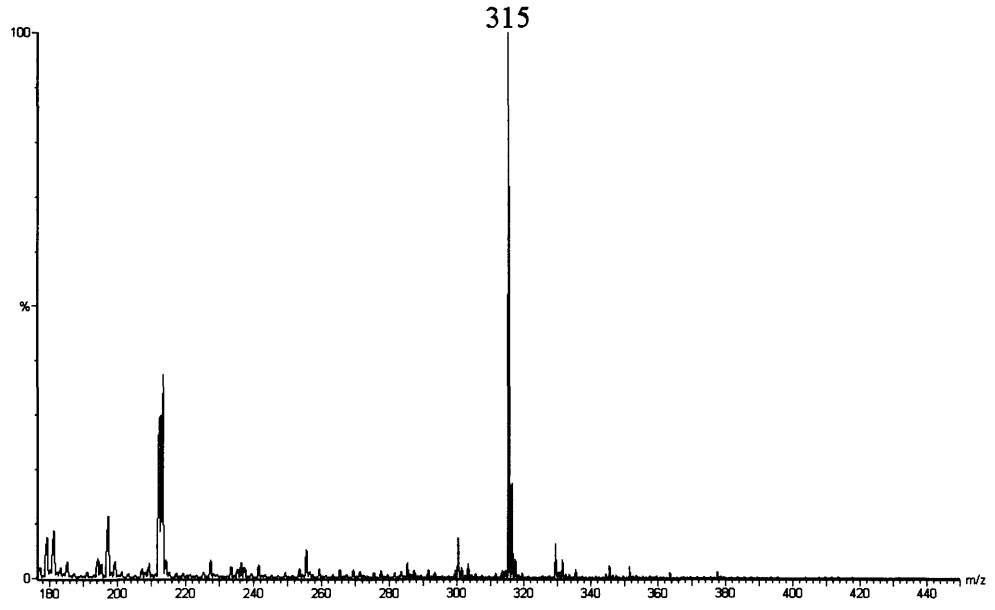
Figure 3.4 *Products of the reaction between quercetin, dimethylsulphide and potassium carbonate. HPLC was carried out on a Varian HPLC system as described in section 2.3.2.1 using chromatographic system A as described in section 2.3.2.4.*



All these synthetic routes were attempted before a commercial supplier of isorhamnetin was

found (Extrasynthese, Genay, France). When the commercially supplied isorhamnetin standard was analysed by HPLC it coeluted with peaks tentatively assigned isorhamnetin previously. When analysed by MS, an ion of m/z 315 was generated, which is consistent with a mono-methylated derivative of quercetin, see Fig. 3.5. Consequently, the synthesis of isorhamnetin was not pursued further.

Figure 3.5 *MS of isorhamnetin standard. m/z of 315.*



Synthesis of quercetin sulphate.

Quercetin has five hydroxyl groups that are all plausible candidates for sulphation. An authentic standard, quercetin 3-O-monosulphate was purchased and further sulphated quercetin conjugates were generated synthetically.

The synthesis, as described in 3.2.3, yielded several different species that absorbed at 375nm, and were thus postulated to be sulphated derivatives of quercetin. Separation of the individual components was carried out on a semi-preparative column, which allowed the injection of larger amounts of reaction products. The products were separated into eight different fractions and concentrated using a rotary evaporator. Fig. 3.6 shows a typical analysis for the separation of the products of the reaction between quercetin and SO_3-TEA. Each fraction was reanalysed using LC-MS to establish the degree of sulphation, i.e. mono or bis conjugation.

Figure 3.6 *Products of an incubation of quercetin with SO_3-TEA. HPLC was carried out on a Varian HPLC system as described in section 2.3.2.1 using chromatographic system B as described in section 2.3.2.4. The numbered peaks represent the fractions collected for MS and NMR analysis.*

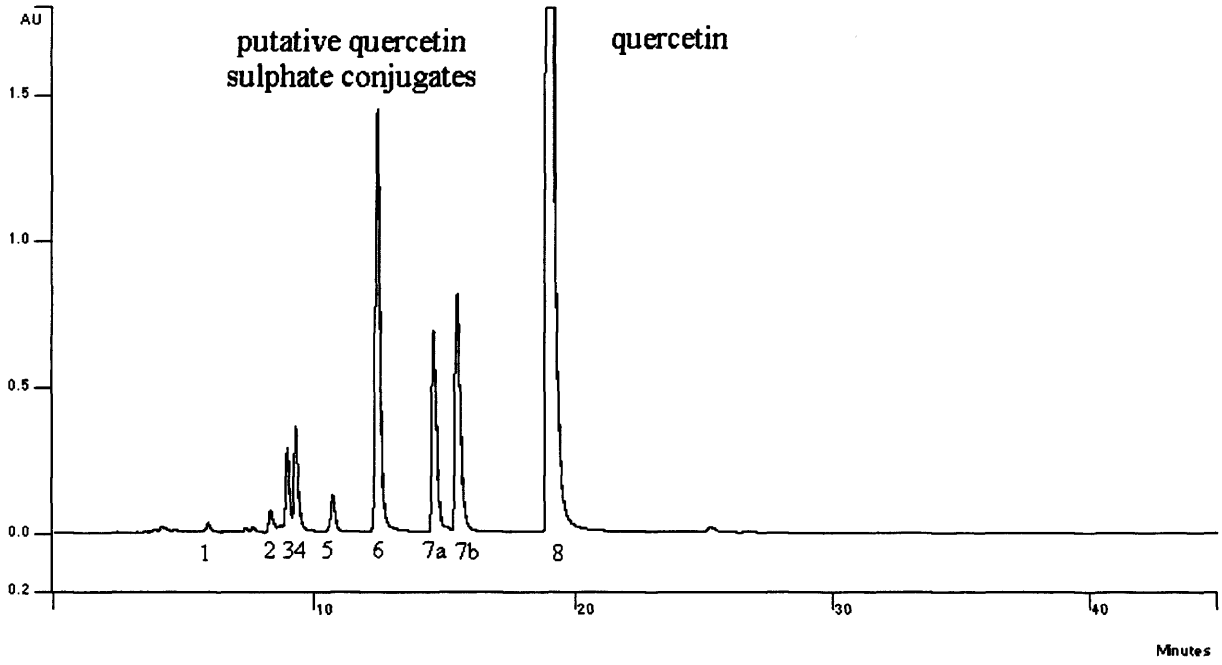


Fig. 3.6 shows a large peak that elutes at 19.3min. Co-elution with a standard and subsequent MS analysis revealed the peak to be quercetin. The other visible peaks that elute prior to quercetin were assumed to be sulphate conjugates of quercetin. This assumption was based on the increased polarity and the absorption at 375nm. Each peak was collected and analysed by MS. The results are shown in Fig. 3.8 and summarised below in Table 3.1.

Figure 3.7 *LC-MS analysis of an extraction of an incubation of quercetin with SO_3-TEA. LC-MS was carried out on a Varian HPLC system coupled to a Micromass Platform mass spectrometer as described in section 2.3.2.1 using chromatographic system B as described in section 2.3.2.4. Shown are chromatograms for (A) bis-sulphate conjugate, m/z 461, (B) monosulphate conjugates, m/z 381, (C) quercetin, m/z 301 and (D) a UV analog channel which measured at 375nm.*

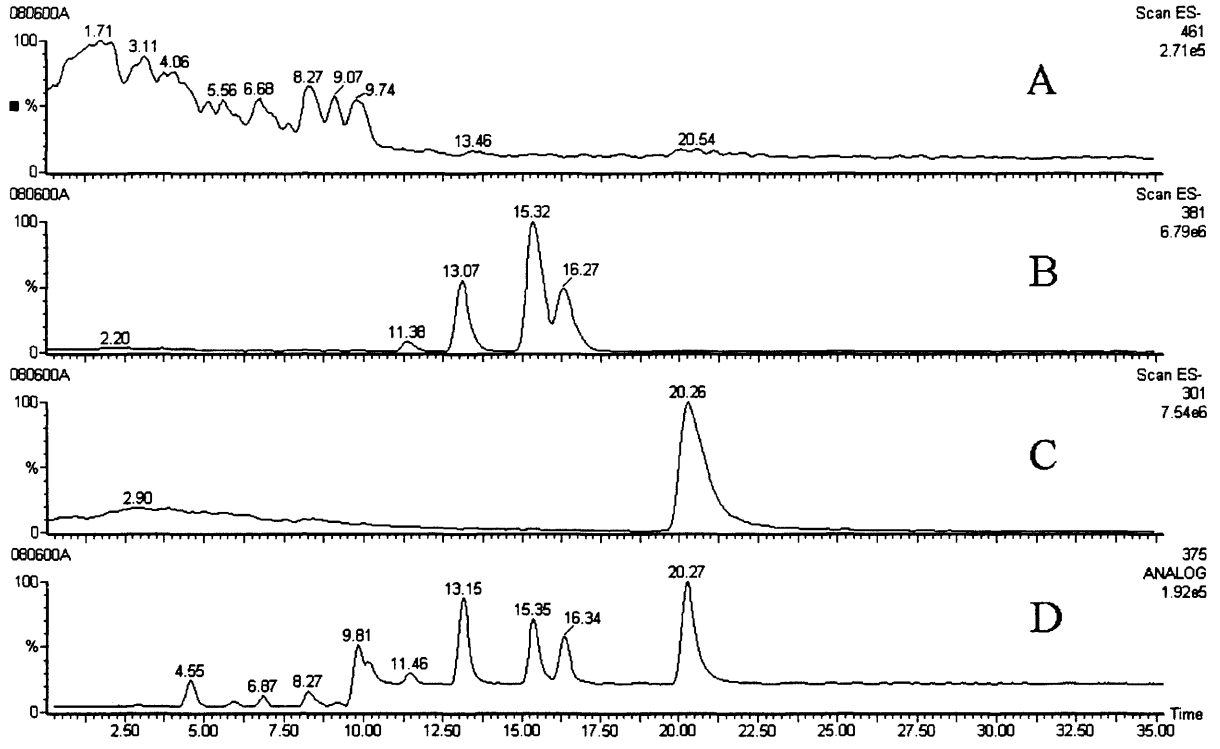


Fig. 3.7 shows the extracted chromatograms and the simultaneous UV chromatogram which allows alignment of the peaks found in UV and MS. Four monosulphate conjugates were synthesised, which is consistent with the data for the conjugation of glucuronic acid to quercetin reported by Day *et al*, according to whom only four of the five hydroxyls were occupied by glucuronic moieties(Day et al. 2000). Using absorption spectra data, Day *et al* deduced that all hydroxyl sites except the 5-position underwent conjugation. The 5-position of flavonols has been shown to react only if it is the only hydroxyl group available(Boutin et al. 1993). The *in-vitro* synthesis of glucuronides of genistein, chrysin and apigenin failed to furnish glucuronides of the 5-position of any of the molecules(Doerge et al. 2000; Galijatovic et al. 2001).

Table 3.1 *Molecular weights of molecules in individual fractions collected from the incubation of quercetin with SO_3-TEA.*

Fraction number	Rt (min)	Molecular weight	Preliminary identification
1	4.5	?	??
2	8.3	462	Quercetin disulphate
3	9.0	462	Quercetin disulphate
4	9.8	462	Quercetin disulphate
5	11.4	382	Quercetin monosulphate
6	13.1	382	Quercetin monosulphate
7a	15.3	382	Quercetin monosulphate
7b	16.3	382	Quercetin monosulphate
8	20.26	302	Quercetin

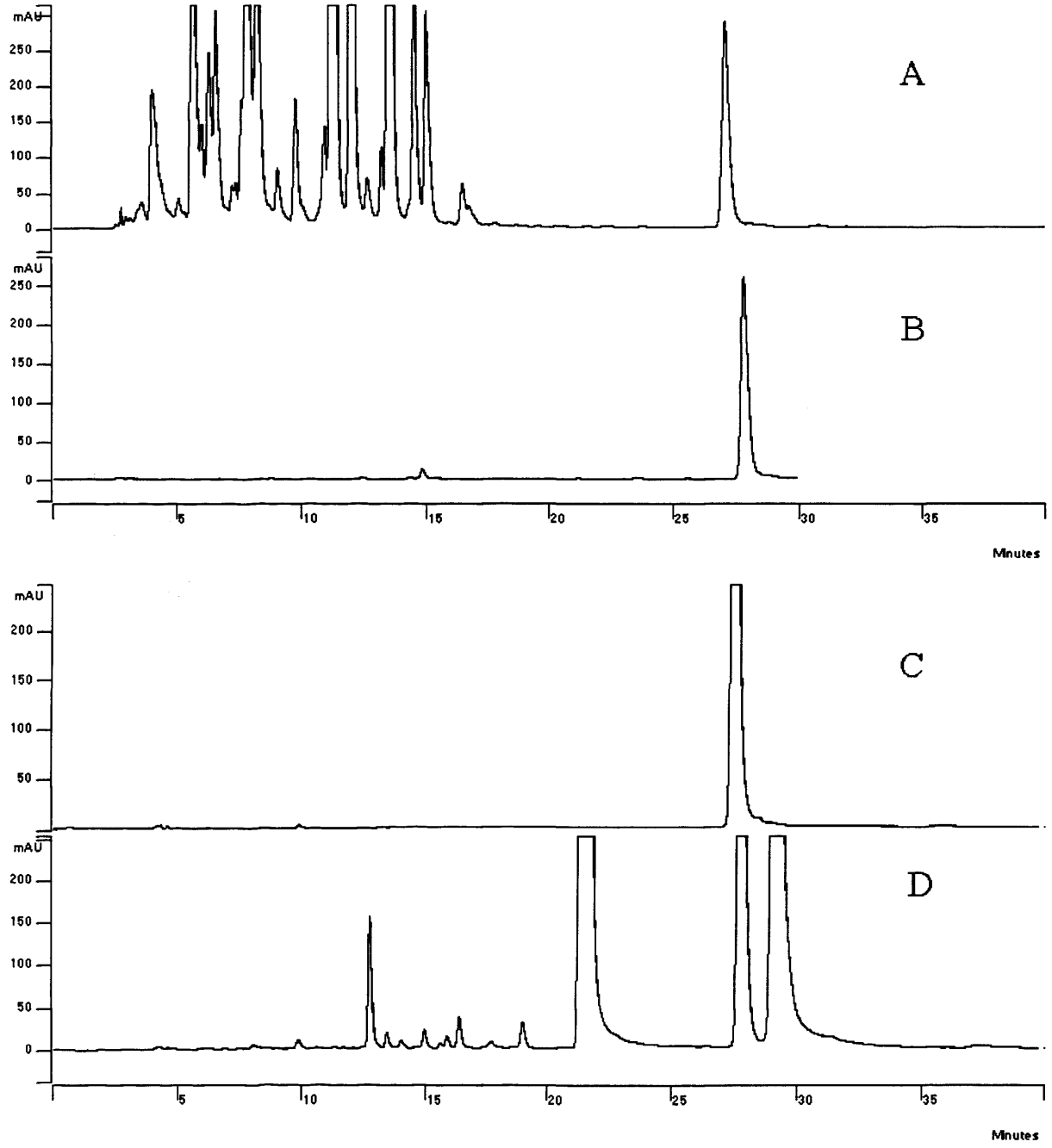
Following MS analysis individual fractions were cleaned up by re-injection onto the HPLC and peaks were recollected. Following evaporation of the mobile phase, each peak was reconstituted in DMSO-d6 and analysed by NMR (discussed later in this chapter).

3.3.3 Enzymatic hydrolysis of quercetin conjugates.

Rat bile obtained 20min after the i.v. administration of quercetin was subjected to enzymatic hydrolysis as described in 3.2.6. The enzyme used was β-glucuronidase which also has sulphatase activity. Thus, the enzyme does not discriminate between glucuronide and sulphate conjugates. Fig. 3.8 shows chromatograms of extracts of bile treated with β-glucuronidase. Also shown are the various control chromatograms that demonstrate the validity of the method. The chromatograms shown demonstrate the disappearance of many of the peaks related to the metabolism of quercetin following enzymatic hydrolysis. Chromatogram D also shows the presence of quercetin (Rt ~ 22min) and isorhamnetin (Rt ~ 29min) which are released following the cleavage of the conjugates. The isorhamnetin peak elutes after the internal standard kaempferol.

These experiments support the notion that quercetin is metabolised to a variety of conjugated metabolites.

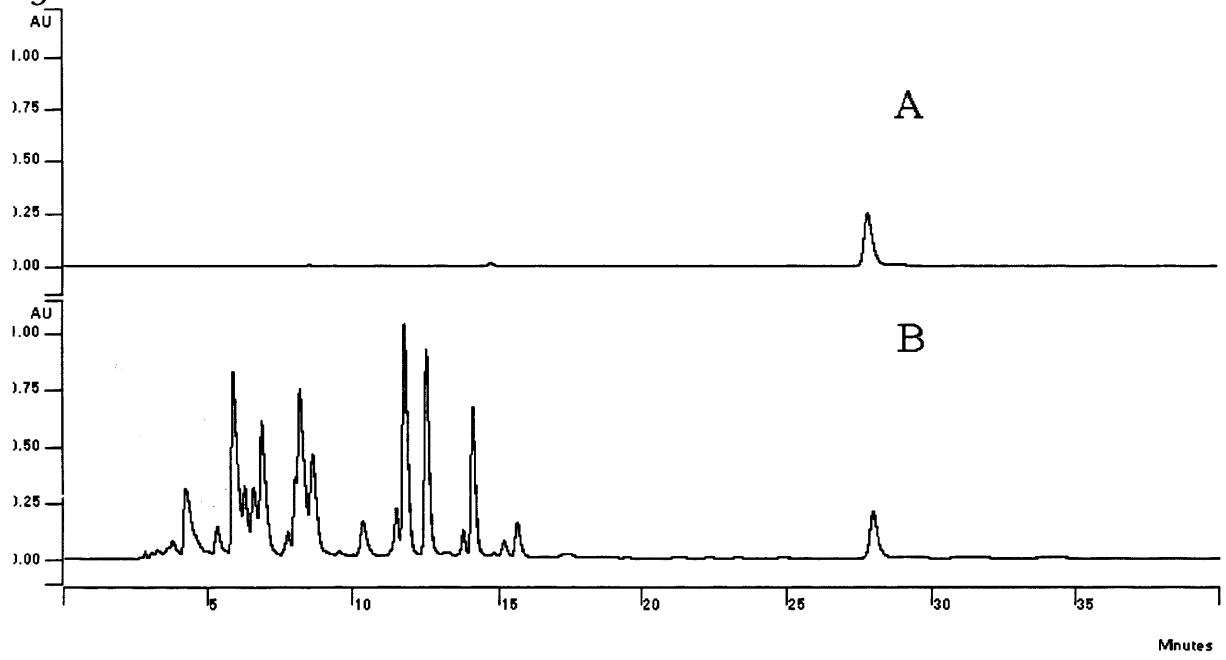
Figure 3.8 *Disappearance of quercetin related metabolites following treatment with β-glucuronidase and sulphatase. Shown are four chromatograms: untreated bile obtained from rats for 20min after quercetin administration (A), untreated bile obtained from rats 20mins prior to quercetin administration (B), bile treated with hydrolytic enzymes obtained 20min prior to quercetin administration (C), and bile treated with hydrolytic enzymes obtained 20min after administration (D). All chromatograms contain a peak for the internal standard kaempferol (Rt 27.7min). HPLC was carried out on a Varian HPLC system as described in section 2.3.2.1 using chromatographic system B as described in section 2.3.2.4.*



3.3.4 Analysis of bile from rats treated with quercetin.

Bile obtained from rats treated with quercetin yielded a large number of metabolites. Shown in Fig. 3.9B is a typical UV-chromatogram of an extract of bile from rats that received quercetin, showing peaks that elute prior to quercetin. Quercetin and isorhamnetin are not present, presumably because of the molecular weight filter of the liver which excludes molecules of less than 350MW entering the biliary duct(Smith 1973).

Figure 3.9 *Separation of quercetin metabolites generated 20mins following the administration of quercetin (chromatogram B). Also shown is a pre-administration bile sample (chromatogram A). HPLC was carried out on a Varian HPLC system as described in section 2.3.2.1 using chromatographic system B as described in section 2.3.2.4.*



Bile was analysed by LC-MS using a mass scan from m/z 200-1000. Shown in Fig. 3.10 are extracted chromatograms that reveal a large number of conjugated derivatives of quercetin and isorhamnetin which are produced in the liver and secreted into the bile. Consistent with previous reports, only four quercetin glucuronides (Fig 3.10C) were generated(Day et al. 2000; Oliveira and Watson 2000). Presumably, the 5-position is not conjugated as this has been shown to be the less preferable position of conjugation(Day et al. 2000). There are five glucuronide conjugates of isorhamnetin (Fig 3.10D). With only four available hydroxyl groups, it is not clear where the fifth conjugation site could be. Other metabolites are double conjugates that contain a sulphate and a glucuronate moiety (Fig. 3.10 I and J) with $[M-H]^-$ of m/z 557 and 571, respectively. There appear to be at least six isomers of quercetin monosulphate-monoglucuronide (m/z 557) and four isomers of isorhamnetin monosulphate-

monoglucuronide (m/z 571).

Figure 3.10 *Extracted chromatograms following LC-MS analysis of bile. Shown below are extracted chromatograms that correspond to conjugated species of quercetin and isorhamnetin. The analysis was carried out on the Micromass Platform mass spectrometer linked to a HP1100 LC system and ran in tandem with a HP1100 UV detector. Chromatographic system B as described in section 2.3.2.4. was used.*

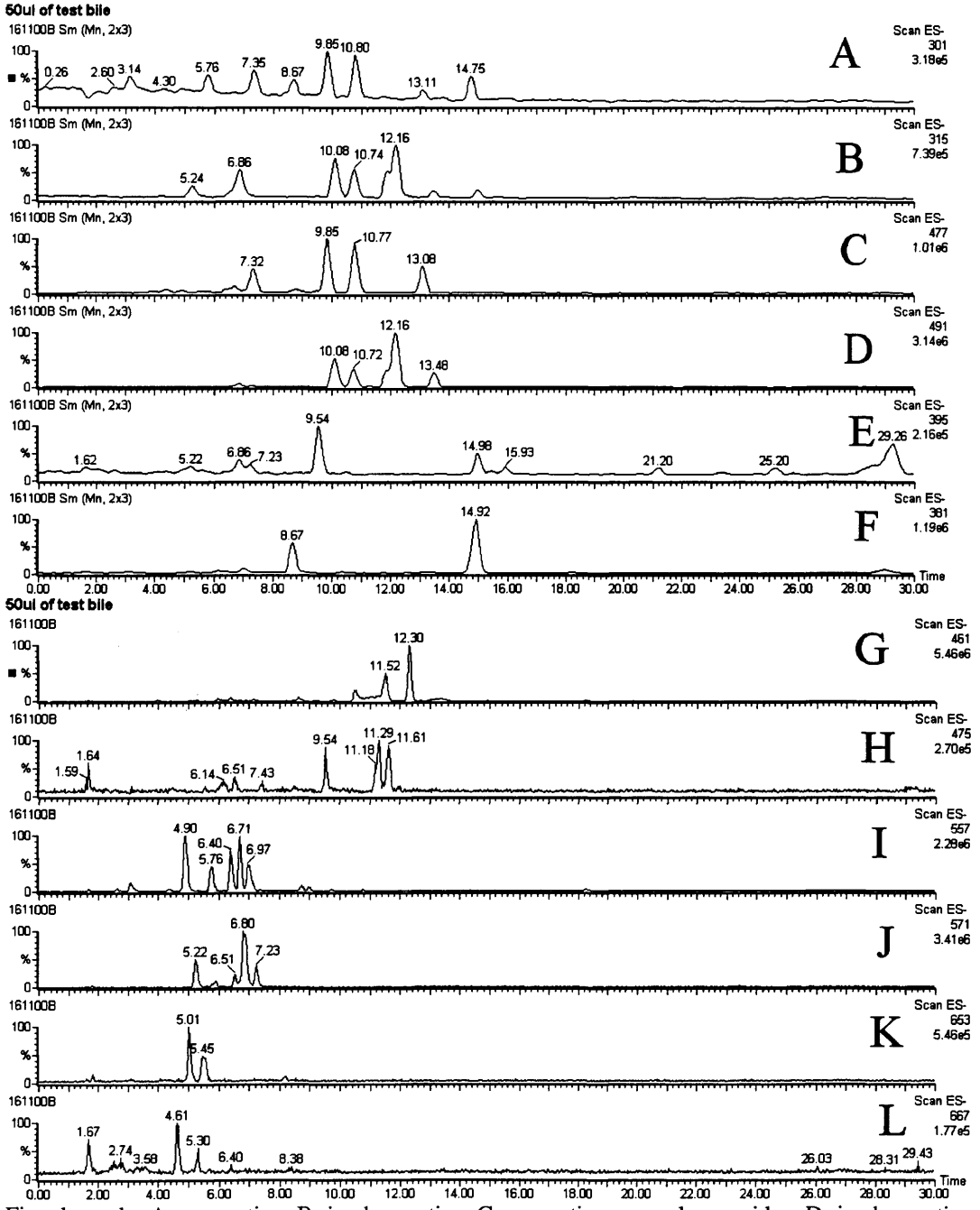


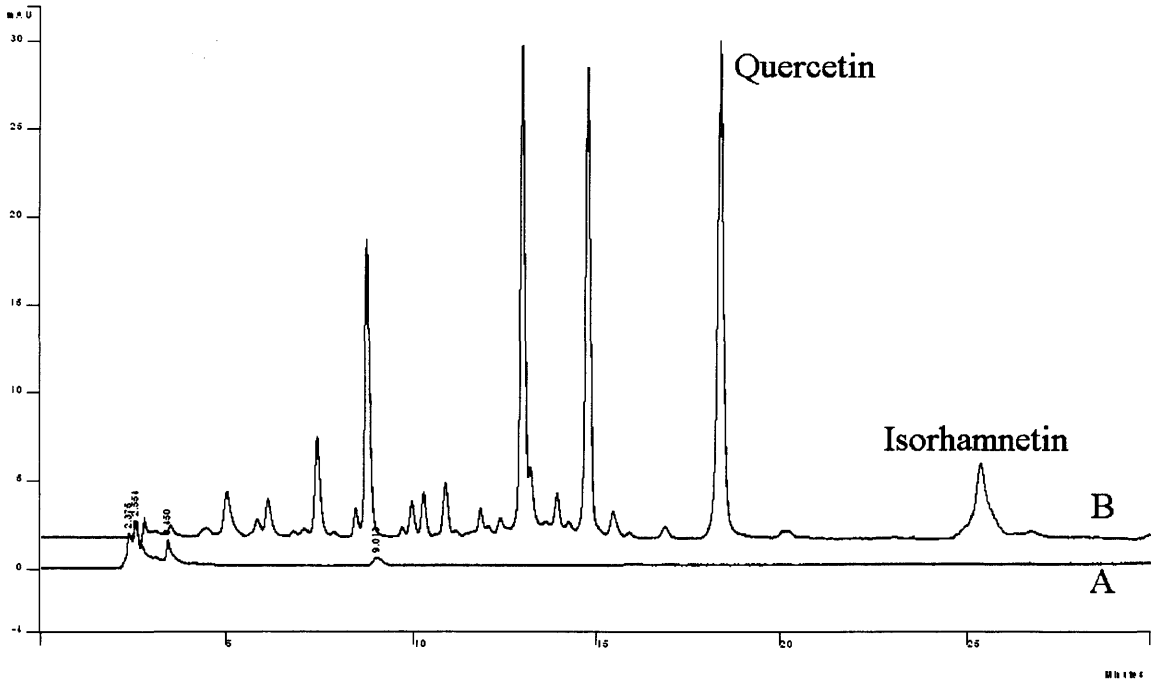
Fig. legend: A=quercetin, B=isorhamnetin, C=quercetin monoglucuronide, D=isorhamnetin glucuronide, E=isorhamnetin monosulphate, F=quercetin monosulphate, G=quercetin disulphate, H= isorhamnetin disulphate, I=quercetin monosulphate-monoglucuronide, J=isorhamnetin monosulphate-monoglucuronide, K=quercetin diglucuronide, L=isorhamnetin diglucuronide.

Mono-sulphation is also a route of metabolism (Fig. 3.10 E and F) but there appear to be fewer isomers of sulphate conjugates than glucuronides. Also, neither of the two sulphate isomers correspond in retention time, to the quercetin sulphate standard (quercetin 3-O-sulphate).

3.3.5 Analysis of rat plasma.

Rats were injected i.v. with quercetin (62.5mg/kg) and plasma collected 5min after administration. Fig. 3.11 shows the metabolite profile obtained following the administration of quercetin. Quercetin and isorhamnetin can be observed in the plasma. An isocratic method was employed (as described in section 2.3.2.4) for the initial HPLC analysis of plasma from rats treated with quercetin. This separation allows the detection of quercetin and isorhamnetin, while also showing the absence of any interfering peaks in plasma from rats treated with dosing vehicle only, chromatogram A, and plasma from rats treated with quercetin, chromatogram B.

Figure 3.11 *Plasma from rats that received dosing vehicle (A) or quercetin (B). The HPLC instrumentation used is described in section 2.3.2.1 and chromatographic system B was used (section 2.3.2.4).*



Using LC-MS, quercetin, isorhamnetin and the conjugated metabolites could be identified as shown in Fig. 3.12.

Figure 3.12 *Extracted chromatograms following LC-MS analysis of a plasma extract. Shown are chromatograms that correspond to different conjugated species of quercetin and isorhamnetin. The analysis was carried out a Micromass Bio-Q instrument coupled to a Varian HPLC system. Chromatographic system B as described in section 2.3.2.4. was used.*

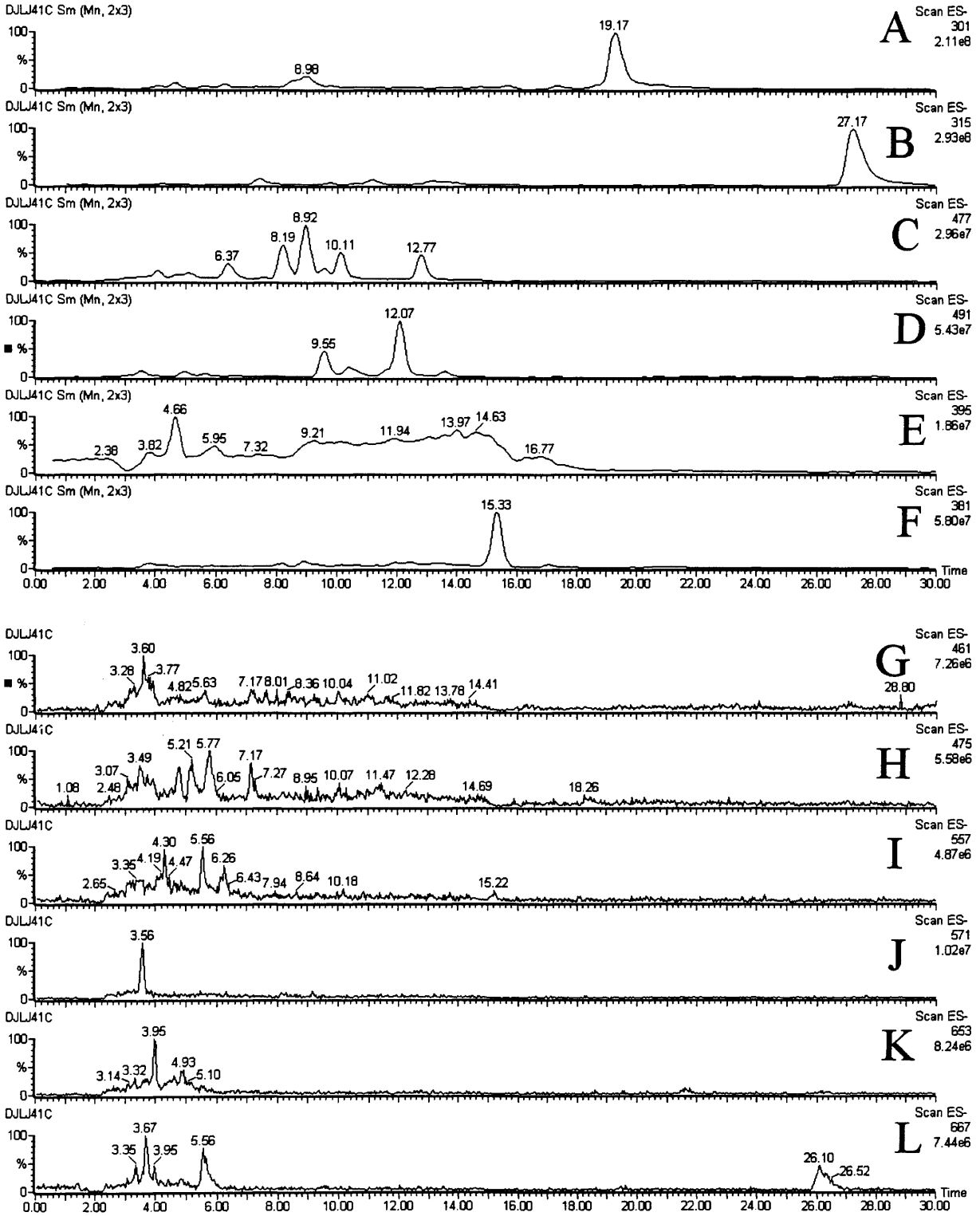


Fig. legend: A=quercetin, B=isorhamnetin, C=quercetin monoglucuronide, D=isorhamnetin glucuronide, E=isorhamnetin monosulphate, F=quercetin monosulphate, G=quercetin disulphate, H= isorhamnetin disulphate, I=quercetin monosulphate-monoglucuronide, J=isorhamnetin monosulphate-monoglucuronide, K=quercetin diglucuronide, L=isorhamnetin diglucuronide.

3.3.6 Analysis of rat urine.

Urine obtained from rats following the oral administration of quercetin (1900-2500mg/kg) was analysed for the identification of quercetin metabolites using LC-MS. Extracted chromatograms obtained from the analysis are shown in Fig. 3.13. Quercetin and isorhamnetin were found in urine (see Fig. 3.13 A and B respectively). There are five quercetin glucuronide conjugates in urine, corresponding to molecules in which each of the five hydroxyl groups are conjugated. In common with the results obtained in bile, there appear to be five separate isomers of isorhamnetin glucuronide.

Fig. 3.13F indicates that monosulphation of quercetin occurs with the formation of two isomers (Rt 8.36 and 15.24). Neither of the quercetin sulphate isomers co-elute with the quercetin 3-O-sulphate standard. There also exist diglucuronides of quercetin and isorhamnetin as shown in Fig. 3.13 K and L.

Urinary excretion of quercetin implies that quercetin was absorbed from the gastro-intestinal tract after oral administration. The urine contains one more quercetin monoglucuronide isomer than bile. This discrepancy may be due to one of two explanations. The rats, in the experiment involving the excretion of quercetin metabolites in urine, received a greater dose of quercetin, than the rats in the experiment involving the excretion of quercetin metabolites in the bile. Consequently, the fifth glucuronide seen in the urine may have been present in the bile, but below the limits of detection. Alternatively, one of the quercetin glucuronide isomers is generated solely in the intestinal tract. There is increasing evidence to suggest that the intestine plays a role in the metabolism of flavonoids and other phenolic compounds(Spencer et al. 1999; Donovan et al. 2000; Kuhnle et al. 1999; Crespy et al. 1999).

Figure 3.13 *Extracted chromatograms from LC-MS analysis of urine of rats which received quercetin. Shown are chromatograms that correspond to different conjugated species of quercetin and isorhamnetin. The analysis was carried out a Micromass Bio-Q instrument coupled to a Varian HPLC system. Chromatographic system B as described in section 2.3.2.4. was used.*

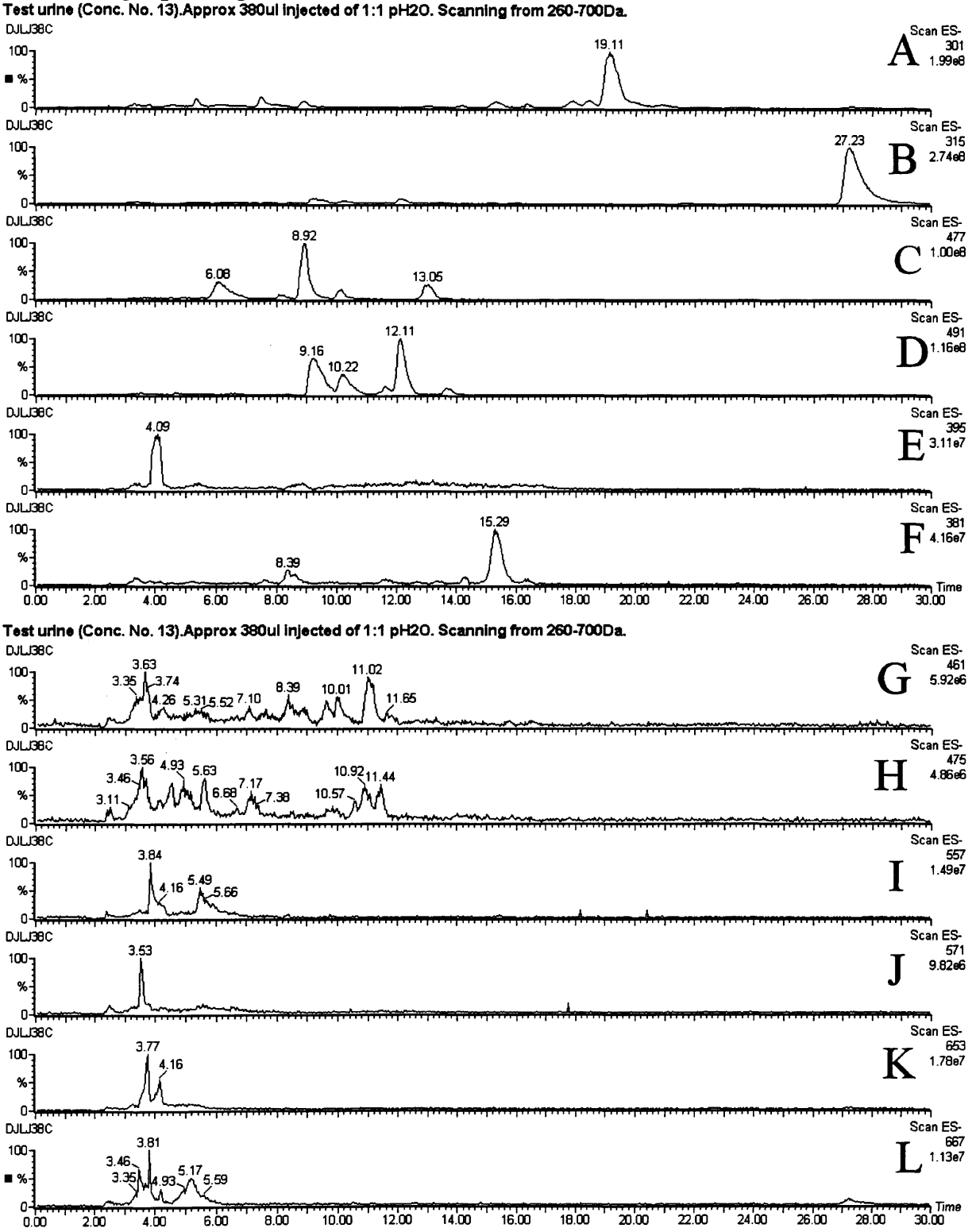


Fig. legend: A=quercetin, B=isorhamnetin, C=quercetin monoglucuronide, D=isorhamnetin glucuronide, E=isorhamnetin monosulphate, F=quercetin monosulphate, G=quercetin disulphate, H= isorhamnetin disulphate, I=quercetin monosulphate-monoglucuronide, J=isorhamnetin monosulphate-monoglucuronide, K=quercetin diglucuronide, L=isorhamnetin diglucuronide.

3.4 Characterisation of individual metabolites.

Synthesised sulphate-conjugates of quercetin.

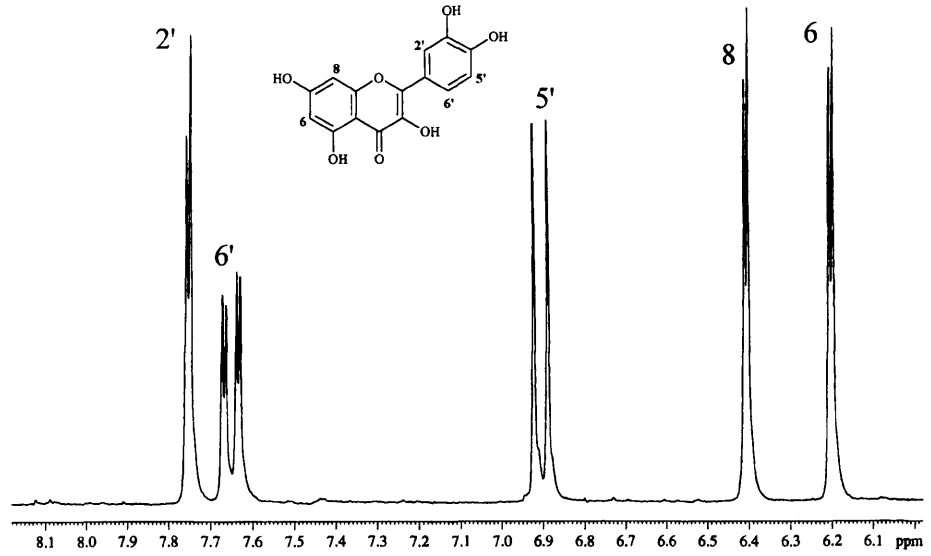
In order to ascertain the precise position of sulphate moiety on the quercetin conjugate molecule, NMR was employed.

All fractions from the HPLC analysis of the extract of the incubate of quercetin with SO_3-TEA were analysed by NMR, and peaks 5,6 7a and 7b gave interpretable results (see Fig. 3.6 for fraction assignment). An authentic sample of quercetin 3-O-sulphate was used for comparison.

Proton (1H), carbon 13 (13C) and 2-D spectra were generated using a Bruker ARX 250 MHz spectrometer, using deuterated dimethyl sulphoxide as the solvent. Fig. 3.14 shows the ^{1}H-NMR of quercetin (A), quercetin-3-O sulphate (B), peak 5 (C), peak 6 (D) and combined peak 7a and 7b (E).

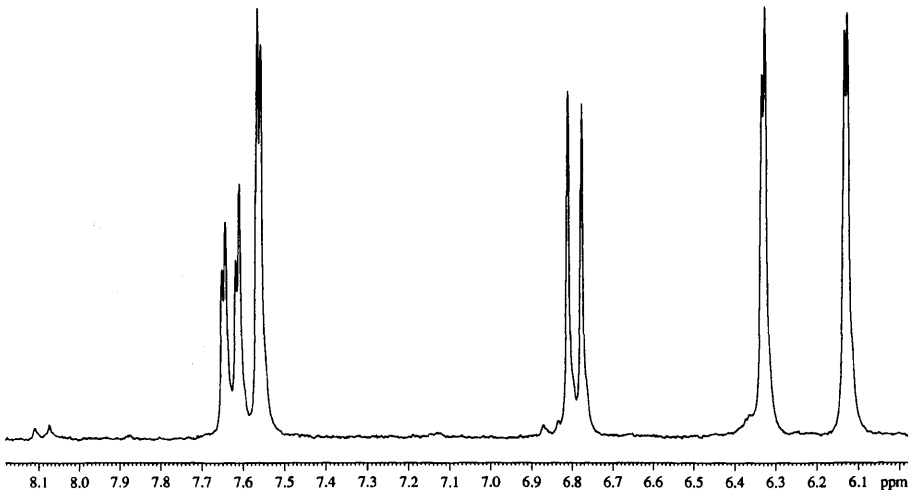
All the peaks were integrated for one proton.

Figure 3.14 *NMR spectra of quercetin standards and sulphate conjugates in DMSO-d6.*



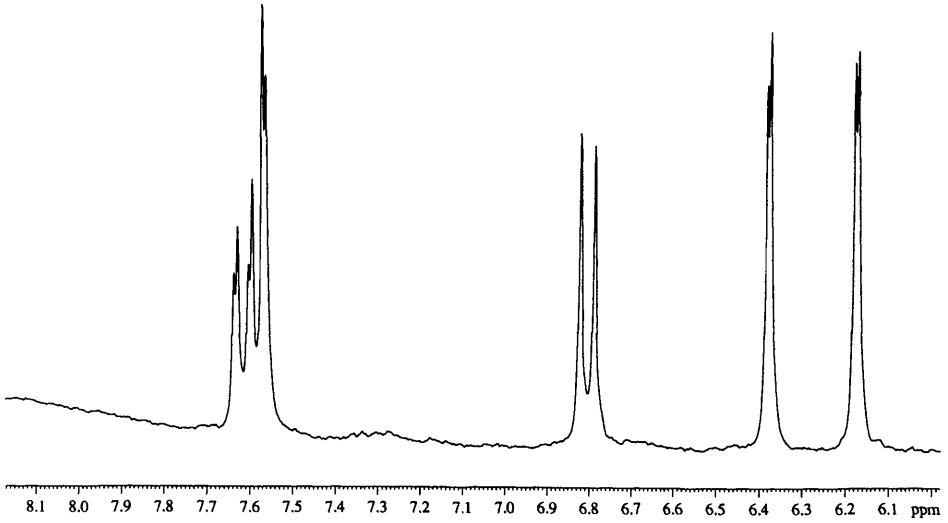
A

Quercetin



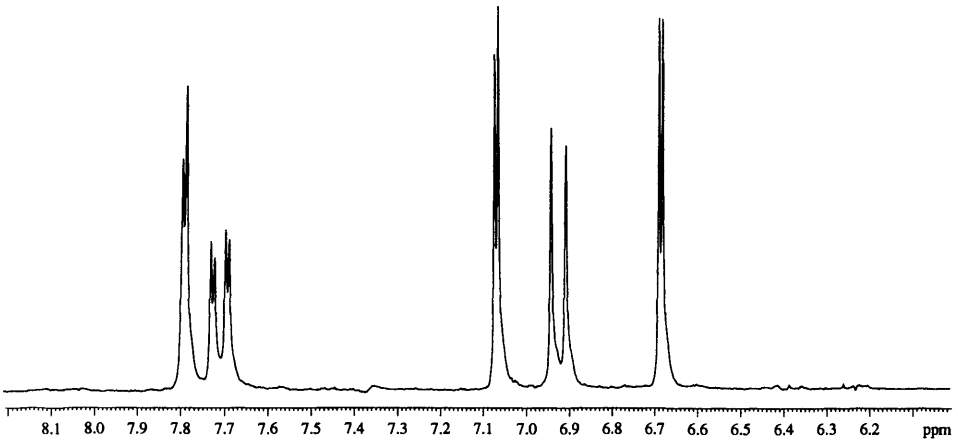
B

Quercetin 3-O-sulphate standard



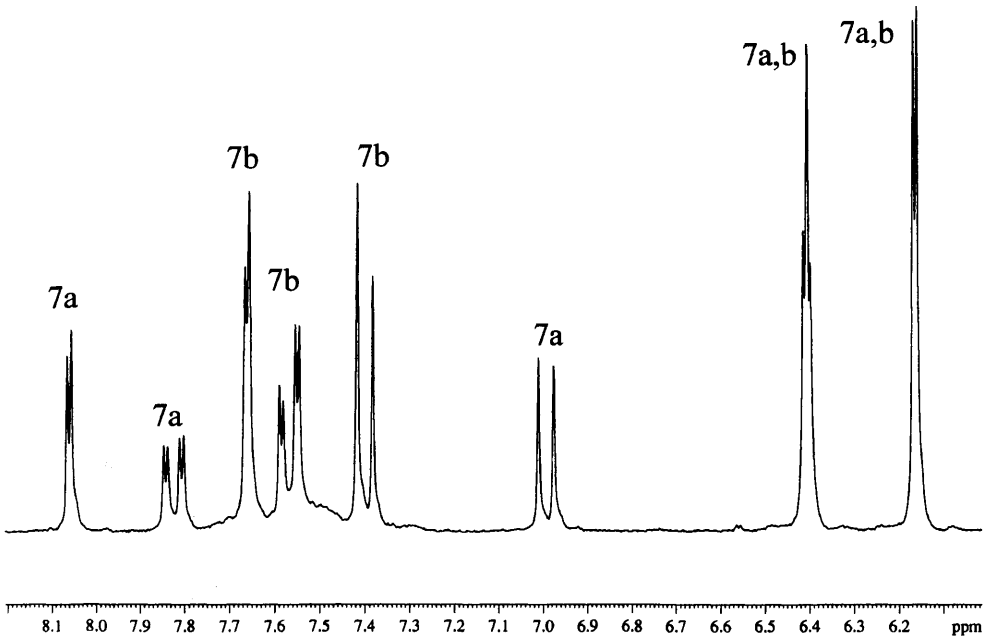
C

Peak 5



D

Peak 6



E

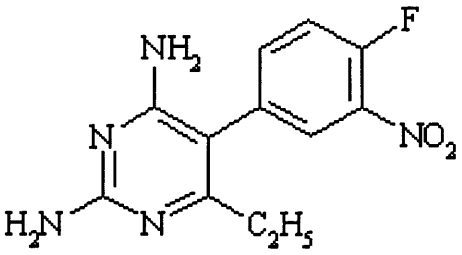
Peak 7a and 7b

NMR spectra interpretation.

Quercetin can be identified by its characteristic proton coupling as shown in Fig. 3.14A. The protons on ring A, 6-H and 8-H, are coupled. These protons appear as a pair of fine (meta-coupled) doublets at 6.2 and 6.4ppm respectively, with a coupling constant of J=2.1Hz. On ring C the 5'-H (6.9ppm) is split by the 6'-H and vice versa. This ortho-coupling results in a large J value of 8.5Hz. The 6'-H is further split by the long range coupling to 2'-H. Consequently, the 6'-H appears as a doublet of doublets (7.65ppm, J=8.5Hz, J=2.12Hz) and the 2'-proton signal appears as a fine doublet (7.75ppm, J=2.14Hz).

Peak 5, the spectrum of which can be seen in Fig. 3.14C, is predicted to be quercetin 3-O-sulphate. The spectrum is identical to that for the authentic sample (Fig. 3.14B). There is an upfield shift of the 2'-H signal. The upfield shift of the 2'-H signal is unusual, as it would be predicted that the conjugation to the 3-position would affect the 6'-position. While the reason for the effect is not clear, the same effect was observed in the identification of quercetin 3-O-glucuronide(Moon et al. 2001). Again, a upfield shift in the 2'-position was noticed when a glucuronide moiety binds to the 3-position. It is likely that quercetin undergoes rotation of the C-2 and C-1' bond. This rotation is analogous to that seen for fluoronitropyrimethamine which exists as two slowly interconverting rotamers(Tendler 1988). Rotation occurs about the C5-C1' bond of this compound (see Fig 3.15). NMR evidence for the existence of two rotamers of quercetin could not be produced, although X-ray crystallography data showed that the B-ring can lie at 0 and 180° to the rest of the structure(Jin, Yamagata, and Tomita 1990). Quercetin 3-O-sulphate and peak five co-eluted when subjected to HPLC. In addition to the MS evidence (Table 3.1), it can be concluded that fraction five is quercetin 3-O-sulphate.

Figure 3.15 *Structure of fluoronitropyrimethamine.*



The major difference between the spectra of quercetin and peak 6 is a change in chemical shift of both the 6- and 8- protons as seen in Fig. 3.14D. Peak 6 is assigned as quercetin 7-sulphate as the shift effect is of a similar magnitude for both 6-H and 8-H.

It proved impossible to resolve peaks 7a and 7b completely by HPLC. The partially separated fractions gave mixed spectra using NMR analysis. It was possible to distinguish between the two components, using the difference in integral values.

Peak 7a, the spectrum of which can be seen in Fig. 3.14E, could be quercetin 3'-O-sulphate. There is a large downfield shift on the 2'-proton that suggests that the substitution of the hydroxyl for a sulphate ester group occurs at the 3'-position.

Peak 7b was assigned as quercetin 4'-O-sulphate. This was deduced by the presence of a large downfield shift of the 5'-proton (δ 0.5ppm). The NMR spectrum is shown in Fig. 3.14E

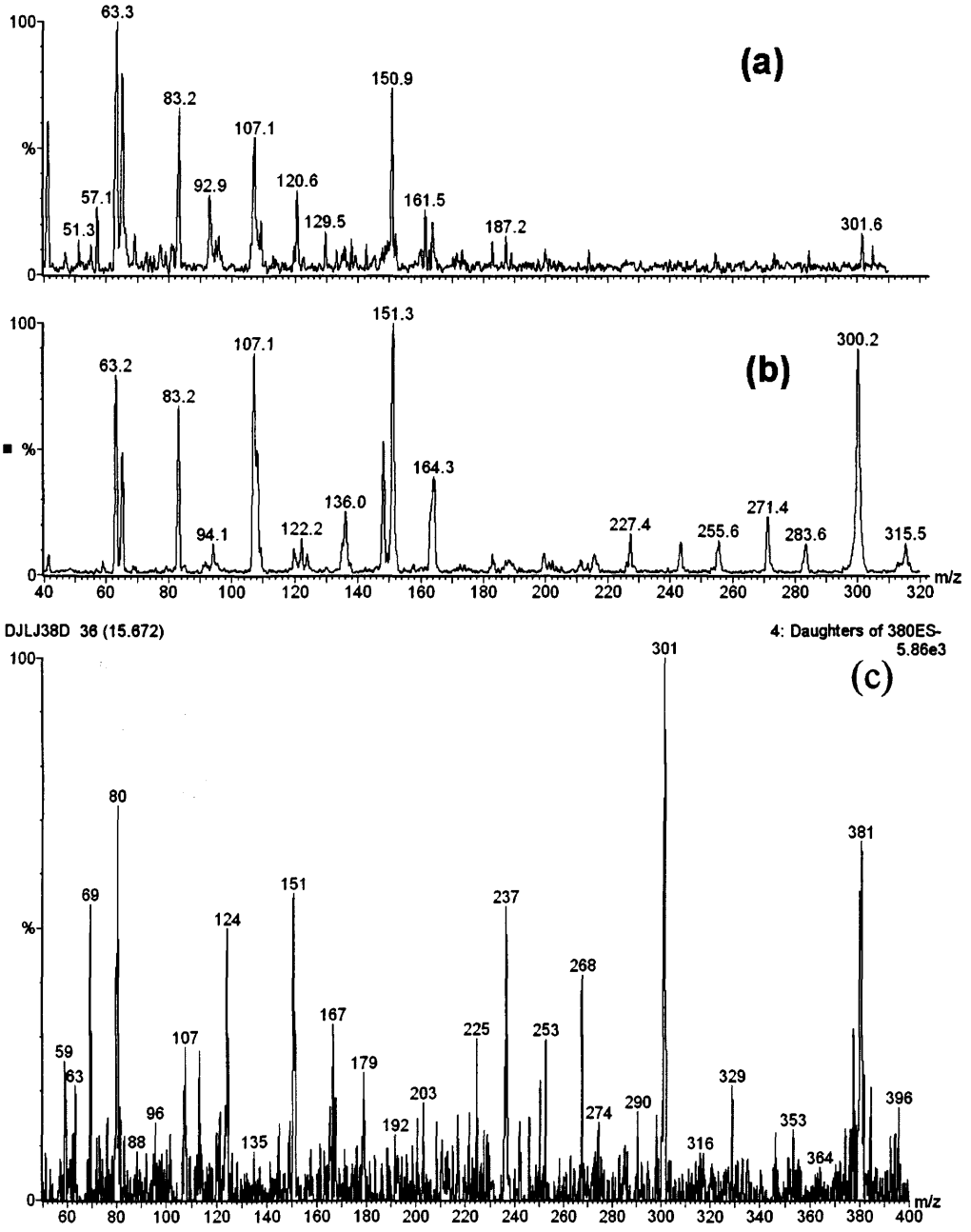
Characterisation of metabolites generated *in vivo.*

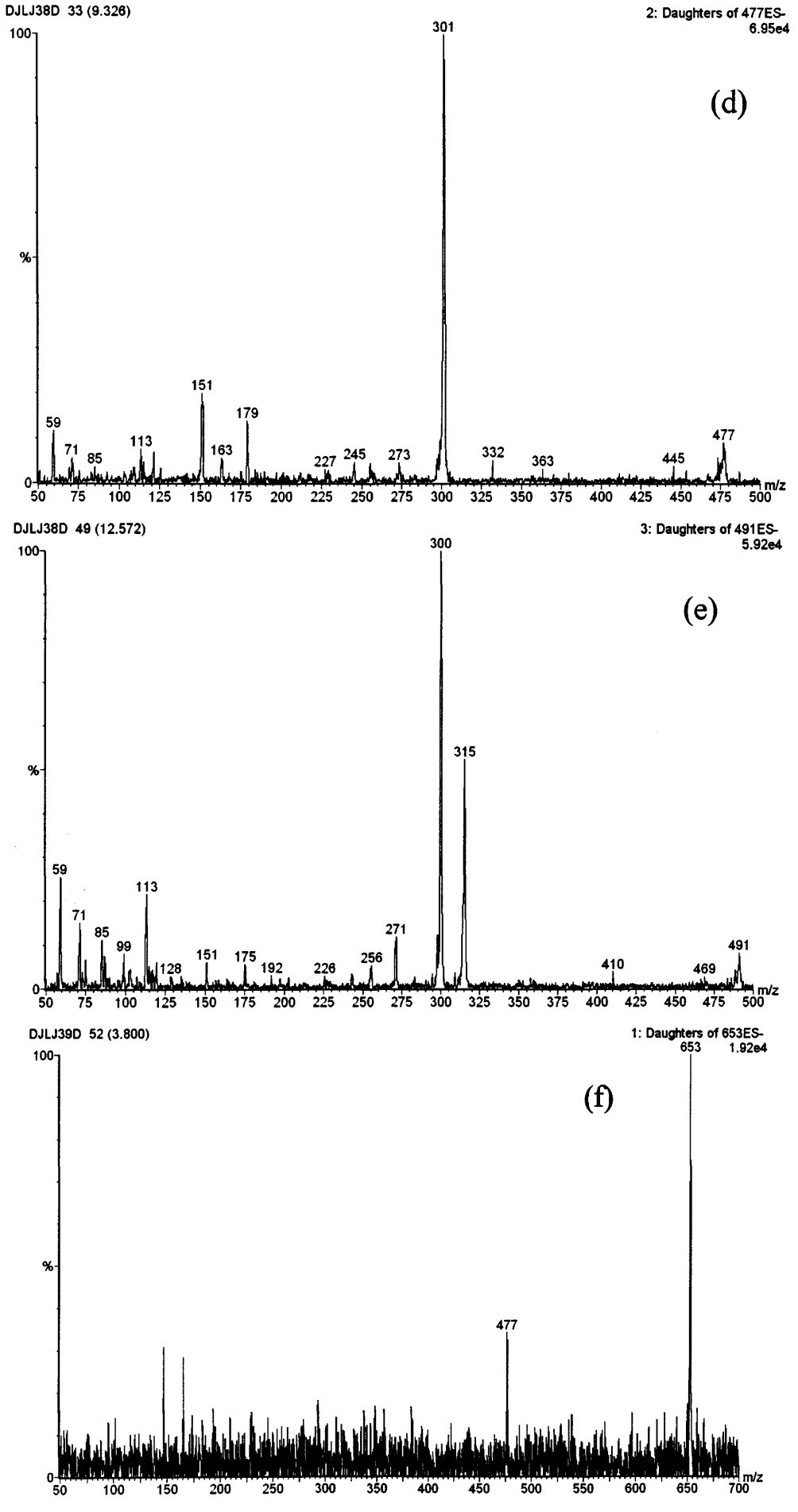
Urine the analysis of which is described in section 3.3.6 underwent further LC-MS/MS analysis. Metabolite ions were selected in the first mass spectrometer and fragmented in the hexapole collision cell. The resultant fragments yielded characteristic product ions. Not all of the metabolites were abundant enough to allow analysis by MS/MS. However, data was generated for the most prominent peaks of putative quercetin and isorhamnetin glucuronides and sulphates.

Figure 3.16(a) shows a product ion spectrum following LC-MS/MS of a peak that co-eluted with authentic quercetin. Shown in the spectrum are the ions that were found in the LC-MS/MS analysis of quercetin. The origin of the individual dissociation ions is described in more detail in section 2.3.3.

The peak eluting after quercetin produced a very similar spectrum to that of quercetin but with an additional ion of $[M-H]^-$ at m/z 315 (see Fig. 3.16(b)). The data suggests a difference of 14 mass units, which is consistent with a methyl group attached to quercetin.

Figure 3.16 *LC-MS/MS of quercetin metabolites extracted from rat urine. Shown are 5 product spectra obtained from individual peaks shown in Fig. 3.13 tentatively assigned quercetin (a), isorhamnetin (b), quercetin monosulphate (c), quercetin monoglucuronide (d), isorhamnetin monoglucuronide (e) and quercetin diglucuronide (f). All spectra obtained on a VG Quattro Bio-Q mass spectrometer using Ar as a collision gas.*




DJLJ38D 33 (9.326)
2: Daughters of 477ES-
6.95e4
(d)
DJLJ38D 49 (12.572)
3: Daughters of 491ES-
5.92e4
(e)
DJLJ39D 52 (3.800)
1: Daughters of 653ES-
1.92e4
(f)
m/z

The $[M-H]^-$ at m/z 477 (Fig. 3.16 D) dissociated to m/z at 301, an ion the presence of which confirms quercetin conjugation with glucuronide. The $[M-H]^-$ of m/z 491 (Fig. 3.16 E) dissociates to m/z 315 confirming the conjugation to isorhamnetin. The $[M-H]^-$ of m/z 653 (Fig. 3.16 F) dissociates to m/z 477. m/z 477 is most likely a species derived from a double glucuronide conjugate after the loss of one of the glucuronide moieties, $[M-176]^-$.

LC-MS and tandem MS allow greater scope for chemical identity of unknown compounds. The data presented in this chapter allows the definitive identification of individual peaks seen in HPLC chromatograms.

3.5 Discussion.

The work described in this chapter identifies and characterises, at least partially, quercetin metabolites. Efforts to biosynthesise metabolites in cellular fractions were unsuccessful. This result is consistent with previously published attempts, which also failed to generate metabolites in cellular fractions(Nielsen et al. 1998).

Attempts were made to generate species derived from quercetin as authentic reference compounds of potentially occurring methyl and sulphate metabolites Quercetin was methylated using chemical and enzymatic methods, and isorhamnetin was obtained but only in small amounts.

The sulphation of quercetin was successfully achieved by incubating quercetin with TEA-SO_3. Four monosulphate conjugates were produced and characterised by LC, MS and NMR. Each of the hydroxyl groups on the quercetin molecule, except that in the 5-position, underwent sulphation. These results are consistent with a report, according to which, quercetin did not undergo conjugation at the 5-position with glucuronic acid *in vitro*(Day et al. 2000). For the *in vitro* synthesis of quercetin glucuronides it was also found that the 7-position appeared to conjugate preferentially. The same was found for sulphation, with quercetin 7-O-sulphate being the predominant product of the chemical sulphation of quercetin.

Quercetin when injected intravenously, undergoes phase II metabolism. Methylation, as shown here and by others(Manach et al. 1997; Shali et al. 1991), is a major route of metabolism for quercetin. A comparison of peaks obtained for quercetin and isorhamnetin following enzymatic hydrolysis, suggests that methylation is quantitatively significant in quercetin metabolism. Quercetin and isorhamnetin undergoes extensive sulphation and glucuronidation

The LC-MS analysis shown in this chapter identifies that there are more isomers of quercetin mono-glucuronides than quercetin mono-sulphates, suggesting that glucuronidation occurs to a greater extent than sulphation. It has previously been shown that at least four hydroxyl sites in the quercetin molecule can be conjugated with glucuronic acid(Day et al. 2000; Oliveira and Watson 2000). The data presented here shows that when quercetin is administered i.v., at

least four quercetin glucuronides are formed. However, only two quercetin sulphate isomers are formed *in vivo*. As described earlier, sulphation can occur at four sites on the quercetin molecule. Consequently, at a chemical level, the quercetin molecule is equally susceptible to sulphation and glucuronidation. The greater extent of glucuronidation may be related to the relatively high levels of quercetin used in this experiment. Often, when a metabolic system is saturated, glucuronidation occurs in preference to sulphation(Gibson and Skett 1994). It has been shown that for genistein, patterns of conjugation (i.e. of sulphation or glucuronidation) were altered by fasting. Piskula found that the plasma of rats fasted prior to isoflavone administration contained significantly more sulphates and fewer glucuronides and mixed sulphate/glucuronide conjugates than the plasma of non-fasted animals(Piskula 1999). It is not clear whether dietary intake of quercetin would yield the same pattern of metabolites as that found after i.v. administration of quercetin.

The extensive phase II metabolism of quercetin is likely to facilitate the swift excretion of the molecule from the body.

4.0 Chapter Four. Occurrence and quantitation of quercetin metabolites.

4.1 Introduction.

There is a paucity of data concerning the metabolism of quercetin in humans (see section 1.5). Moreover, the disposition of quercetin after administration of the pure compound in contrast to a mixture, is poorly defined (as described in section 1.5). In the previous chapter, data on the characterisation of individual metabolites was presented. In the work described in this chapter, the occurrence of quercetin and metabolites in humans and rats was studied, and quercetin was quantified in the bile and plasma of rats. The quantitation and pharmacokinetics of quercetin in humans has been published previously(Ferry et al. 1996).

4.2 Experimental.

4.2.1 Materials and reagents.

Quercetin, HPLC grade dimethyl sulphoxide (DMSO), ammonium acetate, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dithiothreitol (DTT), magnesium chloride, dimethyl sulphide (DMS), S-adenosyl methionine (SAM), pyridine, dioxane and catechol O-methyl transferase, bovine serum albumin (BSA), sodium carbonate, sodium hydroxide, sodium-potassium tartrate solution, copper sulphate solution, phenol, HEPES buffer were all obtained from Sigma (Poole, UK). Disodium ethylenediamine-tetraacetic acid (EDTA), AnalaR grade was obtained from BDH (Poole, Dorset, UK). Triethylamine sulphate (SO_3-TEA), methanol (HPLC grade) and glycerol formal from Fisher (Loughborough, Leics., UK). Isorhamnetin and quercetin 3-O-sulphate was obtained from Extrasynthese (Genay, France).

4.2.2 Analytical chemistry.

Standard HPLC conditions as described in section 2.3 were employed and both chromatographic systems A and B as described in 2.3.2.4 were used.
For electrochemical detection, a Gynkotek model 300 solvent delivery system with an Antec electrochemical detector (Presearch, Letchworth Garden City, Herts., UK) set at an oxidation potential of +0.6V was used. Injection of the sample was *via* a Rheodyne 7125 injector fitted with a 100μL loop.

4.2.3 Sample preparation.

4.2.3.1 Plasma concentration following intravenous injection of quercetin.

Quercetin (6.25mg/kg) was injected into the lateral tail vein of six F344 rats, which had undergone terminal halothane anaesthesia. Five, 10, 20 and 40min after quercetin administration, rats underwent cardiac exsanguination with thoracotomy. Following blood collection, tissues (lungs, kidney and liver) were excised out and immediately frozen in liquid nitrogen.

Plasma was separated from blood by centrifugation. To 196µL of plasma, 4µL of 10mg/mL kaempferol was added and vortexed. To each aliquot, 400µL of DMSO/methanol (1:4, v/v) was added. Following rigorous vortexing, 200µL of the supernatant was removed and added to 200µL of deionised water. Fifty microlitres of this solution was injected onto the HPLC, which was eluted using chromatographic system B, described in section 2.3.2.4.

4.2.3.2 Biliary excretion of quercetin.

F344 Rats were anaesthetised by injecting i.p. 100µl of pentobarbitol (60mg/mL), following which the body cavity was opened and the bile duct located. Using micro-scissors, the bile duct was cut to allow the insertion of polythene cannulae (i.d. 0.28mm; o.d. 0.61mm) which was cut to approximately 30cm in length. The body temperature was maintained using a heat lamp. Bile was collected for 20mins following which, 100µL of quercetin in DMSO (to give a dose of 12.5mg/kg) was injected i.v. via the lateral vein in the tail. Bile was collected in twenty-minute intervals for two hours.

To 196µL of each aliquot of bile, 4µL of 10mg/mL kaempferol was added and vortexed. To each aliquot, 400µL of DMSO/methanol (1:4, v/v) was added. Following rigorous vortexing, 200µL of the supernatant was removed and added to 200µL of deionised water. Fifty microlitres of this solution was injected onto the HPLC, which was eluted using chromatographic system B, described in section 2.3.2.4.

The bile was also subjected to enzymatic hydrolysis by acidifying a 100µL aliquot of bile

with 20μL of 0.5M acetic acid and then adding 100μL of β-glucuronidase (5000U/mL)(Ader, Wessmann, and Wolffram 2000). This mixture was left to incubate for 30mins. After 30min, 196μL of the incubation mixture was removed and 4μL of kaempferol (10mg/mL) was added and the mixture vortexed. To each aliquot, 400μL of DMSO/methanol (1:4, v/v) was added. Following rigorous vortexing, 200μL of the supernatant was removed and added to 200μL of deionised water. Fifty microlitres of this solution was injected onto the HPLC, which was eluted using chromatographic system B, described in section 2.3.2.4. A comparison between conjugated quercetin and unconjugated quercetin could be made by comparing the concentration of quercetin obtained for hydrolysed and non-hydrolysed bile.

4.2.3.3 Urinary excretion of quercetin.

An experiment involving two groups of three F344 rats (200g) was conducted. All rats were anaesthetised using halothane and quercetin was either injected in the lateral tail vein or administered by gavage in DMSO (62.5mg/kg). After quercetin administration, the rats were transferred to metabolic cages that enabled the collection of urine at 8, 12 and 24h.

Urine was extracted using the same procedure as for plasma except omitting an internal standard. Urine was subjected to enzymatic hydrolysis by acidifying 100μL of urine with 20μL of 0.5M acetic acid and then adding 100μL of β-glucuronidase (2000U/mL) (Ader, Wessmann, and Wolffram 2000). This mixture was left to incubate for 30mins. After 30min, 196μL of the incubation mixture was removed and 4μL of 10mg/mL kaempferol was added. To each aliquot, 400μL of DMSO/methanol (1:4, v/v) was added. Following rigorous vortexing, 200μL of the supernatant was removed and added to 200μL of deionised water. Fifty microlitres of this solution was injected onto the HPLC, which was eluted using chromatographic system B, described in section 2.3.2.4.

4.2.3.4 Human plasma following the administration of quercetin.

Two sets of human samples were obtained:
Blood samples were obtained from cancer patients at the Queen Elizabeth Hospital, (Birmingham, UK), who no longer responded to standard therapy. Quercetin was formulated in analytical grade DMSO at a concentration of 50mg/mL. The drug was administered via the

side arm of a polypropylene bag through which RIMSO 50 (50% DMSO/ water, v/v) was slowly infused. A total volume of 50mL of RIMSO 50 was injected with each quercetin administration (250mg/m^2 over 5 min). Blood was obtained in 20mL volumes prior to administration and at 5, 10, 30, 60, 90 and 120min after administration of quercetin. The plasma was separated and stored at -80°C.

A single cancer patient, in whom standard therapy had failed, opted to take pure quercetin capsules three times a day, *per os*. The capsules contained 500mg of quercetin and were purchased from the Bristol cancer centre. Blood samples were taken at various time points before and after administration. Blood was required to mimic control blood for the single cancer patient. Plasma was separated from blood via centrifugation and treated as previously described.

It is relevant to note that quantitative analysis of these samples was not carried out due to the validation of the HPLC method being carried out long after the samples had undergone initial analysis.

Plasma was separated from blood by centrifugation. To 200µL of plasma, 400µL of DMSO/methanol (1:4, v/v) was added. Following rigorous vortexing, 200µL of the supernatant was removed and added to 200µL of deionised water. Fifty microlitres of this solution was injected onto the HPLC, which was eluted using chromatographic system B, described in section 2.3.2.4.

4.2.4 Extraction of metabolites from rat tissue.

Tissues were removed from rats which had recieved quercetin (6.25mg/kg, i.v.). Tissue (1g) was added to 10mL of ammonium acetate and homogenised. Twenty millilitres of DMSO/MeOH (1:4, v/v) were added to the homogenate and vortexed. Typically 5ml was centrifuged at 12000g for 15min. The supernatant was transferred to a clean tube and evaporated down using a nitrogen stream to about 100µL.

4.2.5 Calibration graph.

Quercetin and kaempferol stock solutions were made up in DMSO to give 10mg/mL solutions. Five concentrations of quercetin were made up to give a final concentration of 1, 2, 20, 100 and 200μg/mL. For each concentration of quercetin, 4μL of the solution was added to 192μL of mobile phase, 0.1M ammonium acetate, pH 5.15 / methanol (25:75, v/v). Four μL of 10mg/mL kaempferol was then added to each solution. A further 1mL of mobile phase was added to give a total volume of 1.2mL. Using this calibration graph, an equation for the line was calculated and a concentration for quercetin in samples could be derived. The calculated quercetin concentration is multiplied by 1.11 to take into account the recovery of the drug (i.e. 90% as described in section 2.3.3.1). Additionally, for the calculation of quercetin in bile the figure was multiplied by 1.96 to take into account the increase in volume used as a result of the incubation.

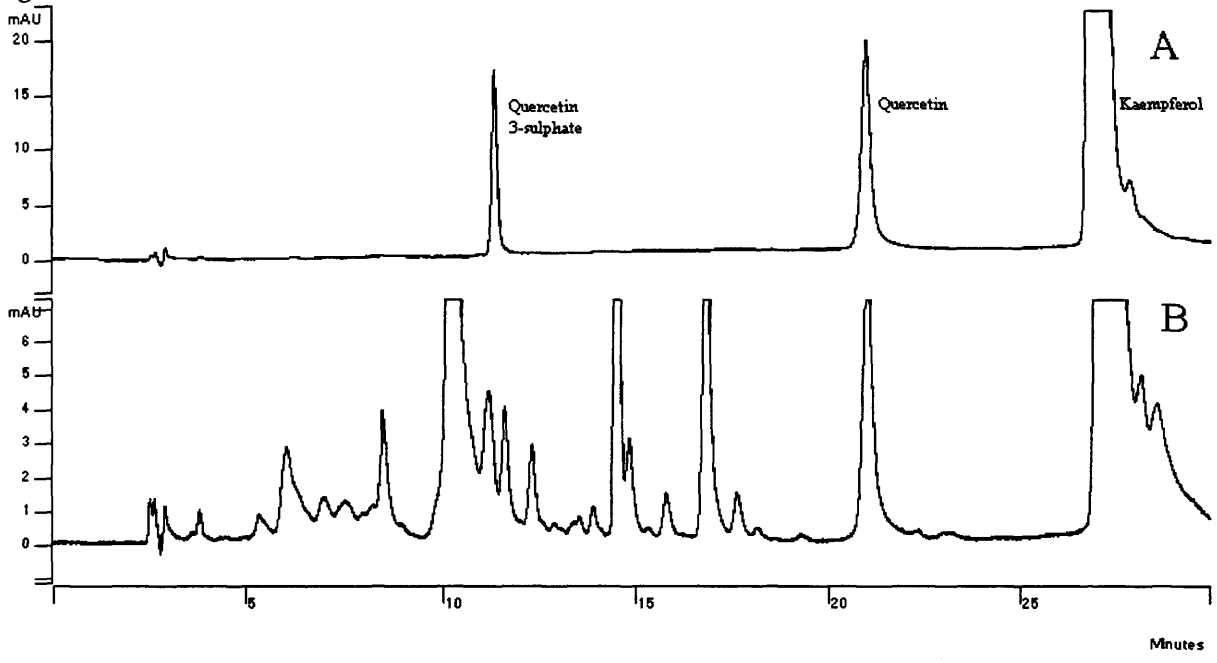
4.3 Results.

4.3.1 Analysis of rat plasma.

Quercetin (6.25mg/kg) was administered via the i.v. route. The analysis of plasma obtained 5, 10, 20 and 40min after administration yielded peaks that were absent in the control plasma and they were tentatively characterised as conjugates of quercetin on the basis of the identification described in chapter 3. A peak consistent with authentic quercetin was not observed at time points beyond 5min, presumably because quercetin was rapidly metabolised (data not shown). Metabolites of quercetin were also observed up to 20min, but beyond this time the peaks attributed to metabolites decreased significantly. The 5min time-point was chosen for subsequent experiments.

Fig. 4.1 shows the metabolite profile in rat plasma 5min after administration of quercetin. Using both the characterisation studies described in chapter three and co-elution of authentic standards, it can be seen that the plasma contains quercetin and metabolites. Quercetin and isorhamnetin are identified by their Rt at 20.9min and 28.5min respectively. Isorhamnetin is the second of the peaks that immediately follow kaempferol. Using kaempferol as the internal standard, the amount of unconjugated quercetin was calculated to be 4.1±1.2μg/mL (13.7±4.1μM; n=6). When the conjugated drug species were enzymatically hydrolysed the total amount of quercetin, i.e. conjugated and unconjugated was 23.2±3.4μg/mL (76.9±11.4μM;n=6). These results suggest that at the time point investigated, of the total quercetin related species circulating in the blood, approximately 80% was conjugated.

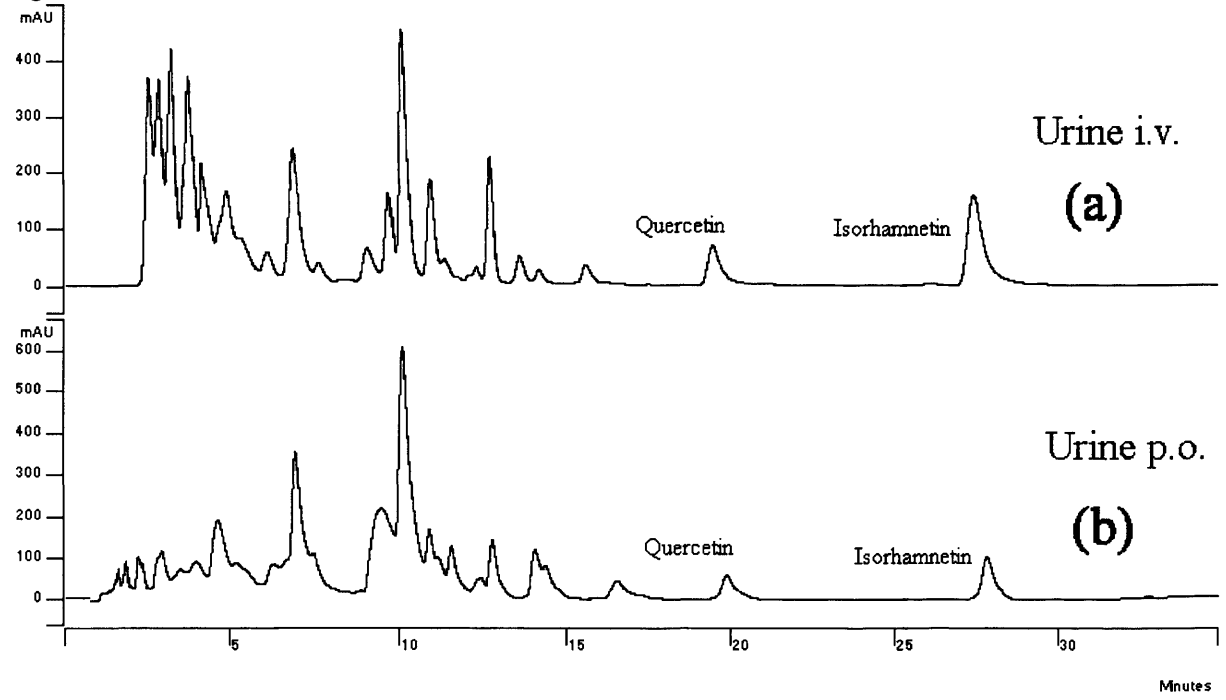
Figure 4.1 *Analysis of extracts of quercetin metabolites from rat plasma obtained following the administration of quercetin (B). Above the plasma extract chromatogram is a chromatogram of standards which contain quercetin 3-O-sulphate, quercetin and the internal standard, kaempferol (A). The internal standard is also present in the plasma sample. Isorhamnetin is the second of the peaks that immediately elute after the internal standard. Chromatographic system B as described in section 2.3.2.4 was used.*



4.3.2 Analysis of rat urine.

The bioavailability of quercetin was investigated by administering the drug (62.5mg/kg) to F344 rats i.v. and p.o. Urine was collected 4, 8 and 24h after administration. The intention was to evaluate the levels of quercetin in the urine for both routes of administration. This evaluation was not possible, because quercetin and isorhamnetin were absent from urine. In a previous experiment quercetin and isorhamnetin had been found in the urine when quercetin was administered by the i.v. (62.5mg/kg) or p.o. routes (1900mg/kg) but quantitation had not been undertaken then (Fig. 4.2). The discrepancy between the two results which were obtained at an interval of 2 years is unclear.

Figure 4.2 *HPLC analysis of an extract of quercetin metabolites from urine collected over 24h following (a) the i.v. administration of quercetin, 62.5mg/kg, and (b) the p.o. administration of quercetin, 1900mg/kg. Chromatographic system B as described in section 2.3.2.4 was used.*



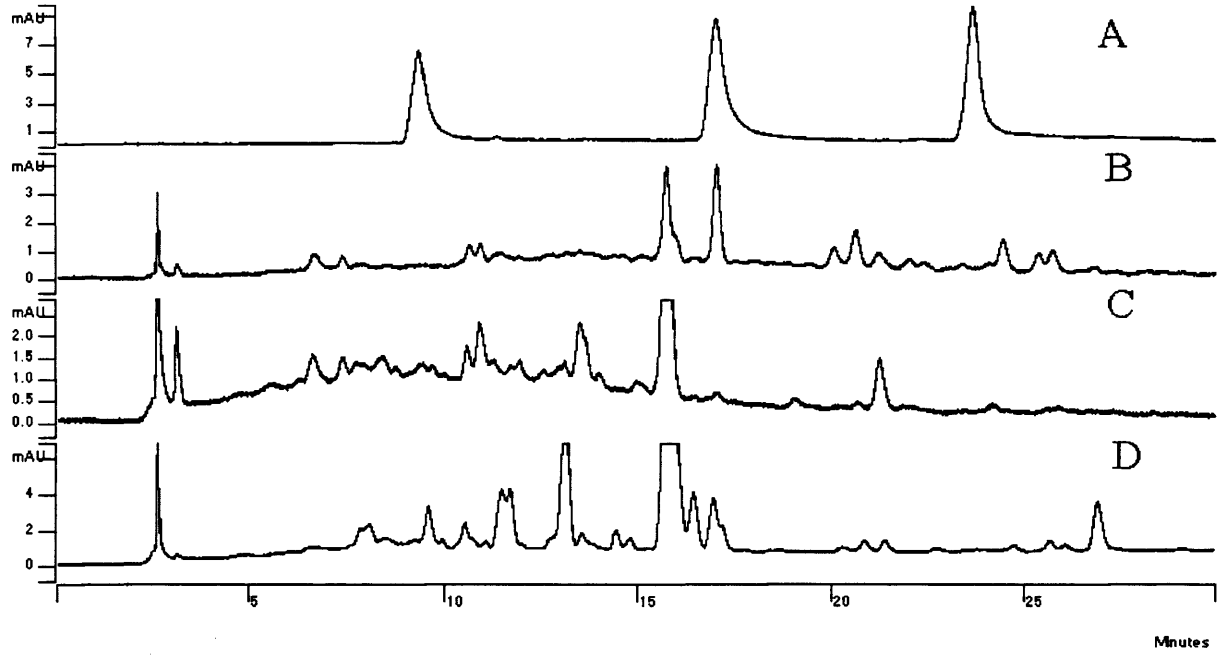
4.3.3 Analysis of rat tissue.

The presence of quercetin and its metabolites was analysed in rat tissues after i.v. administration of quercetin (6.25mg/kg). Tissues were treated as described in section 4.2.4. Fig. 4.3 shows that some tissues contained metabolites that were also present in the plasma. The liver extract yielded more metabolites than other tissues, and there were only trace amounts of quercetin and isorhamnetin. The greater presence of metabolites in the liver is probably related to hepatic conjugation of quercetin and isorhamnetin. The kidney has very low levels of quercetin, and metabolites could not be detected in this tissue. In section 3.3.6, an experiment is described where quercetin underwent renal excretion, as demonstrated by the presence of quercetin and metabolites in urine (as shown in Fig. 4.25). Nephrotoxicity has been attributed to quercetin(National Toxicology Program 1992) and it has been demonstrated that quercetin can be catabolised in kidney cells(Douglass and Hogan 1958). These two strands of evidence demonstrate that quercetin is distributed to the kidney. It may be that the levels are so low that metabolites are difficult to detect.

The extract of lung tissue contained metabolites. Following i.v. administration of a compound through the lateral tail vein, the lung is the first major organ that the compound reaches after the heart. At the 5min time point used, it is conceivable that metabolites have been generated

in the lungs.

Figure 4.3 *Analysis of extracts of quercetin metabolites in tissues removed from rats five minutes after administration of quercetin. The four chromatogram represent (A) three standards (quercetin 3-O-sulphate (Rt 9.8), quercetin (Rt 17.1) and isorhamnetin (Rt 23.7)), (B) extracted quercetin metabolites from lungs, (C) extracted quercetin metabolites from kidneys and (D) extracted metabolites from liver. Chromatographic system B as described in section 2.3.2.4 was used.*

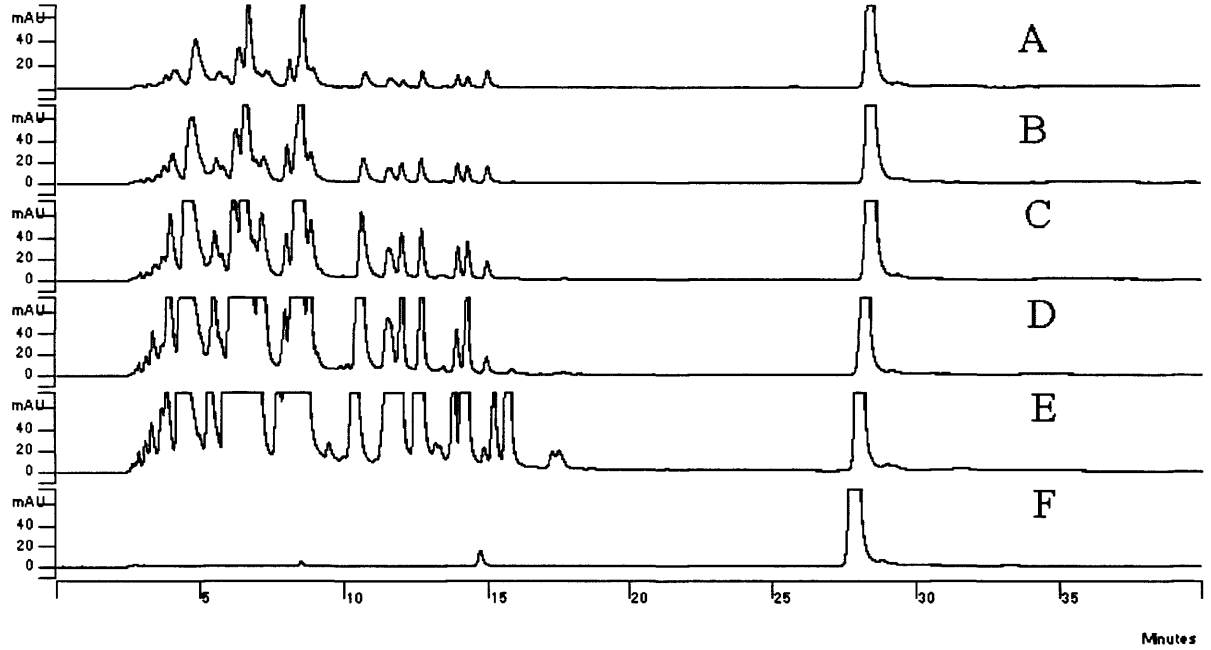


The results presented here indicate that quercetin can be distributed extra-hepatically to other organs thus allowing it greater scope for therapeutic use.

4.3.4 Analysis of rat bile.

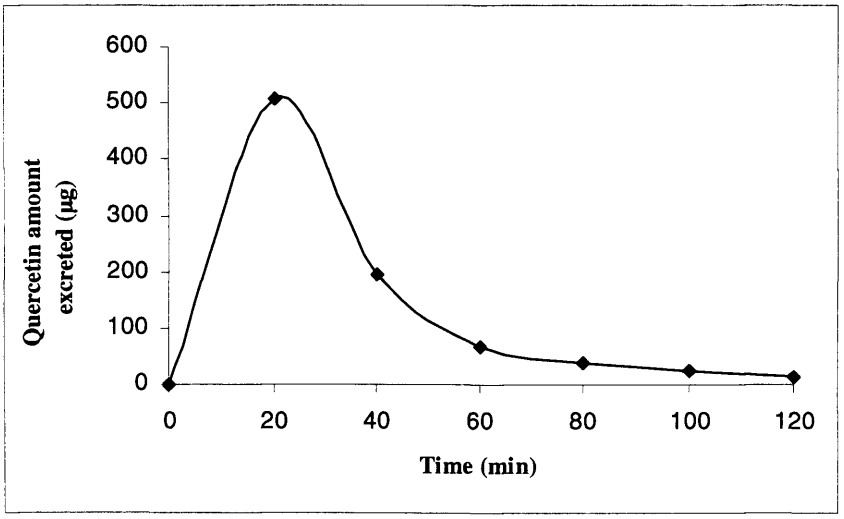
Quercetin (12.5mg/kg) was injected intravenously into the lateral tail vein and bile collected both before administration and afterwards. In Fig. 4.4 are seven chromatograms showing the analysis of bile prior to, and after administration of quercetin. Following administration, bile was collected for 2h, at twenty-minute intervals.

Figure 4.4 *Analysis of extracts of bile showing the biliary excretion of quercetin and its metabolites. Bile was collected for twenty minutes prior to administration and every twenty minutes for two hours after i.v. administration of quercetin in glycerol formal. The six chromatograms depict pre-administration (F), and 0-20, 20-40, 40-60, 60-80 and 80-100 (E-A respectively). Chromatographic system B as described in section 2.3.2.4 was used.*



When bile extract was subjected to β-glucuronidase, both quercetin and isorhamnetin were released (as depicted in Fig. 4.6). Fig. 4.5 shows the amount of quercetin excreted in the bile following quercetin administration and the reduction in the amount of quercetin excreted over time. The total amount of quercetin excreted was 850μg over the 2h.

Figure 4.5 *A graph showing the amount of quercetin excreted in bile over time, following the i.v.administration of 12.5mg/kg of quercetin.*

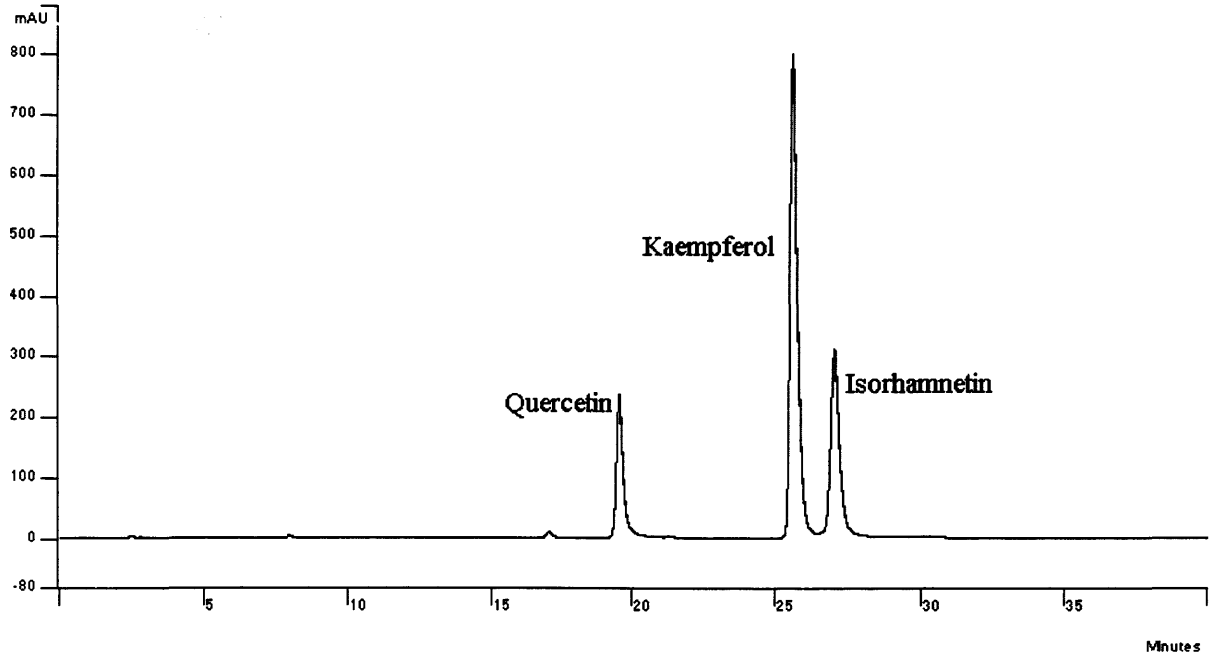


It can be seen in Fig. 4.5, that after 2h, quercetin metabolites were excreted in the bile following intravenous injection, thus suggesting, that sufficient levels of quercetin were

available for metabolism. The fact that so many conjugates are present in the bile is consistent with the notion that quercetin undergoes first pass metabolism. The same observation has been made in humans(Ferry et al. 1996).

It is also pertinent to mention that in the chromatograms of the enzymatically hydrolysed bile, the peak for isorhamnetin was larger than that of quercetin. This observation is demonstrated in Fig. 4.6, which shows a chromatogram depicting hydrolysed bile collected between 40 and 60min after quercetin administration. In terms of peak area, there was approximately 30% more isorhamnetin than quercetin. This relative proportion remained the same for the duration of the observable analyses. Given that equimolar concentrations of quercetin and isorhamnetin realise equal peak areas, it can be concluded that the extinction coefficients of quercetin and isorhamnetin are approximately equal. This evidence suggests that at least an equal amount of isorhamnetin, as compared to quercetin, persists in bile. Chromatographically, tamarixetin (4'-O-methyl quercetin) is distinguishable to isorhamnetin as it elutes approximately 0.8min later than isorhamnetin. Hence, tamarixetin is not found in the rat bile.

Figure 4.6 *Analysis of hydrolysed bile showing the proportion of quercetin to isorhamnetin. Shown are three peaks: quercetin, kaempferol and isorhamnetin. Chromatographic system B as described in section 2.3.2.4 was used.*

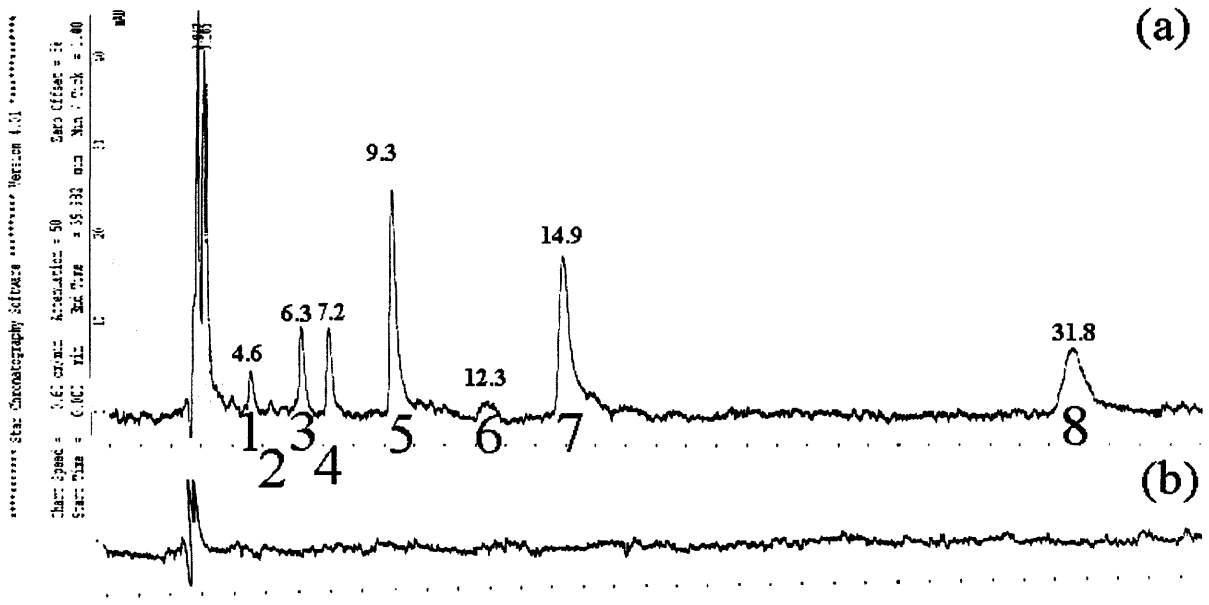


Consequently, it can be concluded that when quercetin is administered i.v. biliary excretion is a major route of metabolism for quercetin.

4.3.5 Human metabolism of quercetin.

Samples of human plasma were obtained in order to identify the metabolites generated when patients are given quercetin. Shown in Fig. 4.7 are a number of metabolites not found in human control plasma. The chromatographic system used was an isocratic system that did not distinguish between many of the conjugates.

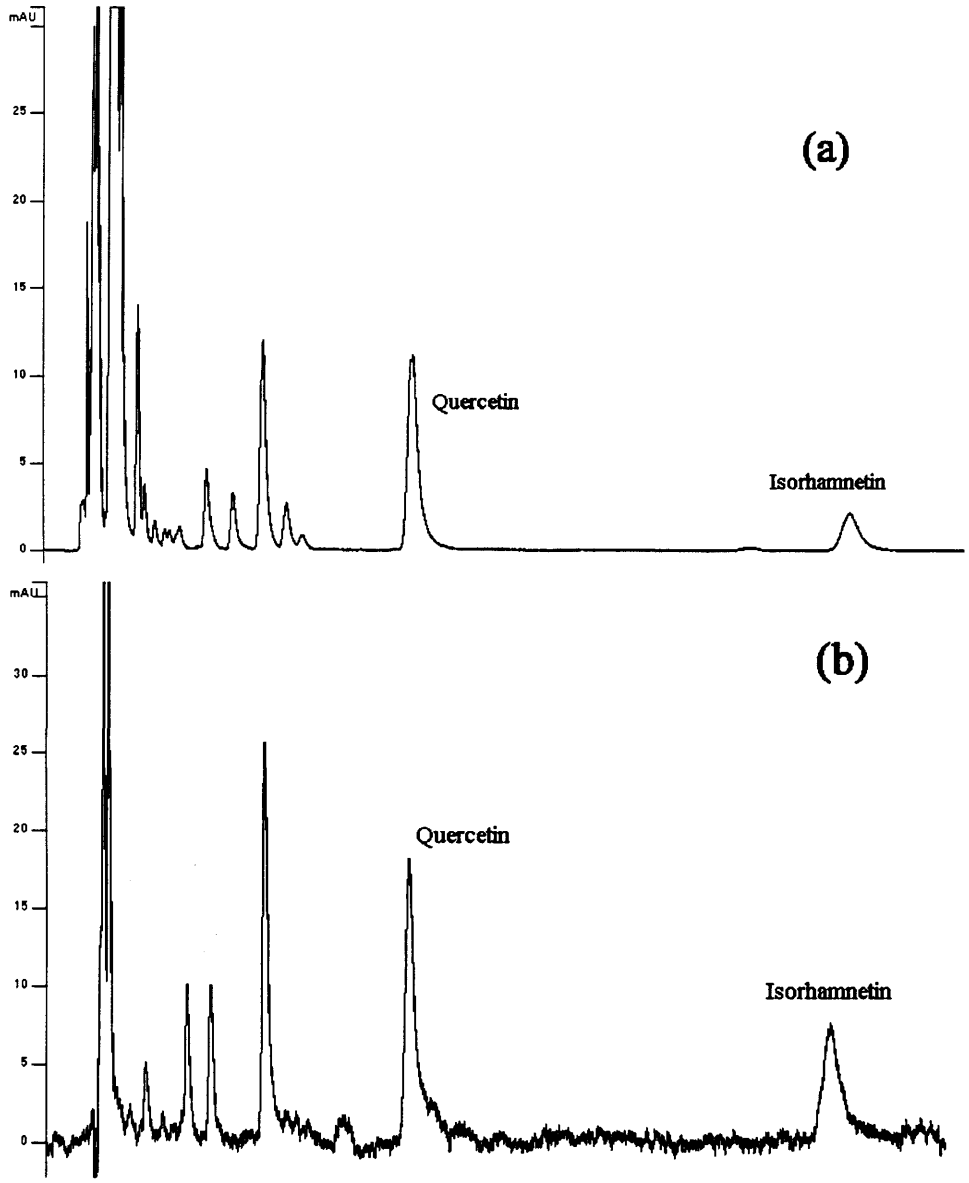
Figure 4.7 *Analysis of extract of quercetin and metabolites in human plasma following administration of quercetin (a). and in control plasma prior to administration of quercetin (b). A Varian HPLC was used as described in section 2.3.2.1. Chromatographic system A as described in section 2.3.2.4 was used for separation.*



The identification of the metabolites found in human plasma was carried out using off-line LC-MS. MS identified peak 7 to be quercetin (m/z 301 and co-elution with a standard), peak 8 is isorhamnetin (m/z 315 co-elution with a standard), peak 5 is a sulphate conjugate (m/z 381). The remaining metabolites can be tentatively assigned as gluco-sulphate conjugates.

The metabolic profile seen in human plasma is very similar to that found in rats. Fig. 4.8 shows a direct comparison between plasma obtained from both humans and rats following the administration of quercetin.

Figure 4.8 *A comparison of the metabolites in human (b) and rat (a) plasma following the intravenous administration of quercetin. A Varian HPLC was used as described in section 2.3.2.1. Chromatographic system A as described in section 2.3.2. was used.*



Quercetin and isorhamnetin are present in both the chromatograms from human and rat plasma, as well as a sulphate conjugate.

The rat plasma would appear to contain more conjugates that were double conjugated with glucuronide and sulphate moieties, which elute close to the solvent front. However, the metabolic profiles produced by rats and humans appear to be similar. The results suggest that F344 rats might be suitable as a qualitative model of human metabolism.

When patients were administered quercetin (250mg/m^2) several plasma samples were taken

after administration (up to 120min). Each of these samples were extracted in exactly the same way as for rats and analysed again using chromatographic system A (described in 2.3.2.4.). Fig. 4.9 shows chromatograms of quercetin control plasma and post-administration plasma, ranging from 5min after administration up to and including 120min after administration.

Figure 4.9 *Analysis of extracts of quercetin and metabolites in plasma following the intravenous administration of quercetin. Chromatographic system A as described in section 2.3.2.4 was used. Chromatogram (a) is an extract of plasma prior to administration and chromatograms; (b-g) are plasma samples obtained 5, 10, 30, 60, 90 and 120min after administration of quercetin respectively.*

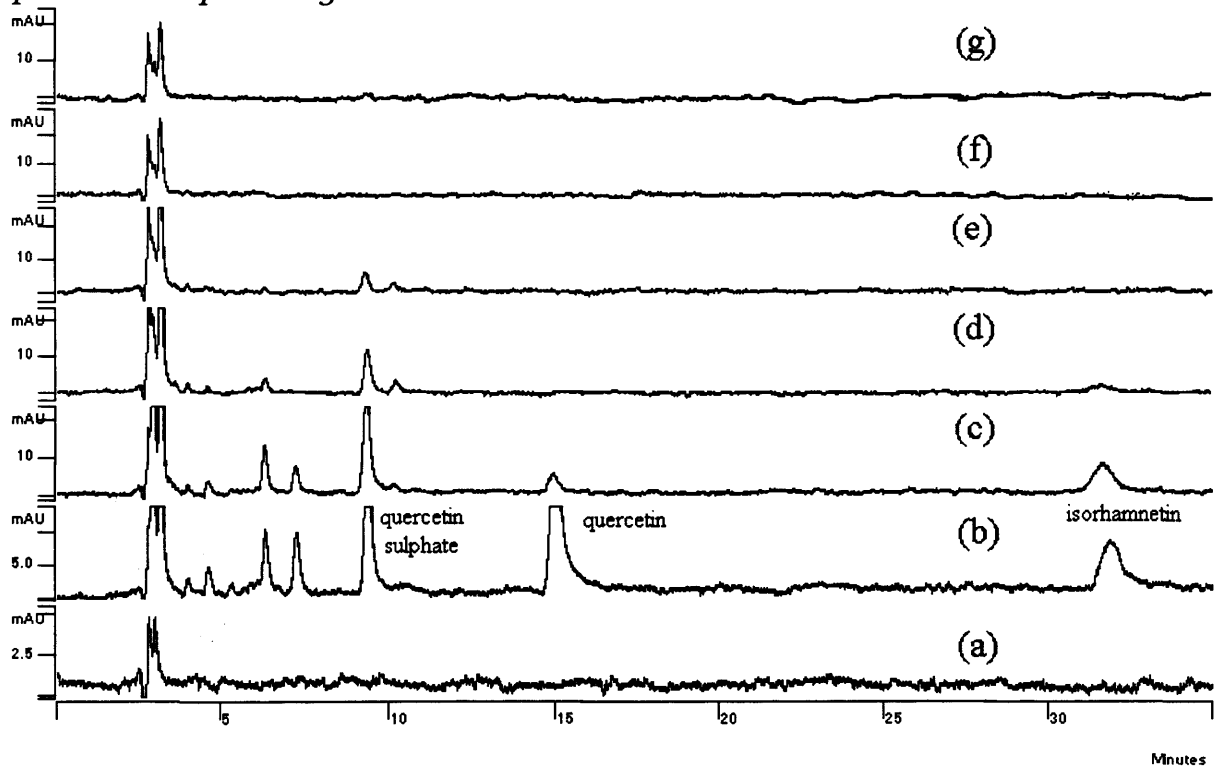


Fig. 4.9 shows the relative change in quercetin and metabolites in human plasma over a time course following i.v. administration of quercetin. The peak co-eluting with quercetin (annotated in chromatogram b) disappeared within 30min. By 10min quercetin and isorhamnetin had decreased significantly.

Peaks that elute prior to quercetin, identified as conjugates in chapter 3, can be seen to be present following quercetin administration. The decline in quercetin levels is accompanied by a decline in conjugate levels. Metabolites of quercetin in humans following i.v. administration are cleared rapidly. After 30min quercetin only persists as conjugates. While quercetin is undetectable after 30min, quercetin sulphate is detectable after 60min. In addition, the polar conjugates seen close to the solvent front persist after 60min (chromatogram e).

Also shown in Fig. 4.9, is the absence of quercetin or its metabolites in chromatogram f, which represents plasma after 90mins. As mentioned previously by Ferry *et al*, the clearance rates are consistent with hepatic flow, indicating a role for the liver in quercetin elimination(Schopke and Bartlakowski 1997).

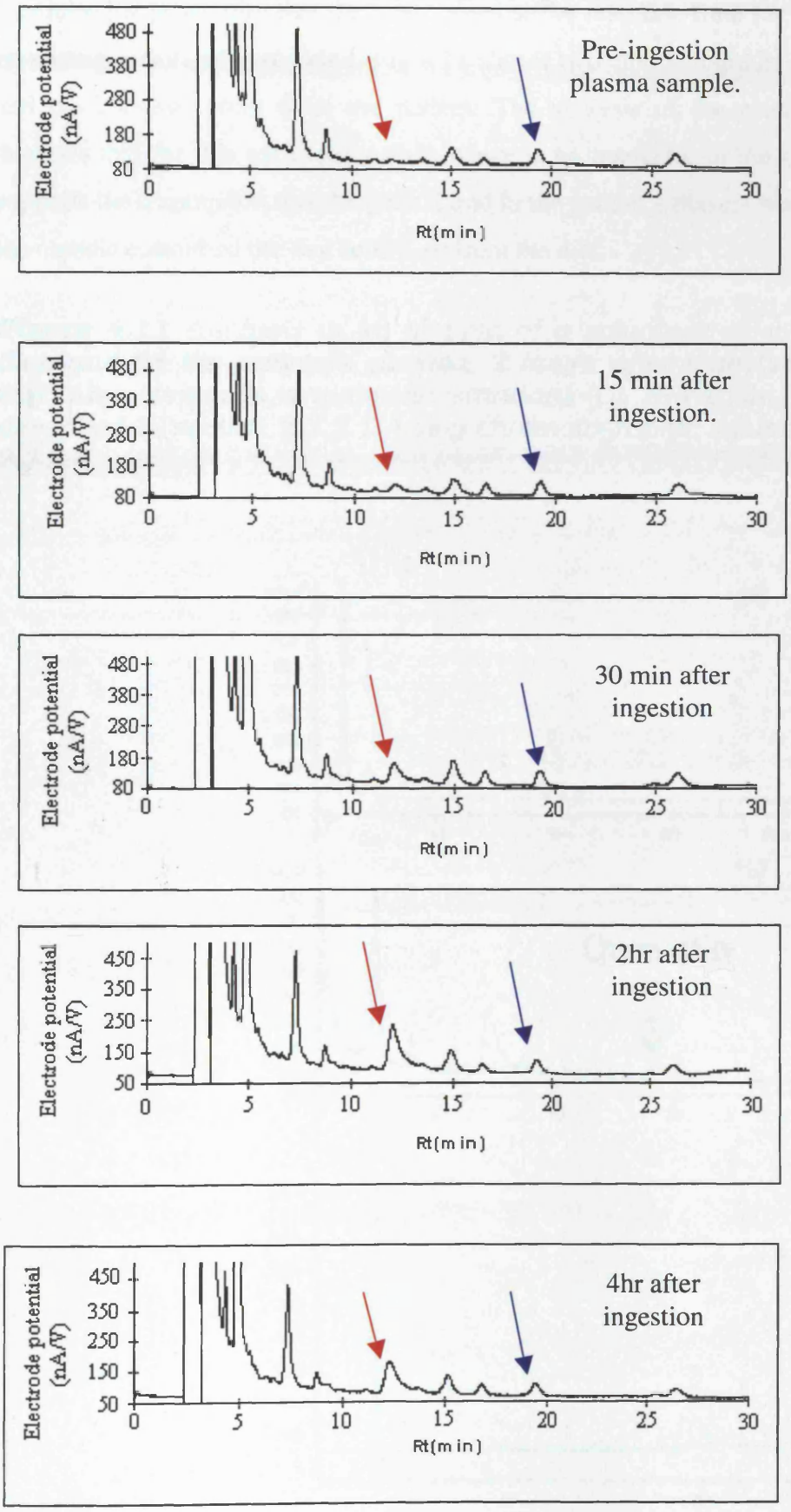
The exposure of a patient to quercetin administered intravenously resulted in quercetin being rapidly conjugated, and persisting in the plasma for a relatively short time. The same trend was also observed for the intravenous injection of quercetin into rats. Subsequently, one patient received quercetin orally and the effect of oral administration on the generation of metabolites and the persistence of quercetin in the plasma was investigated. With the reported low absorption of quercetin (see section 1.5.2), it was suspected that the sensitivity of analysis needed would have to be very high, and consequently electrochemical detection was utilised.

Fig. 4.10 shows ECD-chromatograms of plasma extracts at various times after the oral administration of quercetin. The ECD method had a detection limit for quercetin of 7nM (2ng/mL)(Jones et al. 1998). Fig. 4.8 suggests that the plasma levels of quercetin did not exceed 48nM. The same figure also shows additional peaks in the chromatogram, which were not characterised. While ECD is a very good detection method in terms of sensitivity, it does not have the same specificity as UV detection, at 375nm. Consequently, it is unclear whether the other peaks are related to quercetin. However, the peak with Rt 12.05 appeared to change in peak area during the time course. This peak was not in the pre-administration sample, and gradually increased to a maximum concentration at the 2h-time point, after which it decreased. The presence of the peak suggests the existence of an unidentified metabolite. The metabolite was suspected to be a sulphate conjugate based on the work described in chapter 3.

Quercetin was also detected in the pre-administration sample in this patient. It is unlikely to be an artefact of the assay, as the analysis of the sample was repeated and the same result was obtained. As quercetin ingestion had been part of ongoing treatment, the patient had taken a capsule 12 hours before the start of this study. It is conceivable that quercetin was absorbed slowly through the intestinal wall. Thus, it might be present in the plasma at low concentrations for a very long time due to slow absorption from the intestine.

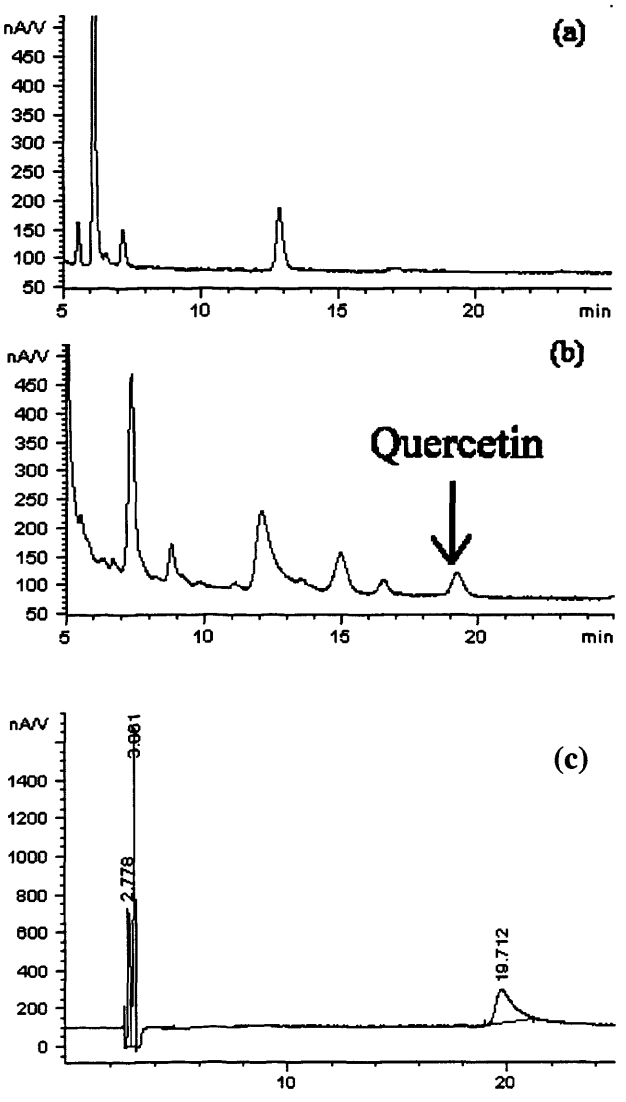
It is also possible that the patient had consumed quercetin in the diet, which would lead to an over-estimation of quercetin derived from the capsule.

Figure 4.10 *HPLC-ECD chromatograms of plasma extracts from a patient who had ingested 500mg of quercetin. A Varian HPLC was used as described in section 2.3.2.1. using chromatographic system A as described in 2.3.2.4.*



To correct for the absence of a suitable negative control, a blood sample was obtained from a volunteer who consumed a quercetin-free diet for a week prior to phlebotomy, so as to minimise the possibility that the peak found at the retention time for quercetin was due to a co-eluting, unrelated compound. Fig 4.11 shows two chromatograms of quercetin-free blood and the 2hr-time point from the patient. The analysis of the plasma from the volunteer suggests that for this particular person, there is no quercetin in the blood. This observation supports the assumption that the peak found in the patient's plasma was quercetin, either from the capsule consumed the day before, or from the diet.

Figure 4.11 *Analysis of (a) plasma of a volunteer on a quercetin-restricted diet, and (b) the patient's plasma, 2 hours after ingestion of quercetin (500 mg). Also shown is a quercetin standard (C). A Varian HPLC was used as described in section 2.3.2.1. using chromatographic system A as described in 2.3.2.4.*



4.4 Discussion.

When F344 rats were treated i.v. with quercetin, the parent compound could only be detected for 5min after administration. After this time, quercetin disappeared, by metabolism to methyl, sulphate and glucuronide conjugates. These conjugates were swiftly cleared until they had disappeared altogether at 90min. Plasma levels of conjugated quercetin reached 23.2±3.4μg/mL at 5min. This figure equates to approximately 77μM. Unconjugated quercetin amounted to 4.1±1.2μg/mL which equates to about 14μM. This level of quercetin is of an order of magnitude sufficient to elicit biological activity (see section 1.4.2).

The plasma levels achieved in rats parallels the levels seen in humans(Ferry et al. 1996). Using formulae derived from toxicity studies of anti-cancer drugs, factors can be used to convert mg/m^2 doses to mg/kg doses(Freireich et al. 1966). If the dose is calculated for the human and rat studies in mg/kg, the plasma level achieved in rats 5min after quercetin administration (4.1μg/mL) is approximately 4.5% of that found in humans immediately after quercetin administration (90μg/mL). The actual quercetin mg/kg dose given to rats (6.25mg/kg) was only one fourth of the dose given to humans (25.54mg/kg). However, if the dose is calculated in mg/m^2, the dose given to rats ($40.6mg/m^2$) is also 4.5% of that given to humans ($945mg/m^2$). Consequently, it can be seen that if dosage is expressed in terms of mg/m^2, the plasma levels achieved in rats mirror those found in humans.

Biliary excretion is the predominant route of metabolism for quercetin in rats, with 850μg excreted in 2h following the administration of 2.5mg (total dose) of quercetin. Eight hundred and fifty μg constitutes approximately one third of the total amount of quercetin administered. Furthermore, when quercetin conjugates in bile were hydrolysed, isorhamnetin existed at levels that were the same as, or greater than those for quercetin. Consequently, at least two thirds of the administered quercetin was excreted in the bile as conjugated forms of quercetin and isorhamnetin. The implication of these observations is that quercetin undergoes extensive first-pass metabolism, a phenomenon which has also been shown for humans(Ferry et al. 1996).

Urine from rats treated with quercetin either i.v. or p.o. in an experiment designed to allow quantitation, did not contain quercetin or isorhamnetin. In contrast experiments in the previous chapter (cf. Fig 3.13) had shown the presence of quercetin and isorhamnetin in urine. The absence of quercetin and isorhamnetin is may have been due to the reduced dose given to

the rats in the bioavailability assessment experiment (62.5mg/kg). Whereas in the characterisation studies described in chapter three between 1900-2500mg/kg of quercetin were administered to rats.

Quercetin was found in lungs and liver, and to a small extent in kidney. This observation shows that following i.v. administration, quercetin is distributed to extra-hepatic tissues.

The apparent predominance of a sulphate metabolite when quercetin is administered orally is noteworthy. Typically, in xenobiotic metabolism *in vivo*, sulphation predominates over glucuronidation when substrate concentration is low(Gibson and Skett 1994). It is conceivable that glucuronidation of quercetin occurs most prominently at higher substrate concentration, suggesting a difference in metabolism depending on the initial concentration of quercetin that the body is exposed to. The impact of quercetin concentration on metabolic profile was not investigated, but it maybe that quercetin preferably undergoes sulphation at low concentrations, such as that achieved after intake with the diet, and becomes more susceptible to glucuronidation at high levels as those achieved after administration of high doses of pure quercetin. The reason for the persistence of the sulphate metabolite is unclear, but it may be due to enterohepatic cycling that cleaves the aglycone and allows it to be reabsorbed and further conjugated. Conjugation may occur at the site of the intestine as described by Ader *et al* for quercetin(Ader, Wessmann, and Wolffram 2000).

Also shown in this chapter is the relevance of using F344 rats as a model for human metabolism. The metabolite profile found in both rat and human plasma following i.v. administration shows sufficient similarity to demonstrate the usefulness of the rat as a model. Also, in line with rat plasma clearance, the disappearance of quercetin, isorhamnetin and metabolites from human plasma was rapid, suggesting tentatively that the relative bioavailability of quercetin is low, which is in accordance with studies in the pig(Ader, Wessmann, and Wolffram 2000), rat(Morand et al. 1999) and humans(Ferry et al. 1996).

The disappearance of quercetin from the plasma and its excretion in urine and bile shows that the levels obtainable in the organism would probably be limited due to its conjugation and subsequent rapid excretion. Poor bioavailability has previously been cited for quercetin (Ader, Wessmann, and Wolffram 2000; Gugler and Leschik 1975; Ferry et al. 1996).

5.0 Chapter Five. Effect of quercetin and its metabolites on PGE-2 levels in HCA-7 cells.

5.1 Introduction.

In section 1.8 the role of COX-2 in malignancy is outlined. A number of cancer chemopreventative agents are thought to exert their activity via interference with COX-2. This chapter will describe experiments carried out to assess the effect that quercetin and its metabolites have on levels of PGE2 in a human colon adenomatous cell line, HCA-7. This cell line is a reasonable model for certain biochemical features of human colon carcinogenesis as the cell line constitutively expresses both COX-1 and COX-2 and produces high levels of PGE2. The aim of the work described in this chapter is to test the hypothesis that quercetin and its metabolites affect COX-2 levels.

5.2 Experimental.

5.2.1 Materials and reagents.

Quercetin, rutin, HPLC grade dimethyl sulphoxide (DMSO), ammonium acetate, were all obtained from Sigma (Poole, UK). Disodium ethylenediamine-tetraacetic acid (EDTA), AnalaR grade was obtained from BDH (Poole, Dorset, UK). Methanol (HPLC grade) and glycerol formal from Fisher (Loughborough, Leics., UK). Isorhamnetin and quercetin 3-O-sulphate was obtained from Extrasynthese (Genay, France). Prostaglandin E2 EIA kit - monoclonal was obtained from Cayman chemical (Michigan, USA).

5.2.2 Cell culture.

HCA-7 Cells (colony 29) were obtained from Dr. Sandra Kirkland (Imperial College, London) who kindly gave permission to use the cell line. Cell medium used was Gibco Dulbeccos MEM medium with Glutamax. The medium is without sodium pyruvate, with 4500g/L glucose and with pyroxidine. Prior to use, medium was supplemented with 10 % (v/v) fetal calf serum (FCS) and penicillin/streptamycin (1000units/mL and 500units/mL respectively).

Cells were passaged by the removal of all cell medium followed by washing twice with 10mL of PBS. Following the removal of the second wash, 5mL of trypsin solution was added. After 5-10mins incubation at 37°C, 5mL of medium was added to the trypsin solution. This mix of trypsinised cells and medium was transferred to a tube and centrifuged for 3min at 200g. The

supernatant was removed and the cells reconstituted in 10mL of medium to form a homogenous suspension. Typically, 1mL of the suspension was added to 19mL of fresh medium to give a 1/20 dilution.

5.2.3 Initial treatment of cells with quercetin.

A pilot study assessed the ability of quercetin to alter the synthesis of PGE2 in HCA-7 cells. Cells were harvested into 5 plates, each containing 10mL of medium. The medium was removed from the plates. To 7.5mL of medium, 75µL of 50mM quercetin in DMSO was added. To all five plates, 9mL of unspiked medium was added. To three of the plates (Q1, Q2 and Q3) 1mL of the spiked medium was added to give a final concentration of 50µM. To one of the remaining plates (untreated) 1mL of unspiked medium was added, while to the final plate (DMSO control), 1mL of medium containing 10µL of DMSO was added. Cells were left to incubate at 37°C.

After 6h, 1mL of medium was removed. One mL of fresh medium was added and another 1mL of sample was taken 24h after the initial quercetin incubation. Both samples once removed were frozen prior to analysis

5.2.4 Dose dependent effect of quercetin on PGE2 in HCA-7 cells.

Five concentrations of quercetin were assessed for their effect on PGE2 levels in HCA-7 cells. 1, 10, 25, 50 and 75µM of quercetin were chosen as the final concentrations. An untreated control and a DMSO control, as used in the pilot experiment were also incorporated into the experiment. Duplicates were used for each concentration and controls. One mL of medium was removed from each of the plates after 6h and analysed.

5.2.5 Comparison of the inhibition of PGE2 by quercetin and its metabolites.

This experiment set out to identify whether the metabolites of quercetin elicited any effect on PGE-2 levels, as compared to quercetin. Five agents were used: Quercetin, isorhamnetin, rutin, quercetin 3-O-sulphate, quercetin 7-O-sulphate and tamaraxetin. Each agent, carried out in duplicate, was incubated in the cells at 10µM. This experiment was repeated three times and the percentage decrease in PGE2 levels as compared to the controls were plotted.

5.2.6 Measurement of PGE2 in cell culture medium.

The measurement of PGE2 in cell culture medium was carried out by using a kit which utilises a competitive enzyme immunoassay (Cayman chemical company). The assay is based on the competition between PGE2 and a PGE2-acetylcholinesterase conjugate (PGE2 tracer) for a limited amount of PGE2 monoclonal antibody. Because the concentration of the PGE2 tracer is held constant while the concentration of PGE2 varies, the amount of PGE2 tracer that is able to bind to the PGE2 monoclonal antibody will be inversely proportional to the concentration of PGE2 in the well. This antibody-PGE2 complex binds to a goat anti-mouse polyclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and the proprietary 'Ellmans reagent' (which contains the substrate to acetylcholinesterase) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of PGE2 tracer bound to the well, which is inversely proportional to the amount of free PGE2 present in the well during incubation.

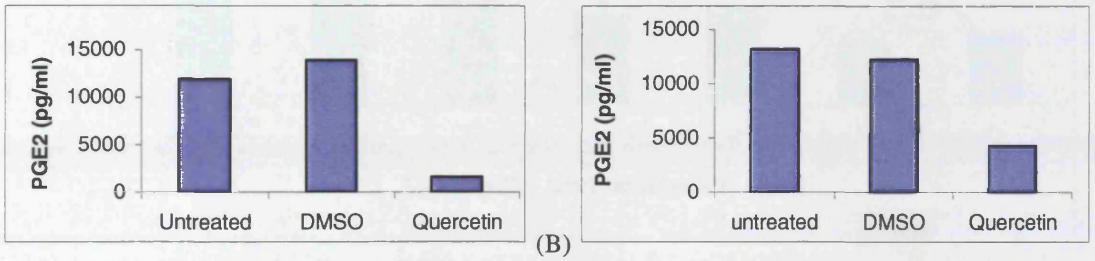
5.2.7 Calculation of PGE2 levels in HCA-7 cells.

The calculation of PGE2 levels was carried out as described in the Bioassay handbook (Cayman Chemical). In essence a calibration line was constructed from which readings were made. For each experiment the calibration line was different due to different times of exposure of the Ellmans reagent. Additionally, the assay is only linear between 20 and 80% of the x-axis. Consequently, any values that fell outside of this range were re-assayed.

5.3 Results.

In a pilot experiment, HCA-7 cells were treated with a solution of 50μM quercetin, which was thought likely to elicit a response. PGE2 levels were markedly reduced by quercetin after 6 and 24h.

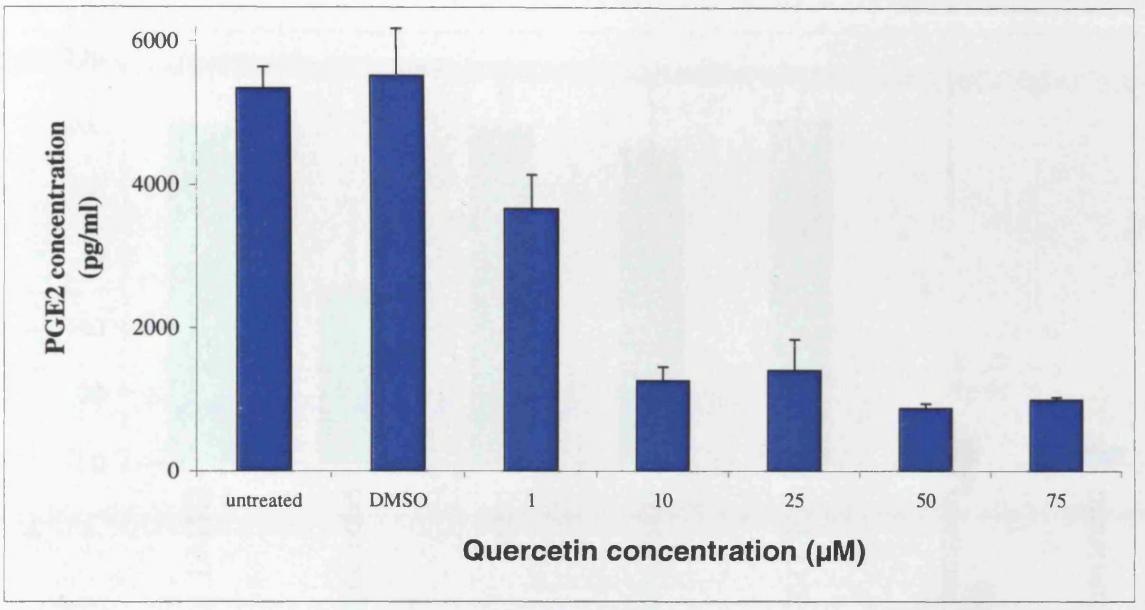
Figure 5.1 *The effect of quercetin on PGE2 levels (pg/mL) for the three sets of plates in HCA7 cells after 6h (A) and 24h.(B).*



The results show that quercetin reduces the levels of PGE2 after 6h to approximately 15% of that found in non-treated or DMSO- treated cells. After 24h, the level of inhibition had decreased and PGE2 levels were approximately 30% of those in control cells. This decrease in inhibition may have been due to instability of quercetin in cell culture medium. Quercetin has previously been shown to oxidatively degrade in cell culture medium(Boulton 1998).

The second experiment used a range of concentrations from 1μM to 75μM to evaluate whether inhibition could occur at levels more reflective of *in vivo* concentrations. The results (Fig. 5.2) show that significant inhibition of PGE2 occurs at concentrations of 1μM and above, after 6h incubation.

Figure 5.2 *PGE2 levels in untreated or treated HCA-7 cells with quercetin in DMSO at various concentrations.*



Consistent with Fig. 5.1, there was little difference between PGE2 levels in untreated and DMSO treated cells. PGE2 levels in cells treated with 50μM quercetin decreased to approximately 16% of that found in untreated or DMSO treated cells. Treatment with 10μM quercetin led to an approximate 76% decrease in PGE2 levels, while 1μM led to an approximate 30% decrease. Thus, the IC_{50} seems to be between 1 and 10μM.

A concentration of 10μM was chosen to investigate the effect of the metabolites of quercetin on PGE2. Four metabolites were compared against quercetin, two sulphate conjugates, and two methylated derivatives. In addition the quercetin glycoside, rutin was included. The results are shown in Fig. 5.3.

Figure 5.3 *The effect of four quercetin metabolites and rutin on PGE2 levels in HCA-7 cells. Q-7-O-SO4 and Q-3-O-SO4 refer to quercetin 7-O-sulphate and quercetin 3-O-sulphate respectively.*

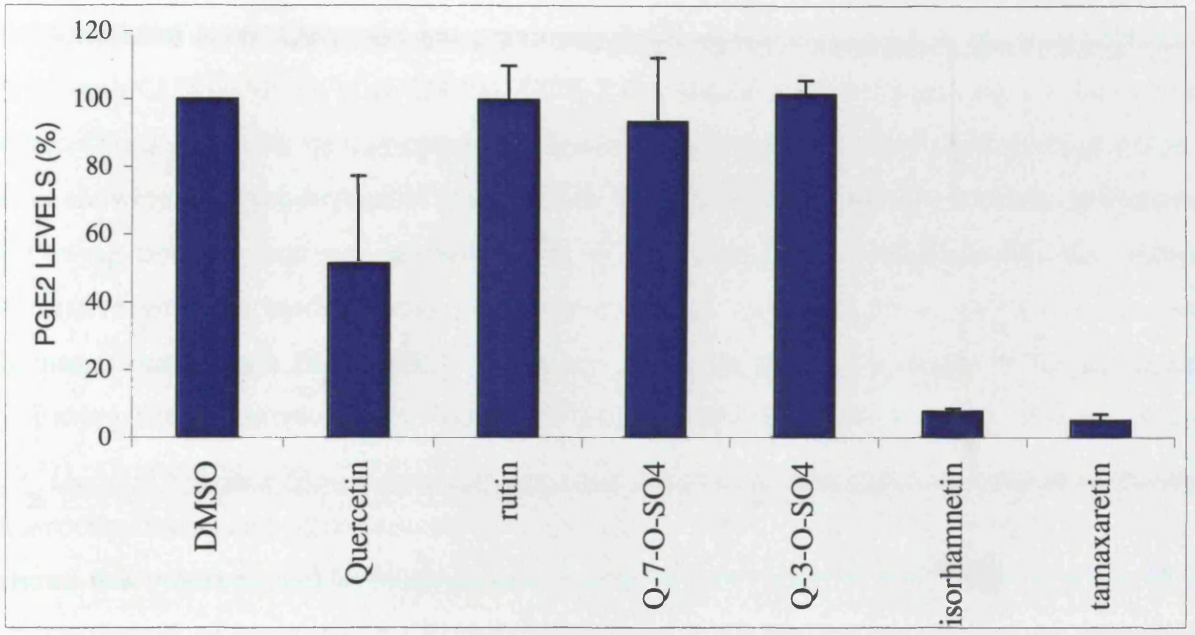


Fig. 5.3 shows that quercetin, at 10μM, causes 51% inhibition of PGE2 levels. Rutin, a glycosidic precursor of quercetin, and two quercetin mono-sulphates had little effect on the levels of PGE2.

The two methyl derivatives, isorhamnetin and tamaxaretin, both caused a dramatic decrease in PGE2 levels in HCA-7 cells. Following the incubation with 10μM isorhamnetin, the levels of PGE2 were down to 8% of those found in the control plates. In the case of tamaxaretin, PGE2 levels were reduced to 5% of those found in the control plates.

5.4 Discussion.

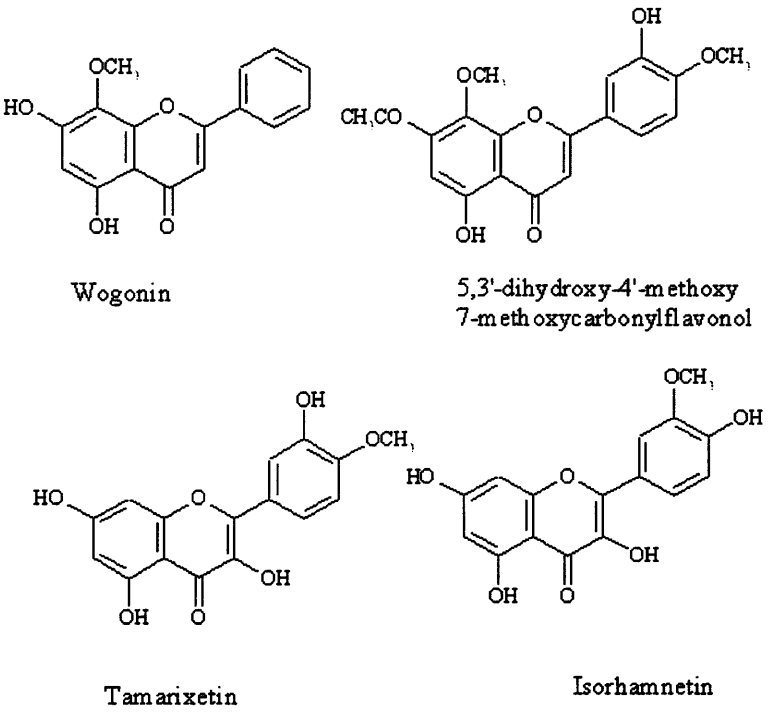
The interaction of cancer chemopreventative agents with COX-2, may occur at the protein or transcriptional level. Quercetin has previously been shown to interact at the transcriptional level with COX-2(Mutoh et al. 2000a). COX-2 is a highly inducible gene and it is likely that quercetin could inhibit its transcription following a 6h incubation. Described in chapter 3 are data showing the metabolism of quercetin to methyl, sulphate and glucuronide derivatives following both i.v and p.o. administration of quercetin in rats. Isorhamnetin, the methyl derivative with the methyl group on the 3'-hydroxyl, was detected in plasma of rats and humans. Tamarixetin (4'-O-methyl quercetin) has been shown by others to be recovered following the administration of quercetin(Ueno, Nakano, and Hirono 1983; Manach et al. 1997). Over-expression of COX-2 is not only seen in human colon tumours but in lung adenocarcinomas and other cancers(Wardlaw, March, and Belinsky 2000). In chapter 3, it was shown that quercetin and isorhamnetin are present in the plasma of both humans and rats. The concentrations of quercetin and isorhamnetin found in rat plasma, as described in chapter 4, indicate that metabolically generated isorhamnetin might contribute to the cancer chemopreventative activity of quercetin.

Both the sulphate derivatives and a dietary precursor of quercetin, rutin, had no effect on PGE2 levels as compared to the control. Even though in this experiment quercetin glucuronides were not available for testing, it can be assumed that they would affect PGE2 levels in a fashion similar to that observed with rutin, a quercetin glycoside. It is possible that a structural motif present on the quercetin and isorhamnetin molecule determines its COX2 ability. The formation of sulphate or glucuronide conjugates may alter the structural characteristics that are responsible for COX2 inhibition. The resorcin-type structure has previously been identified as such a structural motif, in a reporter gene assay of COX-2 promotor activity(Mutoh et al. 2000a). Quercetin, kaempferol, genistein, resveratrol, resorcinol and daidzein were tested in this assay and were all, with the exception of daidzein, shown to suppress COX-2. All, except daidzein, contain the resorcin moiety. It is pertinent to mention that another molecule tested, epigallocatechin gallate, which contains a resorcinol moiety, showed no inhibitory effect on COX-2.

Flavonoids which have been selected for evaluation of effects on COX-2 expression include wogonin (IC_{50}=0.1μM) (see Fig 5.4). Wogonin is a methylated flavonol which additionally has a resorcinol moiety. In a review of COX-2 inhibitors in tumorigenesis(Taketo 1998), it is

noticeable that all compounds tested as specific COX-2 inhibitors contained at least one methyl moiety within the overall structure. There is no clear defining factor with any of the structures shown.

Figure 5.4 *Structure of flavonoids that effect COX-2 expression.*



The series of experiments described in this chapter demonstrates that quercetin and its methylated derivatives can affect COX-2 activity at concentrations that can be achieved *in vivo*. It is thus feasible, that the methylated metabolites of quercetin, via the inhibition of COX-2, may contribute to the cancer chemopreventative activity of quercetin.

6.0 Chapter Six. Glutathione conjugates of quercetin and renal toxicity.

6.1 Introduction.

Quercetin has been demonstrated to be nephrotoxic in both humans and rats. The mechanism responsible for this toxicity has not been identified. The rationale, which underlies the work described in this chapter, is outlined in the general introduction (see section 1.7).

The generation of GSH conjugates was studied *in vivo*, even though work presented in chapters three and four suggest no evidence for glutathione conjugation. It is conceivable that if they exist at very low concentrations, they may be involved in the formation of quercetin nephrotoxicity.

Production of GSH conjugates and subsequent hydrolysis to cysteine conjugates could lead to tissue-specific toxicity in the kidney. In preliminary experiments, potential GSH conjugates of quercetin were generated chemically and bio-synthetically, to provide authentic reference material for subsequent *in vivo* studies. In addition, the hypothesis was tested that quercetin mediated kidney toxicity could be demonstrated in the rat in a short-term experiment with high doses of quercetin.

6.2 Experimental.

6.2.1 Materials and reagents.

Quercetin, fisetin, myricetin, morin, kaempferol, naringenin, mushroom tyrosinase, horse-radish peroxidase, hydrogen peroxide, HPLC grade acetone, HPLC grade dimethyl sulphoxide (DMSO), ammonium acetate, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, reduced glutathione and acivicin (AT125) were all obtained from Sigma (Poole, UK). Disodium ethylenediamine-tetraacetic acid (EDTA), AnalaR grade was obtained from BDH (Poole, Dorset, UK). Methanol (HPLC grade) and glycerol formal from Fisher (Loughborough, Leics., UK). Isorhamnetin and quercetin 3-O-sulphate were obtained from Extrasynthese (Genay, France). The assay kit for γ-GT contained Tris buffer 0.1M, pH 9.0, γ-GT substrate solution (L-γ-Glutamyl-p-nitroanilide), 1.1mmol glycylglycine (in 0.1M Tris buffer, pH9.0), 0.1% sodium nitrite solution, 1.0% ammonium sulfamate solution,

naphthylethylenediamine solution (55mg in 105mL H_2O), γ-GT calibration solution (0.126 mmol/L p-nitroaniline) and 10% acetic acid.

Biochemical assay kit for:

(i) **Glucose**. The glucose reagent, contained glucose [HK] 50 reagent (reconstituted in 50mL deionised H_2O) and glucose standard (1mg/mL),

(ii) **Blood urea nitrogen** (BUN). The BUN reagent, contained BUN acid and BUN colour,

(iii) **Creatinine**. The creatinine reagent, contained sodium hydroxide solution, 1.0M, acid reagent (a mixture of sulphuric and acetic acid), creatinine colour reagent (approximately 0.6% picric acid, sodium borate and surfacant). The alkaline picrate solution was prepared by mixing 5 volumes of creatinine colour reagent, with 1 volume of sodium hydroxide solution. The creatinine standard-A (creatinine, 3.0mg/dL (265μmol/L) was dissolved in hydrochloric acid, 0.02M) and creatinine standard-B (creatinine, 15mg/dL (1326μmol/L), in 0.02M hydrochloric acid).

(iv) **Serum glutamyl pyruvate transaminase (GPT)**. The GPT assay kit, contained sodium pyruvate, 1.5mmol (16.5mg/100mL 0.1M phosphate buffer, pH7.5), alanine-α-ketoglutarate (0.2mol/L DL-alanine + 1.8mmol/L α-ketoglutaric acid which is made up by dissolving 1.78g DL-alanine + 26.3mg α-KG in 100mL 0.1M phosphate buffer, pH 7.5), Sigma colour and 0.4M NaOH (8g/500mL H_2O).

(v) **Alkaline phosphatase (ALP).** The ALP assay contained an ALP reagent (p-nitrophenyl phosphate, 16mmol/L, magnesium ions 4mmol/L, mannitol 274mmol/L and 0.05% sodium azide). γ-GT and biochemical assay reagents were obtained from Sigma (Poole, UK) and the protocol followed as described in the manual.

6.2.2 The *in vitro* synthesis of quercetin-GSH conjugates.

Chemical synthesis.

GSH conjugation of quercetin was initially attempted by generating a quercetin quinone as a reactive intermediate, via oxidation of the catechol ring by sodium metaperiodate. Quercetin (3mM, 100μL) was mixed with sodium metaperiodate (5mg) in 10% acetic acid (5mL). Following vortexing, the red quinone was extracted into chloroform and GSH (10mM) in 10% acetic acid was added. Quinones are strongly electrophilic and thus susceptible to

conjugation with GSH.

For analytical MS characterisation of the products, fast atom bombardment was used as mode of ionisation (conditions described in section 2.4.2.).

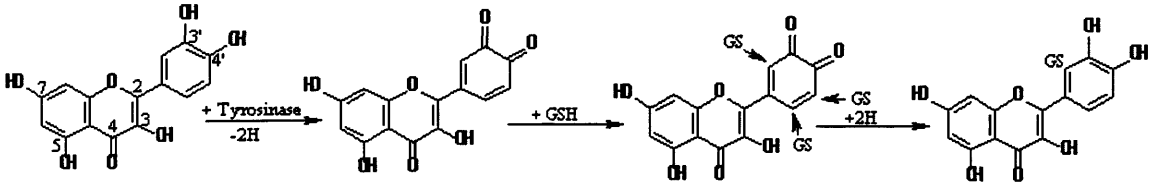
Enzymatic synthesis.

Two different methods were used:

1] Synthesis using mushroom tyrosinase.

Mushroom tyrosinase catalyses the oxidation of molecules such as phenolic catechols, in a one step, two electron oxidation which results in the formation of a quinone. Quinones are highly electrophilic and are susceptible to conjugation with strong nucleophilic agents such as GSH. Once GSH is bound, the quinone can be reduced to a catechol, and the reaction can begin again until all available sites for conjugation are occupied (see scheme 6.1). Mushroom tyrosinase was dissolved in ammonium acetate buffer (0.1M), pH 7.4. Quercetin and GSH were added at equimolar concentrations, typically 0.5 or 1mM. Incubations were carried out in a water bath for 1h at 37°C. The reaction was stopped by the addition of a 1:2 (v/v) of a DMSO/methanol (1:4(v/v)) mixture. The solution was vortexed and centrifuged at 17060g for 15min. The supernatant was removed, diluted 1:1 with water and analysed.

Scheme 6.1 *The proposed reaction of conjugation of quercetin to GSH catalysed by mushroom tyrosinase. The reaction is initiated by the loss of 2H from the 3' and 4' hydroxyl groups to form a quinone. The increased electrophilicity of the B-ring results in it being susceptible to GSH conjugation. GSH can bind to any of the free sites on the B-ring, and on doing so the catechol can reform, promoting another further cycle of oxidation and reduction which can conceivably result in further GSH conjugation.*



2] Synthesis using horseradish peroxidase (HRP).

Horse radish peroxidase (HRP) catalyses the oxidation of compounds using hydrogen peroxide as a cofactor. Typically, the reaction involves a two-step, two electron mechanism. It was predicted that adduction of GSH would occur at the B-ring and this would result in the reformation of a catechol group. In a previously described method, ammonium acetate buffer (0.1M, pH 6.4) was used as a reaction medium(Davies et al. 1995), and was thus used in subsequent studies. GSH and quercetin were incubated at equimolar concentrations (0.5 or 1mM). Hydrogen peroxide, 250μM (H_2O_2) was used as substrate for HRP.

A chromatographic peak that was suspected to be a quinone was analysed by LC-MS. Incubations were also carried out in the presence of DTT (80mM), a reducing agent, to observe the effect on the suspected quinone peaks following HPLC.

Incubation volumes were increased to 10mL. Following incubation, samples were evaporated to dryness overnight, and then reconstituted in 3.5mL of water. The samples were subjected to solid phase extraction as described in section 3.2.3.1. Step 8 of the SPE-protocol was altered, with samples being reconstituted in 1.0mL of 50% water/methanol (v/v).

6.2.3 Analysis of glutathione conjugates of quercetin.

The HPLC was carried out on Varian instrumentation as described in 2.3.2.1. using gradient chromatography as described in section 2.3.2.4. This allowed the analysis of quercetin and its thioether metabolites, and conditions for mass spectrometry and tandem mass spectrometry were the same as those described for quercetin metabolites in section 2.3.3.1.

HPLC fractions corresponding to peaks were collected and analysed for the presence of GSH conjugates of quercetin by determining the UV spectrum (from 200-600nm) using a Perkin Elmer UV/Vis Lambda-2 spectrophotometer. Only one of the fractions gave interpretable results.

For some of the work described in this chapter an alternative gradient to that described in section 2.3.2.4 was used. The column was eluted at time zero with 75 % solvent A, 0.1M amonium acetate, pH 5.15 and 25% solvent B, methanol. Solvent B then increased to 45%

over 20min and the remained isocratic for a further 30min. The flow rate was 1.0mL/min.

6.2.4 Detection of quercetin-GSH conjugates *in vivo.*

GSH-conjugates are formed in the liver, and excreted into the bile. Therefore, for the detection of quercetin-GSH conjugates, a bile cannulation of F344 rats with intravenous injection of quercetin was performed. Rats were anaesthetised by the i.p. injection of 100µl of pentobarbitol (60mg/mL), following which the body cavity was opened and the bile duct located. Using micro-scissors, the bile duct was cut to allow the insertion of a polythene cannulae (i.d 0.28mm; o.d. 0.61mm), which was cut to approximately 30cm in length. The body temperature was maintained using a heat lamp. Bile was collected for 20mins following which, quercetin in glycerol formal (12.5mg/kg) was injected i.v. via the lateral vein in the tail. Bile was collected in twenty-minute intervals for a total of two hours. Quercetin and its metabolites were extracted by the addition of a 1:2 (v/v) of a DMSO/methanol (1:4(v/v)) mixture. The solution was vortexed and centrifuged at 17060g for 15min. The supernatant was removed, diluted 1:1 with water and analysed.

In another experiment, a selective inhibitor of γ-GT, acivicin was used. The reason for this was to prevent the metabolism of GSH conjugates of quercetin to cysteine derivatives, thus increasing the amount of GSH-conjugates observed in the urine. Three groups of F344 rats were used, each group consisting of five rats. Groups one and two were dosed i.p. with 5.0mL/kg of PBS. Group three was dosed i.p. with 10mg/kg acivicin dissolved in PBS. After 60min group one was dosed i.v. with glycerol formal, 0.5mL/kg rat. Groups two and three were dosed i.v. with 62.5mg/kg quercetin dissolved in glycerol formal. Animals were placed in metabolism cages for 24h. Urine and faeces were collected at 4, 12 and 24h, and analysed for GSH-conjugate formation.

6.2.5 Renal toxicity of quercetin induced *in vivo* in rats.

The solubility of quercetin in DMSO was determined and it was found that 500mg could be dissolved per mL of DMSO. One mL of this solution, was injected p.o. to a 200g rat to yield a dose of 2500mg/kg. Also lower doses of 1900 and 2300mg/kg were administered.

Each dose of quercetin was administered to four animals i.v., via the lateral vein of the tail.

Rats were placed in metabolism cages and urine, blood and faeces were collected after 24h.

Six parameters were chosen as indicators of nephrotoxicity. The experimental procedures were derived from the Sigma protocols, which are described in the following text.

Gamma-glutamyl transpeptidase (γ-GT) activity in the urine(Sigma 1989).

The activity of γ-GT was measured using an assay based on the transfer of the glutamyl group from L-glutamyl-p-nitroanilide to glycylglycine catalysed by γ-GT. The activity of γ-GT would be expected to increase in the presence of GSH conjugates, and consequently acts as an indicator of their presence.

Urinary Glucose concentration(Sigma 1995a).

Glucose concentration in the urine was estimated by using the Sigma glucose (hexokinase) assay. An increase in urinary glucose concentration is indicative of chronic renal failure. Failure to filter glucose indicates severe tubular damage.

Glucose is converted to glucose-6-phosphate by hexokinase in the presence of ATP. Glucose-6-phosphate (G-6-P) is further converted with NAD to 6-gluconate and NADH in a reaction catalysed by G-6-P dehydrogenase. The formation of NADH was monitored at 340nm.

Plasma and urine creatinine concentration(Sigma 1992).

Creatinine is a diagnostic marker for kidney damage, and indicates changes in the glomerular filtration rate. The principle of the test is that normally creatinine excretion is relatively constant, whereas in the presence of renal disease, the rate of creatinine excretion is increased, allowing estimation of the extent of impairment of renal function.

Creatinine on reaction with alkaline picrate forms a yellow/orange colour. The resultant colour is destroyed by acid pH and the difference in colour intensity, measured at 500nm, is proportional to creatinine concentration.

Blood urea nitrogen (BUN)(Sigma 1990a).

BUN is a standard diagnostic marker for kidney damage. Evaluation of blood urea nitrogen is probably the most common procedure for indirectly evaluating glomerular filtration rate. The failure of the kidney to filter such low molecular weight proteins is indicative of tubule damage. The principle behind the test utilises the reaction of urea and diacetylmonoxime, which forms a pink complex which can be monitored at 535nm.

Alkaline phosphatase (ALP)(Sigma 1995b).

ALP is a diagnostic marker of a number of physiological disorders. As ALP is an enzyme found in the brush-border membrane of the proximal tubular cells, it can be used to study the toxicity of compounds against proximal tubular cells. As the proximal tubular cells are also rich in γ-GT, any toxicity exhibited by the GSH-conjugates of quercetin would probably be localised to the proximal tubules.

The principle behind this assay involves the measurement (at 405nm) of the formation of p-nitrophenol, a product of ALP catalytic hydrolysis of p-nitrophenyl phosphate.

p-nitrophenyl phosphate + H_2O $\xrightarrow{ALP}$ p-nitrophenol + phosphate

Hydrolysis occurs at alkaline pH, and the increase in absorbance at 405nm is directly proportional to ALP activity of the sample.

Serum glutamate pyruvate transaminase (GPT)(Sigma 1990b)*:*

GPT is an enzyme assay useful in the assessment of hepatotoxicity.

The enzyme catalyses the transfer of α-amino groups from specific amino acids to α-ketoglucaric acid to yield pyruvic acid.

e.g. Alanine + a-ketoglutaric acid $\xrightarrow{GPT}$ Pyruvic acid + glutamic acid

The pyruvic acid formed is reacted with 2,4-nitrophenylhydrazine. The colour intensity of the resulting phenylhydrazones is proportional to the transaminase activity.

6.3 Results.

6.3.1 Synthesis of GSH-conjugates of quercetin.

Chemical synthesis.

When quercetin was mixed with sodium metaperiodate in acetic acid, the mixture turned red which is tentatively characteristic for the formation of a quinone. On the addition of chloroform, the unidentified red compound was extracted into the chloroform layer, giving the organic layer a red appearance. The addition of GSH caused the red colour to disappear. The chloroform layer was analysed by MS.

The spectrum in Fig. 6.1 contains an ion of $[M-H]^-$ at m/z of 301, consistent with quercetin. Ions that correspond to the theoretical m/z for GSH-conjugates of quercetin (i.e. $[M-H]^-$ at m/z 606, 911 or 1216) were not found.

Fig. 6.1 shows that the most abundant ion has a $[M-H]^-$ at m/z of 611, which is consistent with that of oxidised glutathione, (GSSG). MS/MS of the ion $[M-H]^-$ at m/z 611 reveals two ions with $[M-H]^-$ at m/z 611 and 306; consistent with oxidised and GSH respectively (Fig. 6.2). The mass spectrometric analysis suggests that the major constituent of the extract is GSSG.

Figure 6.1 *FAB-MS analysis of a chloroform extract of the incubation of quercetin with GSH and sodium metaperiodate. The spectrum was obtained on the VG SEQ-70 MS instrument in FAB mode.*

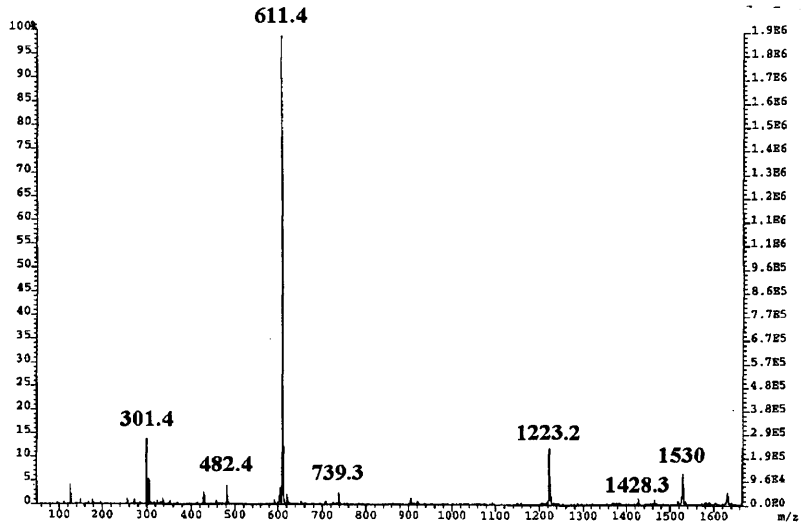
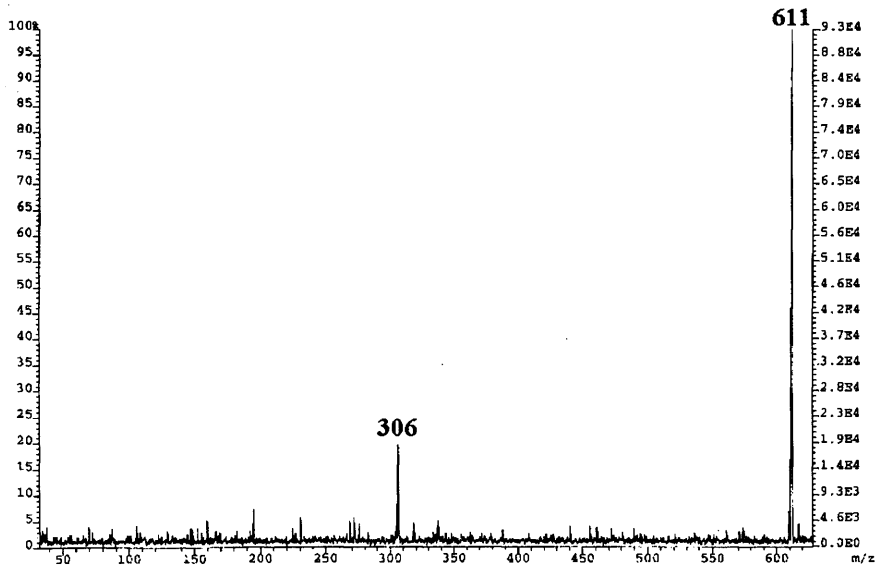


Figure 6.2 *MS/MS analysis showing the product ions formed following tandem mass spectrometry of [M-H]⁻ at m/z of 611. There are two abundant ions, namely m/z 611 and 306. The spectrum was obtained on the VG SEQ-70 MS instrument in FAB mode.*

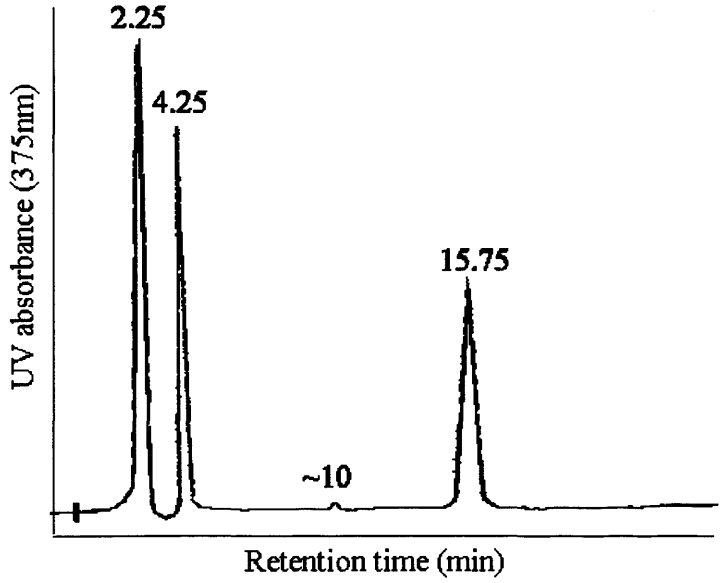


Consequently, the chemical method did not appear to generate GSH conjugates of quercetin. Therefore, an enzymatic approach to the synthesis of GSH conjugates was attempted.

Biochemical synthesis using mushroom tyrosinase:

The products of the enzymatic synthesis of GSH-conjugates of quercetin were analysed by HPLC. The chromatogram in Fig. 6.3 shows three peaks obtained from an extract of an incubation of quercetin with GSH and tyrosinase, which were absent in the control samples. Peak 3 had a retention time (Rt 15.75) that corresponded to a quercetin standard and when analysed using MS gave a $[M-H]^-$ of m/z 301, which is also consistent with quercetin. GSH was not detected in this system. The two peaks (RT of 2.25 and 4.25 respectively) were investigated further as to their likelihood of being GSH conjugates of quercetin. Tyrosinase does not absorb at 375nm, and is unlikely to be extracted using chloroform, hence, it can be excluded as an interfering peak.

Figure 6.3 *HPLC analysis of an extract of an incubation of quercetin, GSH, and tyrosinase. Each peak is annotated with its respective Rt (min). Chromatogram obtained using a Pye unicam HPLC instrument as described in section 2.3.2.1 using chromatographic system A as described in section 2.3.2.4.*



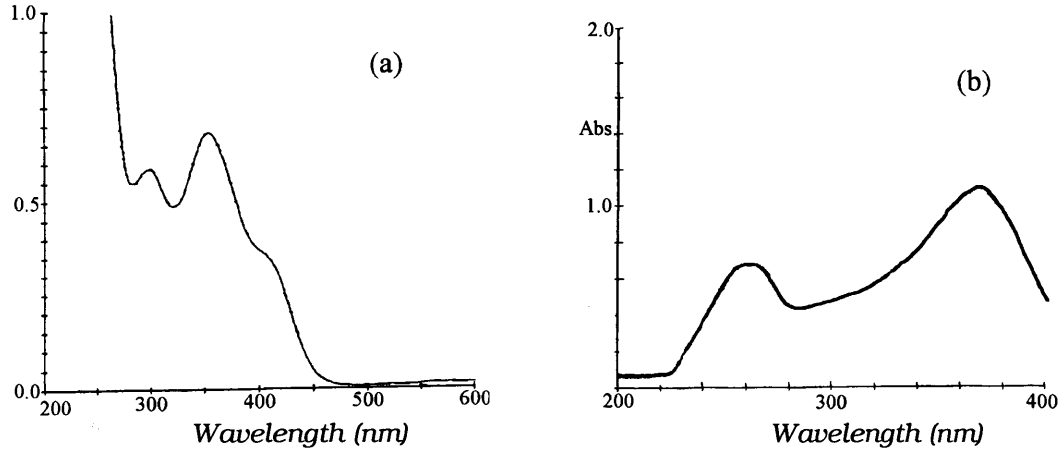
The characterisation of the species giving rise to peaks at Rt of 2.25 and 4.25 was carried out by scaling up the incubation volumes, followed by HPLC analysis. Fractions corresponding to peaks were collected and subjected to mass spectrometry. Peaks one and two (Rt 2.25 and 4.25 respectively) were collected as well as a third smaller peak (Rt~10). Peak one contained two prominent ions of $[M-H]^-$ at m/z 306 and 611. These ions correspond to GSH and GSSG respectively. As GSH, whether reduced or oxidised, does not absorb at 375nm, it would seem

unlikely that the peak could be attributed to GSSG.

Peak 2, as the putative quercetin-GSH conjugate gave two major ions. The prominent ion had a $[M-H]^-$ of m/z 606, which is consistent with a mono-GSH conjugate of quercetin. Peak 3 had a $[M-H]^-$ at m/z of 306, consistent with GSH.

Peak 2 was analysed by UV-spectrophotometry (Fig. 6.4).

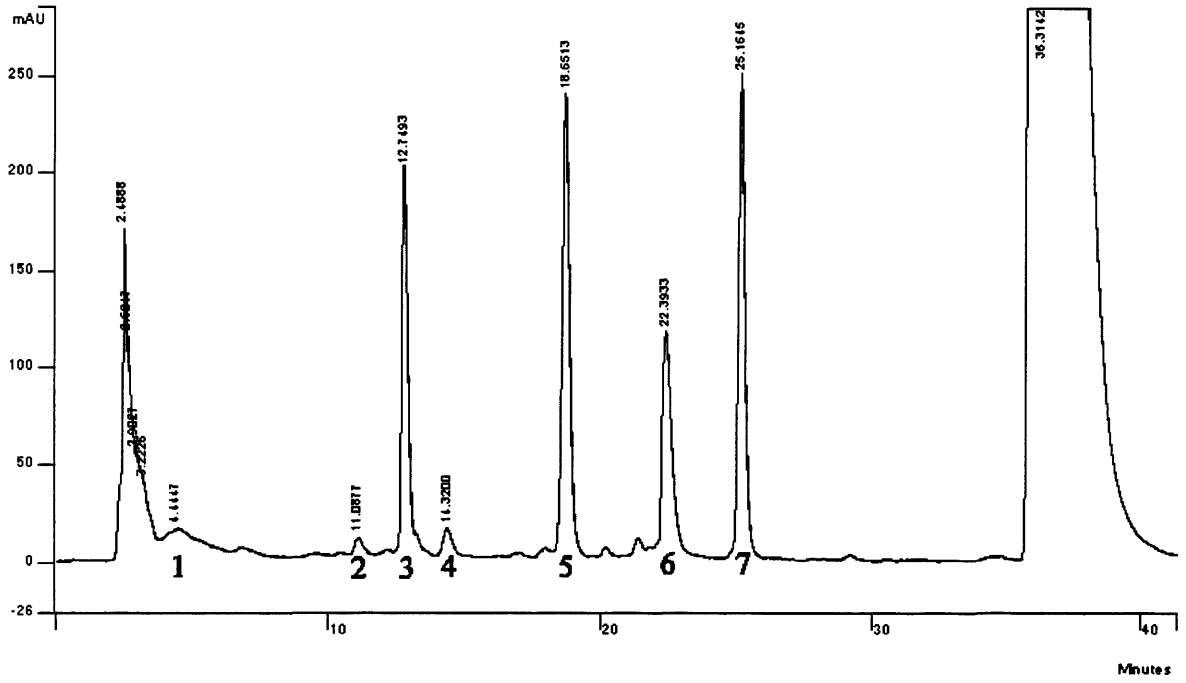
Figure 6.4 *Spectrophotometric analysis of peak 2 (a) and quercetin standard (b). Quercetin shows an absorbance maxima of between 370-375nm, while spectrum (a) shows a hypsochromic shift to between 350-360nm.*



A hypsochromic effect is seen following the conjugation of GSH to quercetin. Usually a hypsochromic shift in absorbance suggests decreased conjugation, such as the loss of a double bond. One possible mechanism would involve the binding of GSH to one of unsubstituted positions in the B-ring of the quercetin molecule, and by doing so reducing the quinone to a catechol with a double bond (see scheme 6.1). Fig. 6.4 indicates that GSH binds to the ring without reformation of the double bond between the 3'-C and 4'-C. Alternatively, the isocratic chromatography employed may not have separated the various conjugates produced. Thus, the UV-spectrum may show a mixture of products rather than one.

In an attempt to carry out more detailed mass spectrometric characterisation, the incubation volume was increased 10-fold. Additionally, a gradient HPLC method (described in 6.2.3.) was used, as it was suspected that the two peaks (Rt 2.25 and 4.25) could in fact contain other molecules. Fig. 6.5 shows a typical example of the separation obtained.

Figure 6.5 *HPLC analysis of an incubation of quercetin with GSH catalysed by tyrosinase. For explanation of the numbers underneath the peaks see text. HPLC was carried out using the Varian HPLC system described in section 2.3.2.1 using chromatography described in section 6.2.3.*

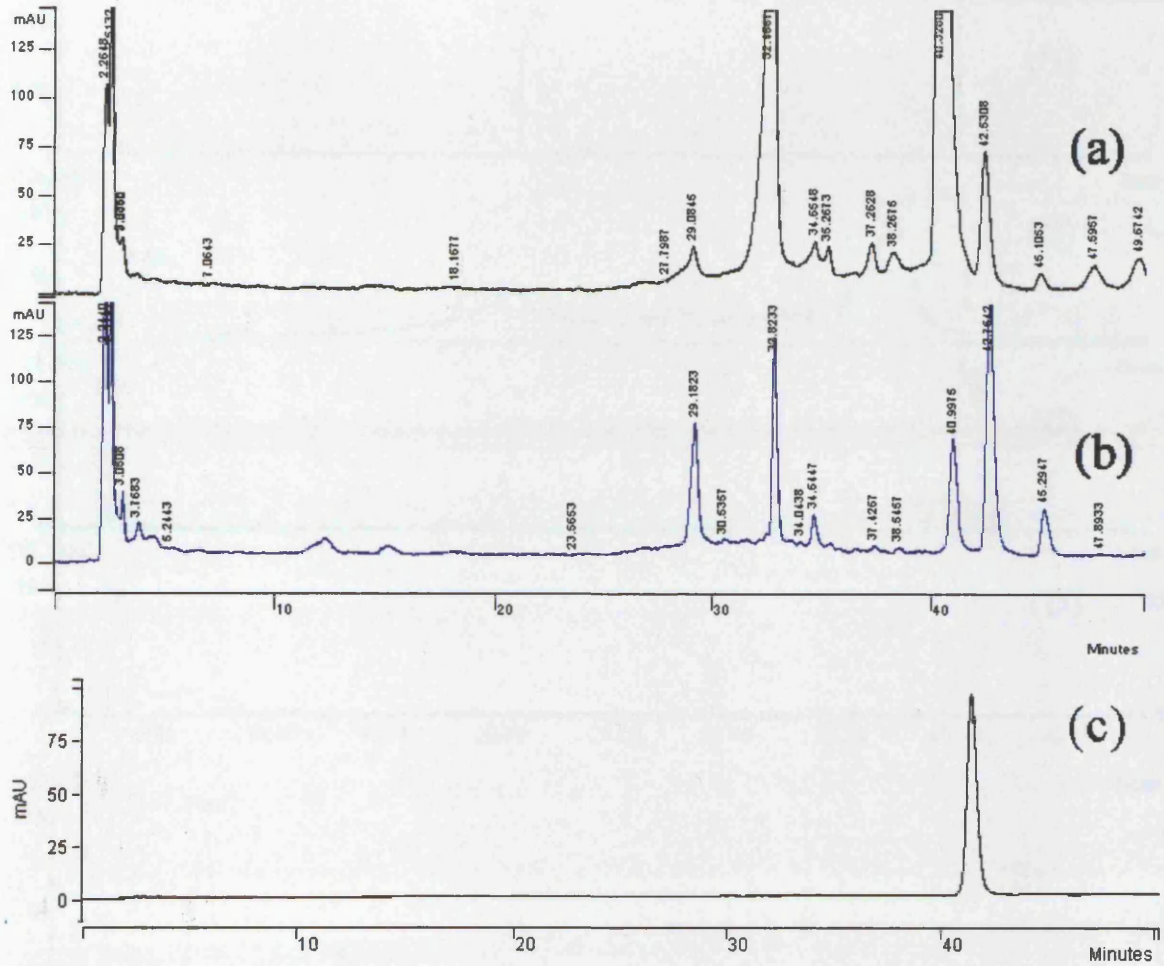


The large peak with a Rt of 36.3 coeluted with quercetin. There are seven other peaks of interest. There seem to be three groups of peaks. A peak close to the solvent front, three peaks eluting between 11.1 and 14.3min, and the remaining three peaks eluting between 18.7 and 25.2min. The addition of GSH to the quercetin molecule conceivably produced a species that was more hydrophilic than quercetin, which using rp-HPLC would elute before the parent compound. The addition of more than one GSH molecule to one quercetin molecule should increase the hydrophilicity of the resultant conjugate proportionally to the number of GSH substituents. As chromatographic elution is a function of hydrophilicity, it was thought possible that peak 1 was a tris-conjugate, peaks 2-4 were bis-conjugates and peaks 5-7 were mono-conjugates. In order to investigate whether this was the case, each peak was collected and analysed by off-line LC-MS. Initial examination revealed peaks 5 and 7 to contain abundant ions of m/z 608 in electrospray positive mode, corresponding to isomeric forms of mono-GSH conjugates of quercetin. Peak 3 contained an abundant ion of m/z 913 in electrospray positive mode, consistent with the predicted m/z of quercetin-GSH bisconjugate.

Biochemical synthesis using horseradish peroxidase:

Tyrosinase catalyses a one-step, two-electron oxidation of compounds to quinones. To investigate whether other oxidising enzymes can catalyse the formation of a quinone from quercetin, horseradish peroxidase (HRP) was used. Oxidation by HRP is a two step reaction, ultimately generating a quinone via a semi-quinone(Metodiewa et al. 1999). Under assay conditions similar to those used for the tyrosinase activation, a comparison between tyrosinase and peroxidase oxidation of quercetin and subsequent GSH conjugation was carried out. In Fig. 6.6, chromatograms of both tyrosinase and peroxidase incubation products are compared. The peak profiles in terms of retention time are similar, however, there are substantial differences in amounts of products formed. There are two peaks that elute between 40.3 and 42.8min. Co-elution with quercetin demonstrated that the earlier peak is quercetin (Fig. 6.6 (c)). The peak eluting directly after quercetin was suspected to be a quinone because of its longer retention. The formation of a quercetin-quinone in the presence of peroxidase or tyrosinase is in agreement with previous reports(Metodiewa et al. 1999; Robak, Kisiel, and Wolbis 1991; Awad et al. 2000; Boersma et al. 2000).

Figure 6.6 *HPLC analysis of extracts from incubations of quercetin with GSH catalysed by tyrosinase (a) and peroxidase (b). A quercetin standard can also be seen (c). HPLC instrument as described in section 2.2.3 using chromatography as described in section 6.2.3.*



Further MS analysis of the incubation extracts revealed the identity of several of the peaks found in the chromatograms. Fig. 6.7 shows 8 individual ion chromatograms obtained following a scan analysis of the eluant from the separation of a tyrosinase incubation using LC-MS. The selected ions can be tentatively assigned to quercetin (A), *O*-quinone quercetin (B), oxidised GSH (C), GSH (D), bis-glutathionyl-S-quercetin (E), *O*-quinone-bis-glutathionyl-S-quercetin (F), and mono-glutathionyl-S-quercetin (G).

Figure 6.7 *LC-MS of extracts of the enzymatic synthesis of GSH conjugates of quercetin. Shown are extracted chromatograms from a scan of m/z 200-1300. The HPLC system described in 2.3.2.1. Chromatographic system B as described in 2.3.2.4. was used.*

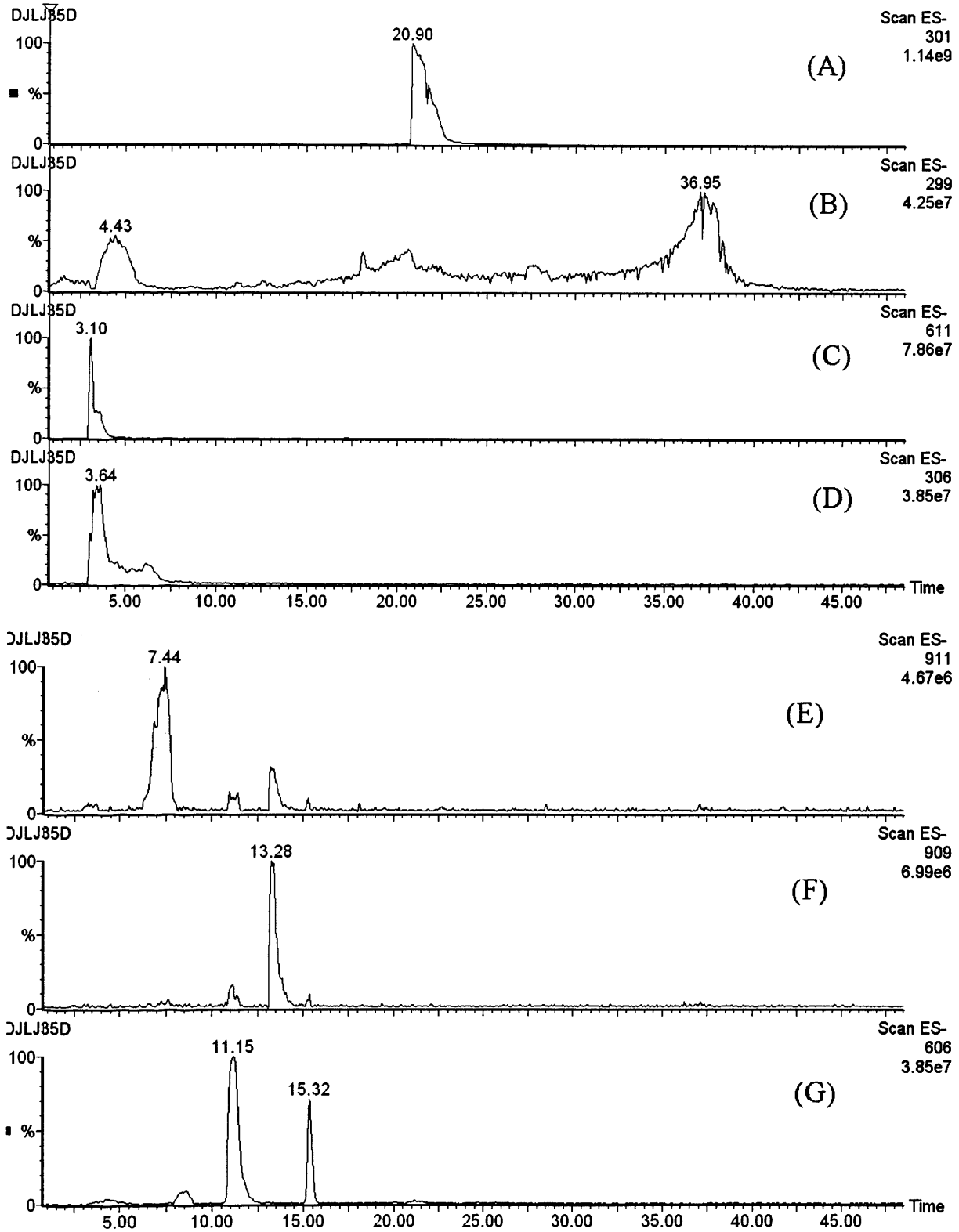


Fig. legend: Quercetin (A), *O*-quinone quercetin (B), oxidised GSH (C), GSH (D), bis-glutathionyl-S-quercetin (E), *O*-quinone-bis-glutathionyl-S-quercetin (F), and mono-glutathionyl-S-quercetin (G).

A feature of Fig. 6.7 is the presence of peaks that contain abundant ions with m/z values that are consistent with quinone derivatives. That is to say, quinoid derivatives can be tentatively assigned to quercetin and bis-GSH quercetin

In each case the quinone eluted after its parent compound. For example, the predominant ion for m/z 909, consistent with a *O*-quinone-bis-GSH quercetin eluted at Rt 13.28, while the largest peak for its catechol parent, bis-GSH quercetin, with an $[M-H]^-$ at m/z 911 had an Rt value of 7.4min. Furthermore, while quercetin eluted at 20.9min the quercetin quinone derivative eluted at 36.9min. The formation of a *O*-quinone quercetin would result in the flavonol structure decreasing in polarity, thus eluting later than its catechol congener. To further substantiate the existence of the quinone, an attempt at reducing it using DTT was carried out (Fig. 6.8).

Figure 6.8 *HPLC analysis of extracts of the incubation of quercetin, GSH and peroxidase with (a) and without DTT (b). HPLC instrument as described in section 2.3.2.1 using an alternative gradient chromatographic system as described in section 6.2.3.*

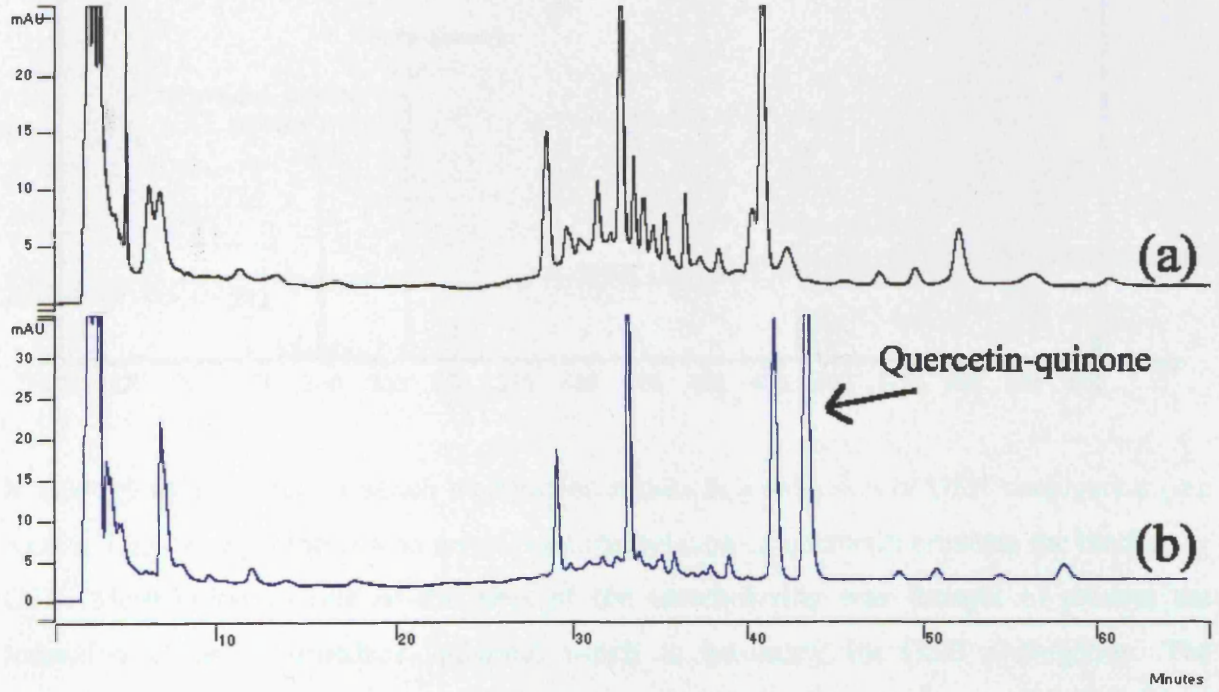
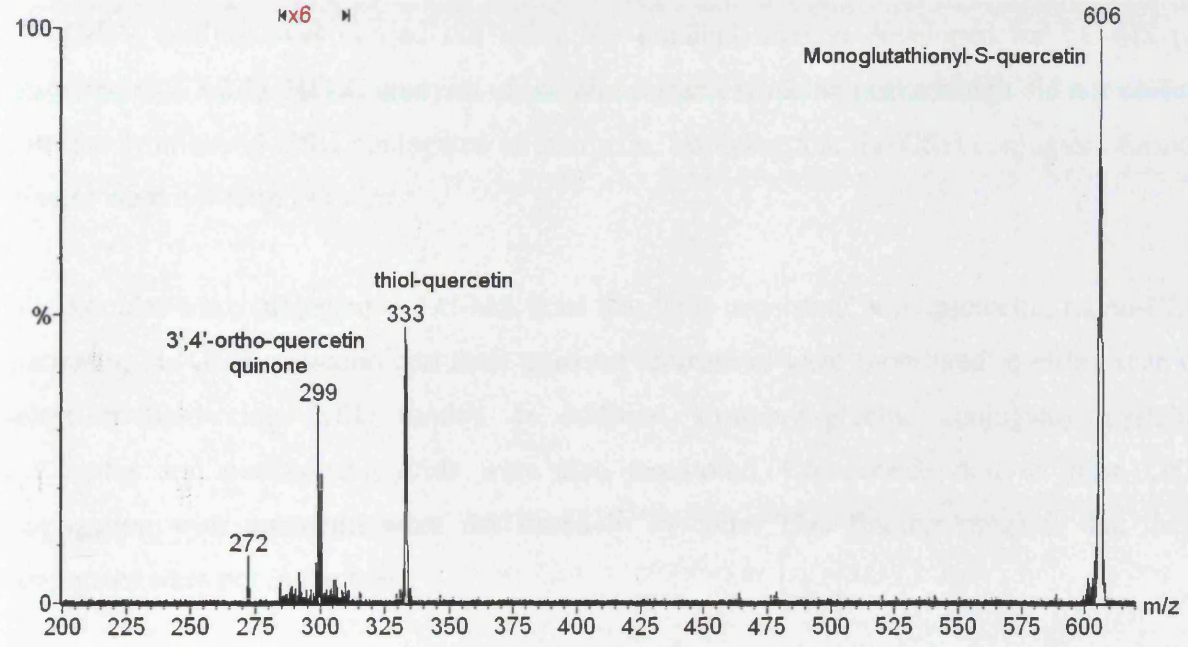


Fig. 6.8 shows the disappearance, on the addition of DTT, of a peak that elutes after quercetin which might be a quercetin quinone. The disappearance of this peak was accompanied by a increase in the peak area of quercetin, thus supporting the notion that the peak corresponds to a quinone that is reduced back to the catechol structure following DTT exposure.

The products from a peroxidase incubation were subjected to on-line LC-MS/MS. Fig. 6.9

shows a typical spectrum for an ion which corresponds to a mono-GSH conjugate of quercetin ($[M-H]^-$ at m/z 606). On fragmentation of the putative mono-GSH conjugate of quercetin, a quercetin quinone was formed ($[M-H]^-$ at m/z 299). Additionally, a fragment of $[M-H]^-$ at m/z 333 was observed. The $[M-H]^-$ at m/z 333 corresponds to the quinone thioether of quercetin, which can be derived from GSH conjugates. The thioether is formed following cleavage of the cysteinyl-S moiety and the sulphur remains attached to quercetin. Consistent with this possibility is the small ion of $[M-H]^-$ at m/z 272 which is perhaps the remainder of the GSH molecule.

Figure 6.9 *Product ion spectrum of $[M-H]^-$ m/z 606 ion obtained using tandem mass spectrometry on the Quattro Bio-Q instrument.*



In analogy to oestrogen in which methylation results in a reduction of GSH conjugation (see section 1.7), the hypothesis was tested, that methylation of quercetin prevents the binding of GSH. Methylation of one of the sites of the catechol ring was thought to prevent the formation of an intermediate quinone, which is necessary for GSH conjugation. The conjugation of isorhamnetin with GSH was explored. The results obtained showed that conjugates were not formed (data not shown). The absence of conjugates was probably the consequence of the presence of the *O*-methyl group at the 3'-C of the B-ring, which prevents the formation of the intermediate quinone, and ultimately of a GSH-conjugate.

6.3.2 Detection of quercetin-GSH conjugates *in vivo.*

The presence in rat bile and urine of the conjugates characterised above was explored. The liver was considered to be a suitable source of GSH conjugation. Consequently, an experiment involving the collection of bile following the administration of quercetin was carried out, to investigate whether GSH conjugates were formed *in vivo.*

Two rats were bile-duct cannulated, injected with quercetin intravenously and bile collected. GSH conjugates can be metabolised to cysteine derivatives in the biliary duct due to the relatively high concentration of γ-GT found in the bile duct tissue. The presence of cysteine derivatives of quercetin in the bile was also investigated.

HPLC-UV analysis was carried out using the gradient method developed for LC-MS (as described in 2.3.2.1). HPLC analysis of the bile extracts revealed peaks which did not coelute with the synthesised GSH conjugates of quercetin, implying that the GSH conjugates formed *in vitro* were not found *in vivo.*

Bile samples were subjected to LC-MS. Ions that were consistent with quercetin, mono-GSH quercetin, bis-GSH quercetin and their quinone derivatives were monitored in either scan or select-ion-monitoring (SIR) modes. In addition, cysteinyl-glycine conjugates, cysteine conjugates and mercapturic acids were also monitored. Compounds derived from GSH conjugation with quercetin were not found in the bile. This finding suggests that these conjugates were not in the bile.

However, the absence of GSH conjugates in the HPLC chromatograms does not preclude the possibility that they were formed. For example, trichloroethylene (TCE), a potent nephrotoxicant, is metabolised to a very minor extent (<0.01%) by conjugation with GSH(Green et al. 1997). This conjugate is converted to *S*-(1,2-dichlorovinyl)-L-cysteine (DCVD) which in turn, is activated by β-lyase to induce nephrotoxicity(Green et al. 1997). It is conceivable that quercetin undergoes an analogous metabolic pathway. If the hypothesis is correct, quercetin might form GSH conjugates at undetectable levels. These metabolites might be degraded to quercetin cysteine species, which in analogy to DCVD might exert nephrotoxicity. The possibility of detrimental, yet undetectable, levels of GSH conjugates is in theory, supported by the multiple sites on the quercetin molecule available for conjugation. All of these conjugates together may elicit toxicity following hydrolysis.

In order to test the hypothesis that quercetin nephrotoxicity in the rat might be associated with GSH conjugation, an experiment was designed in which kidney toxicity was to be induced using high concentrations of quercetin. If nephrotoxicity could be induced, the potential role of inhibitors of enzymes involved with GSH-associated nephrotoxicity could be used to alleviate the nephrotoxicity.

Six markers of nephrotoxicity present in either urine or plasma, were tested and results are summarised in Table 6.1.

Table 6.1 *Six markers of nephrotoxicity following oral administration, to rats, of different concentrations of quercetin.*

Quercetin concentration (mg/kg)	**0**	**1900**	**2300**	**2500**
Nephrotoxic marker.	**Units.** (presented as mean±s.d.)			
Gamma-glutamyl transpeptidase(*[1])	**2.79**±0.07	**4.30**±0.24	**4.58**±0.04	**4.0**±0.09
Glucose(*[2])	**30.9**±0.58	**27.2**±0.99	**28.4**±1.20	**25.7**±0.89
Blood urea nitrogen(*[3])	**9.66**±0.85	**13.27**±2.29	**11.86**±0.80	**12.02**±0.49
creatinine (urine) (*[4])	**0.87**±0.052	**0.83**±0.052	**0.92**±0.17	**0.87**±0.04
creatinine (plasma) (*[5])	**0.091**±0.017	**0.097**±0.095	**0.196**±0.061	**0.079**±0.013
alanine transaminase(*[6])	**34.89**±5.40	**31.35**±4.60	**35.07**±15.98	**36.62**±1.72
alkaline phosphatase(*[7])	**88.78**±10.7	**81.67**±10.9	**86.52**±7.7	**60.06**±10.6

Units = *[1], enzyme unit; *[2], μg/mL; *[3], mg/100mL; *[4] and *[5], mg/dL; *[6] (Sigma-Frankel units/mL); *[7], enzyme units.

The urine and plasma of rats treated with quercetin displayed either subtle or no change in the levels of markers of nephrotoxicity, as compared to control rats. Elevated γ-GT and BUN values were seen at all three dose levels. Notably, neither of these were dose-dependent. Urinary glucose levels showed a slight reduction in concentration, but again a dose dependent relationship was not evident. Quercetin reduces ALP activity at the higher dose (2500mg/kg). Creatinine in urine was similar in both untreated and treated animals. In plasma, all four groups displayed similar concentrations of creatinine, except at 2300mg/kg quercetin, which showed an increase. The ALT values, a marker for hepatotoxicity, did not differ between treated and control.

The effect of quercetin on the nephrotoxic markers were not marked enough to warrant the use of the F344 rat as a short-term nephrotoxic model under these conditions.

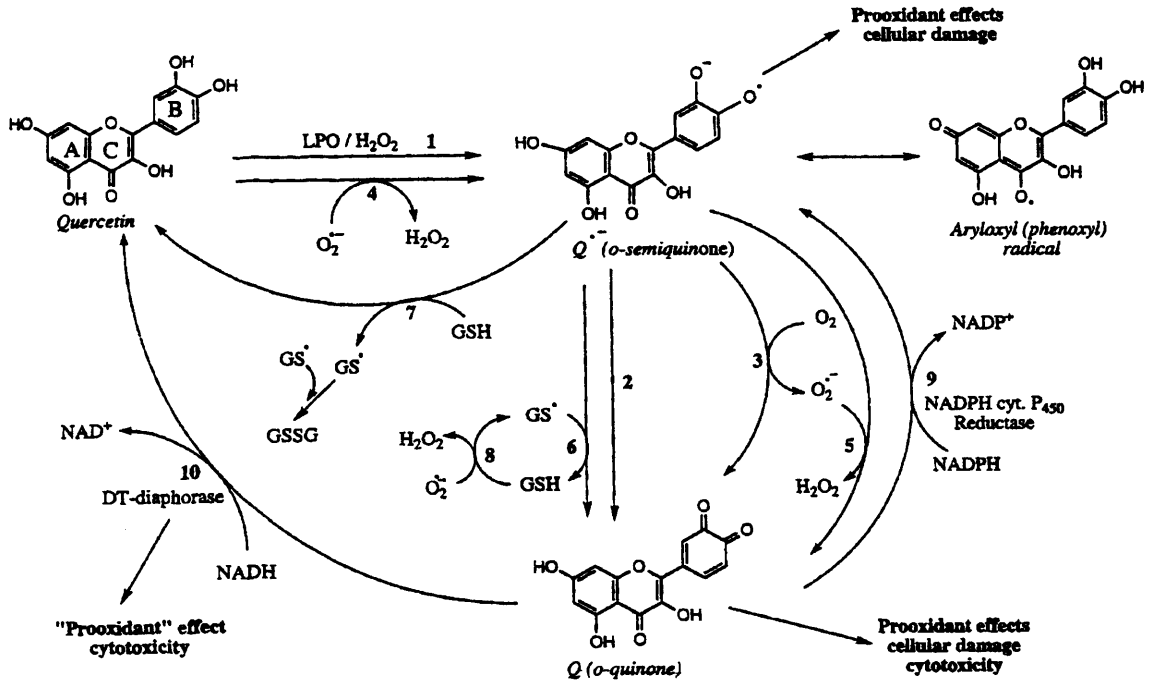
The urine obtained in this experiment was analysed for the presence of GSH-conjugates of quercetin. Following analysis by LC-MS, GSH-conjugates could not be detected in the urine. Consequently, this particular approach was not pursued any further.

If GSH conjugates of quercetin were being generated *in vivo*, it could be possible that the conjugates were metabolised further to cysteine- and possibly quinone-thioether- derivatives. Consequently, an attempt was made to minimise metabolism that would effectively reduce the concentration of GSH-conjugates of quercetin. This approach to detecting GSH-conjugates of quercetin involved using the γ-GT inhibitor acivicin. Acivicin would inhibit the formation of cysteine derivatives, thus in theory leading to a greater concentration of the precursor GSH-conjugates of quercetin in the urine. Rat urine was monitored using HPLC and LC-MS, but failed to detect the presence of GSH-conjugates of quercetin. Since this experiment would have demonstrated their presence in the urine, this data indicates that GSH-conjugates of quercetin are not formed *in vivo*.

6.4 Discussion.

A mechanistic explanation for quercetins nephrotoxicity has thus far not been proffered. In chapter one, a hypothesis was proposed for quercetin-mediated nephrotoxicity. Quercetin may be conjugated with GSH and subsequent metabolism of these quinone thioethers could be responsible for the production of toxic metabolites that targets the kidney and gives rise to nephrotoxicity/carcinogenicity. The effect of quercetin on cellular levels of GSH is unclear and appears to depend on cell type. Recently, the *in vitro* activation of quercetin to semiquinone and quinone derivatives has been described(Metodiewa et al. 1999). Interestingly, the authors report the formation of a semi-quinone, which can react with oxygen (O_2) to form a quinone and subsequently generate superoxide radical. The production of superoxide radicals by flavonoids has been proposed previously(Hodnick et al. 1988). Alternatively, the semiquinone may be reduced back to quercetin via strong electrophiles such as GSH. Thus, quercetin might undergo a two-step, one- electron oxidation to quercetin *O*-quinone with the generation of a semiquinone intermediate. This pathway may provide the reason why quercetin can demonstrate prooxidant effects in the presence of molecular oxygen. The authors also gave evidence for the two-steps one-electron reduction of quercetin *O*-quinone to the semiquinone catalysed by NADPH cytochrome P450 reductase.

Scheme 6.2 *The activation of quercetin to semiquinone and quinone derivatives following peroxidation using lactic peroxidase. Scheme adapted from Metodiewa* et al*(Metodiewa et al. 1999).*



The formation of quinones and semiquinones typically results in highly reactive species which even though present at low concentrations may have significant toxicological effects.

Since the work described here was completed separate papers have been published on GSH-quercetin adducts(Awad et al. 2001; Galati et al. 2001; Awad et al. 2000; Boersma et al. 2000). These workers indicate that contrary to their expectation, GSH conjugates predominate on the A-ring, presumably involving intermediate quinone methides(Galati et al. 2001; Awad et al. 2000; Boersma et al. 2000). Another paper indicated that pH is a critical factor in the site of conjugation on the quercetin molecule(Awad et al. 2001). It was presumed that GSH conjugation would occur on the B-ring with the formation of *o*-quinones. If conjugation occurs at the A-ring of quercetin, one could assume that isorhamnetin should be susceptible to conjugation with GSH. The failure of isorhamnetin to conjugate to GSH, as shown in this chapter, was presumed to be due to the catechol no longer being present on the B-ring and thus preventing any further reaction with GSH. The absence of isorhamnetin conjugates also infers that GSH conjugation of quercetin occurs on the B-ring.

The *in vitro* incubation of quercetin and GSH with either tyrosinase or peroxidase yielded a variety of GSH-conjugates of quercetin that could be identified by HPLC and LC-MS (see Fig. 6.7). The quinone/semiquinone identified by LC-MS appears to correlate with the previously proposed uncharacterised quinone described by Metodiewa *et al*(Metodiewa et al. 1999). The formation of a quercetin quinone was confirmed by the LC-MS detection of an ion of m/z at 299. Also the HPLC peak assigned to quercetin quinone disappeared on the addition of a strong reducing agent, DTT. The formation of a quinone from the catechol increases the electrophilicity of the B-ring and consequently makes the ring susceptible to conjugation with GSH at one of the free sites, of which there are three. On binding, the ring can either revert back to the catechol structure or alternatively the quinone can be reduced to form a semiquinone as described by Metodiewa *et al*(Metodiewa et al. 1999). Quinones which undergo redox cycling can cause cellular ATP depletion that occurs prior to loss of cellular viability, and can accompany, or precede NADH loss thus promoting cellular oxidative stress(Gant and T 1988). Alternatively, quinones can cause toxicity by arylation of thiol moieties. It has been shown that the *O*-quinone quercetin molecule is a substrate of NADPH-cytochrome P450 reductase, which catalyses a two-step one-electron reduction of the quinone to form the semiquinone intermediate(Metodiewa et al. 1999).

Interestingly, in CHO-cells that expressed a four-fold higher level of DT-diaphorase,

cytotoxicity of quercetin was markedly increased as compared to wt-CHO cells(Metodiewa et al. 1999). DT-diaphorase is an enzyme that protects against semiquinone radical formation from quinone. DT-diaphorase acts as a two-electron quinone reductase, which promotes the formation of NAD^+ from NADH, thus depleting the cell of reducing power(Metodiewa et al. 1999).

GSH conjugate formation was studied as a possible explanation for the toxicity described in the clinicals trial and the long-term rat study. The results presented here show that quercetin can in principle form GSH conjugates and more importantly, stable quinone precursors of those conjugates are also formed *in vitro*. In the toxicity study model chosen only subtle effects on kidney physiological markers were shown. GSH-conjugates of quercetin, which might be conceivably generated and metabolised to potentially nephrotoxic quercetin-cysteine conjugates, were not detected *in vivo*. The conclusion of the work shown here is that either this metabolic pathway is not relevant for quercetin, or alternatively the level of quercetin-GSH conjugates generated is too low to allow analytical detection. The rat species used, the F344 rat, was not a sensitive model for the study of short-term induction of nephrotoxicity by quercetin, and ultimately for the investigation of the mechanism that underlies the nephrotoxicity.

It is also conceivable that the conjugates could bind to macromolecules within the kidney following metabolism, thus avoiding excretion and detection. However, it is equally feasible that GSH conjugates of quercetin were not formed and some other mechanism is responsible for the renal toxicity exhibited in rats and humans(Ferry et al. 1996; National Toxicology Program 1992).

Potentially, the kidney lesions might have been caused by the accumulation of large concentrations of sulphate and glucuronide conjugates of quercetin and isorhamnetin, that somehow, could lead to localised cytotoxicity in the kidney. In analogy to oestrogen which undergoes hepatic methylation and glucuronidation, it may be that quercetin undergoes conjugation reactions in the liver, the products of which maybe transported to the kidney. Evidence has been described where in the case of oestrogen, deconjugation occurs specifically in the kidney, effectively releasing the parent compound which leads to tissue specific toxicity(Zhu et al. 1996). It is conceivable that a similar mechanism is occurring for quercetin.

7.0 Chapter Seven. General Discussion.

Clinical trials of new anti-cancer agents can ultimately be used to provide pharmacokinetic and pharmacodynamic information together with endpoints indicating efficacy or toxicity.

Flavonoids are of considerable interest, as they are dietary components that exhibit biological properties potentially exploitable for medicinal purposes. One of the aims of the work described in this thesis, was to add to the body of work currently available on the metabolism of pure quercetin. There are gaps of knowledge concerning the metabolism of pure quercetin, when used as a therapeutic agent. The work described in this thesis attempts to address some of these gaps. Furthermore, the quercetin-related renal toxicity observed in rats and humans has been investigated. Also, an insight to a possible biological effect of quercetin and its metabolites was explored. The alteration of PGE-2, a marker of COX-2 activity, by quercetin and its metabolites was studied, as COX-2 is a potential therapeutic target for quercetin when used as a cancer chemotherapeutic or chemopreventative agent.

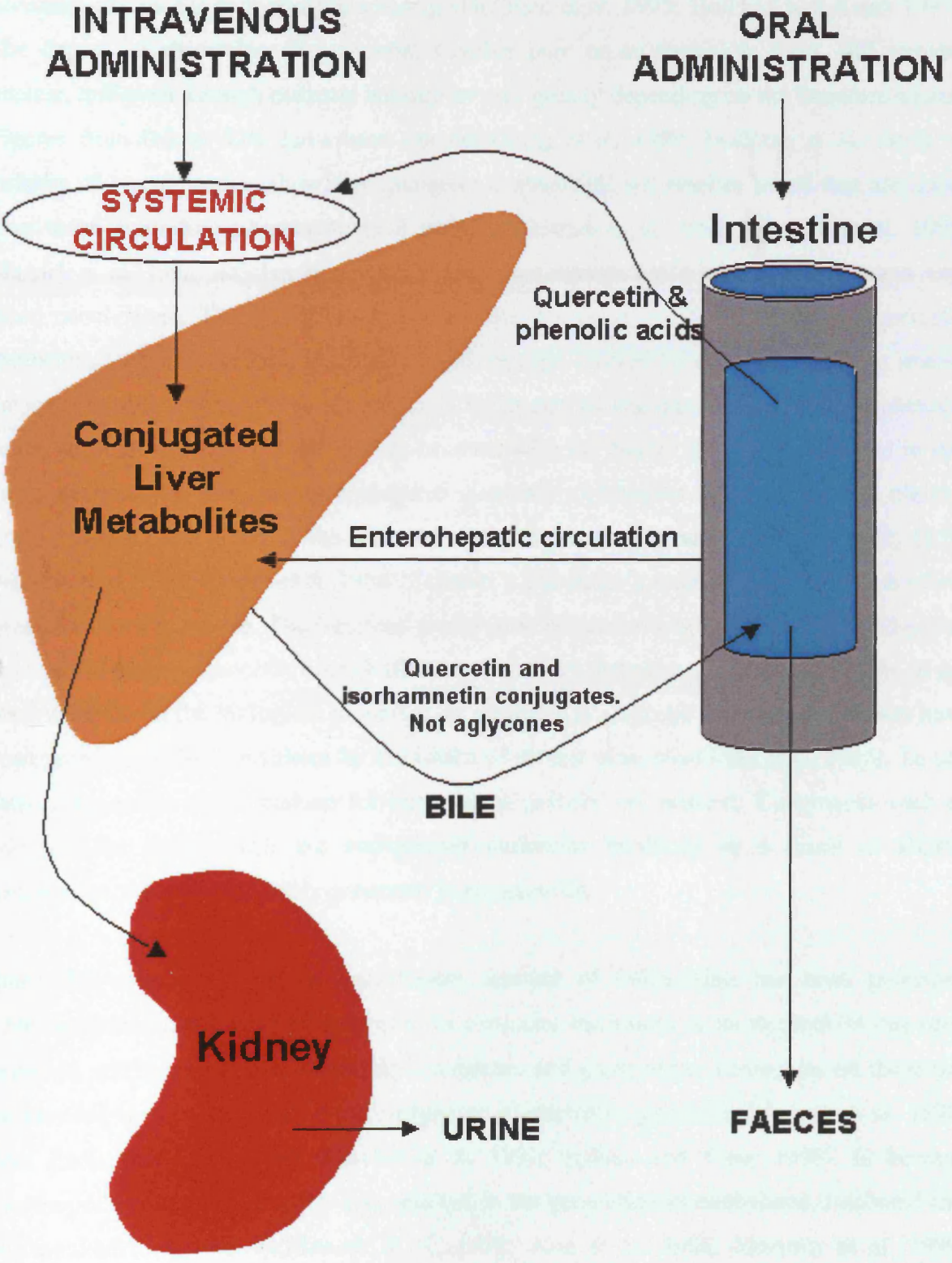
It might be useful to juxtapose what is known about the absorption, metabolism and bioavailability of quercetin including the results provided in this thesis, with the knowledge of the absorption, metabolism and bioavailability of quercetin when it is ingested in glycosidic form with the diet.

In chapter four the metabolism of quercetin after oral administration is described. As discussed in chapter one, it is thought that quercetin is absorbed only to a minor extent. Consequently, any effects of quercetin, whether as a component of the diet or as a pure drug, would most likely be confined to tissues of the gastrointestinal tract. Quercetin and its metabolites were identified in the urine of rats, confirming that it is absorbed, although the extent of absorption could not be measured directly in rats. In chapter four, a pilot study in a patient also had consumed pure quercetin was described. The pilot study suggests that a very low concentration of quercetin circulates in the plasma, and its concentration slowly increases after repeated ingestion of quercetin tablets. This observation is consistent with previous work that showed quercetin to accumulate following multiple doses(Hollman et al. 1997b; Hollman et al. 1997a). The level of quercetin measured in human plasma did not reach values that would be sufficient to alter cellular processes associated with chemoprevention or anti-neoplastic activity. However, it is possible that quercetin, even at low levels, together with other dietary antioxidants, contributes to the cellular antioxidant system.

An overall summary of the disposition and elimination of quercetin is shown in scheme 7.1.

In the scheme, the starting point is pure quercetin, but the scheme also applies to glycosidic quercetin following cleavage of the sugar residue.

Scheme 7.1 *The metabolism of quercetin in humans. This is a summary of results of many publications and of the work presented in this thesis.*



Quercetin occurs naturally in various glycosidic forms, the composition of which differs from foodstuff to foodstuff. Consequently, the actual levels of quercetin within the body depend not only on endogenous factors such as the individual rate of metabolism of glycosidic-quercetin to aglycone but also on the dietary composition of different glycosides. An additional factor affecting quercetin's bioavailability is the differential susceptibility of quercetin glycosides to hydrolytic cleavage(Hollman et al. 1995; Hollman and Katan 1997). The degree of absorption of quercetin, whether pure or in glycosidic form, still remains unclear, and even a rough estimate appears to vary greatly depending on the literature source. Figures from 0.3 to 52% have been quoted(Young et al. 1999; Hollman et al. 1995). A number of recent studies show that quercetin is absorbed, but reaches levels that are lower than those exhibiting pharmacological activity(McAnlis et al. 1999; Manach et al. 1998; Manach et al. 1995; daSilva et al. 1998). The work demonstrated here is in agreement with these conclusions. The gut is an important site for the metabolism of dietary quercetin glycosides (see scheme 7.1). Quercetin glycosides are cleaved by colonic bacteria to release the aglycone(Kim et al. 1998). Quercetin is either further degraded to a number of phenolic acids, absorbed through the gut wall or excreted with the faeces. The work described in this thesis suggests the presence of conjugated quercetin metabolites in bile, urine and plasma. Little attention was given to the phenolic acids described previously(Kallianos et al. 1959; Petrakis et al. 1959; Booth et al. 1956). Scheme 7.2 includes a variety of intestinal catabolites generated from quercetin. The intestinal metabolites of quercetin are absorbed as adjudged on the basis of their presence in urine(Kallianos et al. 1959; Petrakis et al. 1959). The role of the phenolic acids in the biological properties of quercetin is yet to be determined, but they have been proposed to be biomarkers for the intake of dietary quercetin(Gross et al. 1996). To use these compounds as biomarkers for quercetin is perhaps not prudent. Compounds such as homovanillic acid, which are endogenous molecules produced as a result of DOPA metabolism, are not exclusively generated from quercetin.

Since this research began, a considerable amount of information has been published concerning the metabolism of quercetin. In particular the nature of its metabolites has been explored, and it is now clear that methyl-, sulphate- and glucuronide- conjugates are the major metabolites in rats following dietary exposure to quercetin glycosides(Manach et al. 1995; Zhu, Ezell, and Liehr 1994; Manach et al. 1997; Piskula and Terao 1998). In humans consumption of a quercetin-rich diet, resulted in the generation of methylated, sulphated and glucuronidated derivatives(Manach et al. 1998; Aziz et al. 1998; McAnlis et al. 1999; Hollman et al. 1995). Quercetin, when administered i.v., as outlined in the work presented

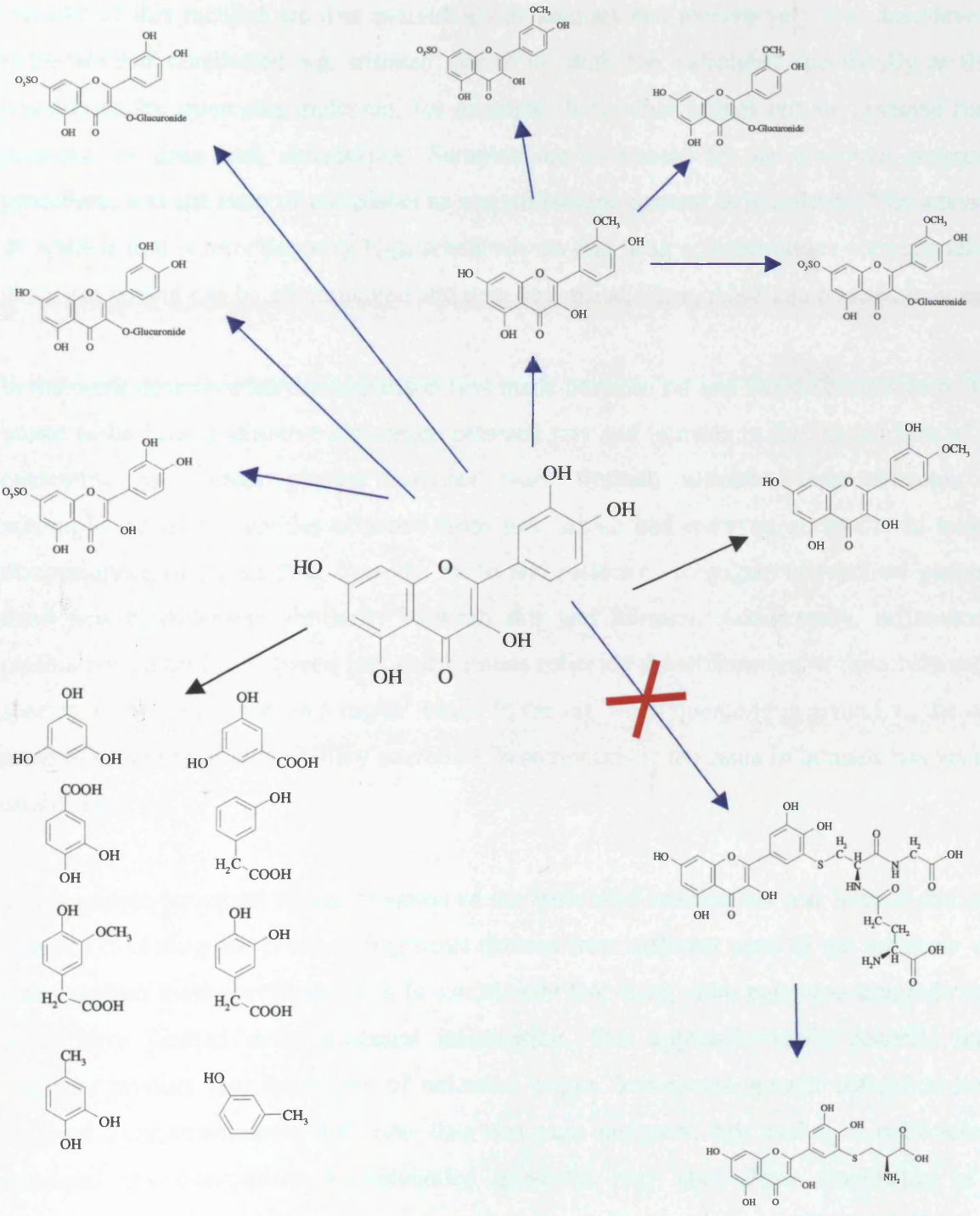
here was rapidly removed from the blood and excreted in both the bile and urine as methylated, glucuronic and sulphated derivatives.

Typically, conjugation of xenobiotics results in the molecule acquiring increased hydrophilicity, and as a consequence, the molecule is more readily excreted. Methylation, on the other hand, results in the molecule becoming less polar and consequently less susceptible to excretion. In the case of quercetin it has been proposed that methylation constitutes a detoxification step(Zhu, Ezell, and Liehr 1994). Quercetin is a potent mutagen, a property that is apparently alleviated by methylation(Brown 1980). The methylation of quercetin first found in hamsters by Zhu *et al*(Zhu, Ezell, and Liehr 1994) has been confirmed here by mass spectrometry in rats and humans (chapter 3). Methylation is a major route of metabolism for quercetin as shown by the levels of isorhamnetin present in the bile. Prior to the inception of work leading to this thesis, the characterisation of the conjugated metabolites had not been reported. A particular contribution made in this work is a comprehensive assessment of the pattern of conjugation which quercetin and isorhamnetin undergo. It is clear from chapter three that each hydroxyl group on either quercetin or isorhamnetin may become glucuronidated. In the case of quercetin, there is evidence to suggest tentatively that intestinal metabolism is responsible for at least one monoglucuronide. This conclusion is drawn by the presence of only four quercetin monoglucuronides in bile and five quercetin monoglucuronides in urine. The role of intestinal metabolism in the conjugation of quercetin and other flavonoids has been explored previously(Crespy et al. 1999; Spencer et al. 1999). Human UGT 1A8, an isoenzyme of UGT (uridine diphosphate glucuronyl transferase) is found only in the gut and not in other tissues and would appear to glucuronidate flavonoids(Cheng, RadominskaPandya, and Tephly 1998). Intestinal mucosa, kidney and other tissues possess enzymatic activity for metabolism such as glucuronidation, O-methylation and hydroxylation. It is possible that the intestinal tract contributes to the overall metabolism of quercetin. When the metabolism of epicatechin, a flavonoid similar to quercetin, was studied it was reported that UGT activity was strongest in the intestine(Moon et al. 2001). Quercetin aglycone and quercetin glycosides have been shown to be absorbed from the intestinal mucosa after which they are mostly converted to conjugated metabolites in the absorptive cells before entering systemic circulation(Moon et al. 2001).

Furthermore, a number of bisconjugates, including mixed sulphate and glucuronide conjugates of quercetin and isorhamnetin have been identified. The net result of the conjugations is that quercetin is very quickly cleared from the body and excreted either in the

bile or urine, as in general phase II metabolism increases the polarity of xenobiotics, thereby facilitating their excretion. Scheme 7.2 outlines comprehensively the metabolic fate of quercetin.

Scheme 7.2 *Metabolism of quercetin. The structures of the sulphate and glucuronide conjugates of quercetin and isorhamnetin represent single isomers of the individual conjugates, of which there are multiple isomers. Blue arrows indicate metabolic routes studied in this thesis. The red cross indicates that quercetin-GSH conjugates were not found in vivo. Also shown are the catabolites produced as a result of bacterial metabolism.*



In chapter four, a qualitative assessment of the distribution of quercetin within the body is presented. Quercetin and its metabolites were found in all tissues analysed, suggesting that, while excretion of quercetin is indeed very rapid, it nevertheless does reach tissues. The quantitative distribution of quercetin in humans is not fully understood. In future such a study may be conducted using the technique of acceleration mass spectrometry (AMS). The salient features of this method are that individuals or animals can receive very low dose levels of radio-labelled compound e.g. tritiated quercetin, with the radiolabel specifically at the 7-position of the quercetin molecule, for example. Individual tissues can be assessed for the presence of drug and metabolites. Samples are processed by an elaborate preparative procedure, and the ratio of radiolabel to normal isotope content is monitored. The advantage of AMS is that it provides very high sensitivity so that drug concentrations corresponding to dietary exposure can be administered and then quantified. Thus, AMS can be used in humans.

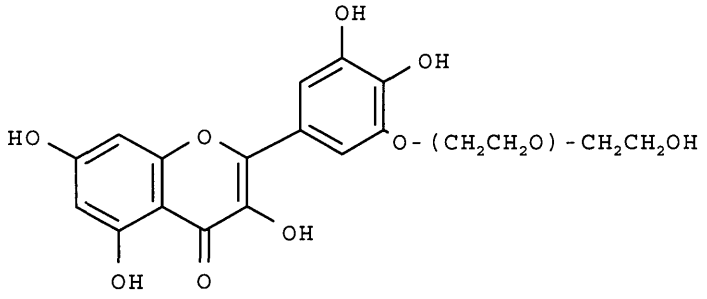
In the work described here, comparison was made between rat and human metabolism. There seems to be little qualitative difference between rats and humans in the metabolism of pure quercetin. As human plasma samples were limited, ultimate characterisation was accomplished using samples obtained from rats, which had received quercetin. In terms of disappearance of parent drug from the blood and pattern of conjugate metabolites generated, there was considerable similarity between rats and humans. Additionally, differences in plasma concentration between rats and humans reflected the differences in dose between the species, when calculated on a mg/m^2 basis. In the rat, when quercetin is given i.v., the major route of elimination is via biliary excretion. Whether this is the same in humans has yet to be established.

The complete structural characterisation of the individual metabolites was limited due to the inability to distinguish between fragments derived from different parts of the molecule when using tandem mass spectrometry. It is conceivable that using more mass spectrometric stages could have yielded more structural information. This approach would generate further fragment product ions from ions of unknown origin. Sometimes, greater resolution can be obtained using instruments with more than two mass analysers, thus leading to more accurate structural characterisation. Radiolabelled quercetin may also allow elucidation of the quercetin molecule by mass spectrometry. Alternatively, Nuclear Magnetic Resonance spectroscopy could have been used to yield structural information regarding quercetin metabolites. NMR was not attempted for metabolites generated *in vivo*, due to drug concentration being a limitation.

As described in chapter 5, results did not support the hypothesis that GSH conjugation of quercetin is responsible for nephrotoxicity. GSH conjugates of quercetin could be synthesised *in vitro*, which is consistent with recent studies(Awad et al. 2001; Galati et al. 2001). However, such molecules were not detected *in vivo*. One might speculate that the nephrotoxicity displayed by quercetin in the clinical trial may be attributable to the considerable amounts of sulphate and glucuronide conjugates or the parent compound in the kidney. In support of this idea, results in the clinical trial suggested that the kidney toxicity was abrogated by i.v. pre-hydration of patients. The nephrotoxicity observed in the long-term feeding study(Dunnick and Hailey 1992) might have been precipitated by accumulation of drug metabolites specifically in very old rats as damage was only seen at the termination of the study. The role of quercetin and its conjugates and the confounding effect of age need to be investigated further.

It is clear from the literature and tentatively from the work presented in chapter four that the overall bioavailability of quercetin needs to be improved if the effects seen *in vitro* can be achieved *in vivo*. Bioavailability could be altered in a number of ways. A chemical derivatisation could add a functional group to generate a quercetin prodrug. One example is attachment of a polyethylene glycol (PEG) group to quercetin via an ether bond (see Fig. 7.1). PEG is increasingly being used to derivatise hydrophobic compounds in order to increase their hydrophilicity(Harris 1992; Graham 1992).

Figure 7.1 *A quercetin molecule attached to polyethylene glycol (PEG).*



The advantage of PEG is its inherent versatility. PEG is soluble in water and in most organic solvents. Molecules linked to PEG have increased water solubility, and appear to demonstrate more controlled ester/ether bond hydrolysis.

Another strategy to increase the hydrophilicity of lipophilic compounds is their reaction with sugars, a sugar moiety which increases their absorption. The conjugation of sugars to

quercetin has been attempted using myo-inositol 2-phosphate, which led to greater solubility of the quercetin molecule(Calias et al. 1996). Thus, the oral bioavailability might be improved by increasing the absorption through the intestinal wall. It is important to note that in the case of the glucose-rhamnose glycoside of quercetin (rutin), the bioavailability for quercetin is similar for the glycoside as compared to the pure compound(Manach et al. 1995). In contrast, it has also been shown that the glycosides are not absorbed as well as the aglycone(Walgren, Walle, and Walle 1998; Crespy et al. 1999). The attachment of a sugar moiety may only improve the bioavailability of the molecule in the case of specific glycosides, but not in the case of others. It has been suggested previously that the disposition of quercetin in humans, when ingested as glycosides, depends primarily on the nature of the sugar moiety of the glycoside(Graefe et al. 2000). Another approach to improving absorption is to attach saponin groups which have previously been shown to increase the solubility of quercetin(Schopke and Bartlakowski 1997).

Ferry *et al* attached a 3'-(N-carboxymethyl) carbamoyl group to quercetin, which cleaved when in the presence of water(Mulholland et al. 2001). The oral bioavailability was not improved, presumably due to the rapid release of quercetin when reaching the stomach. The relative bioavailability when given i.v. appeared to be enhanced by the presence of the 3'-(N-carboxymethyl) carbamoyl group.

In the phase I clinical trial, tyrosine kinase inhibition by quercetin in lymphocytes was evaluated(Ferry et al. 1996). Quercetin at a dose of 60mg/m^2 inhibited lymphocytic tyrosine kinase. This result suggests that quercetin at high concentrations elicits responses related to interference with cellular signal pathways. At present, there exist a few reports that investigated the biological activity of the metabolites in relation to the parent compound. Most of these reports investigate, through a variety of means, the inherent antioxidant potency of quercetin metabolites and quercetin. These reports were more concerned with addressing the question of whether quercetin can act as a dietary antioxidant. The work in chapter five, addressed the question whether quercetin metabolites, which are present *in vivo*, might contribute to the inhibition of COX-2 shown by quercetin *in vitro*. Extrapolating from the potent *in vitro* inhibition of COX-2 shows that quercetin and its methylated analogues may inhibit COX-2 activity *in vivo* which may be of therapeutic relevance. Quercetin was shown to reach plasma levels of just below 14μM when administered i.v. to rats at a dose of 6.25mg/kg. These levels in plasma are theoretically sufficient to elicit a response in a number of biological assays that are described in Table 1.3.

Described in this thesis is the first chemical synthetic method for the generation of quercetin sulphate metabolites. The products were successfully identified and characterised so as to allow their use in the PGE2 assay.

Overall, quercetin has been shown to demonstrate some therapeutic potential when tested in a variety of *in vitro* biochemical tests. However, for quercetin to be used as a therapeutic agent, more work is required to clarify its metabolism. Factors affecting its absortion have not been clarified, the role of intestinal metabolism has not been elucidated and concentrations achieved in different tissues have not been assessed. Moreover, the development of a quercetin prodrug might be useful for the future of quercetin as a therapeutic agent. Attempts so far have not been successful in improving oral bioavailability. The work carried out on COX-2 inhibition by quercetin and its metabolites may be relevant in light of the fact that COX-2 is implicated in gastrointestinal malignancies, and quercetin may be localised to the gastrointestinal tract because of its poor absorption.

The work described in this thesis attempts to generate data on the metabolism of a specific flavonoid, quercetin, a molecule which has therapeutic potential. The optimisation of a variety of analytical techniques described here might allow similar studies on analogous compounds. In recent years, a great deal of interest has been raised on compounds derived from plants that offer therapeutic benefit. The flavonoids, in particular, are worthy candidates for drug development. Work from this thesis might aid any future studies in this field.

8.0 Chapter Eight. References.

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Determination of Quercetin in Human Plasma by HPLC with Spectrophotometric or Electrochemical Detection

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A reversed-phase high-performance liquid chromatographic method for the determination of quercetin in human plasma following intravenous infusion is described. Quercetin in plasma was extracted with methanol–dimethyl sulphoxide (4:1 v/v) and separated on a C_{18} Hypersil-BDS column with 44% (v/v) methanol in 0.1 M ammonium acetate (pH 5.15) containing 0.27 mM EDTA as the mobile phase. The drug was detected specifically and sensitively at its absorption maximum of 375 nm, or electrochemically, with a detection limit of 80 ng/mL and 2 ng/mL, respectively. © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

Quercetin (Fig. 1) is a flavonoid found ubiquitously in photosynthetic plants. It is estimated that approximately 25 mg/day is consumed as part of a normal healthy diet (NTP report, 1992). It has recently undergone phase I clinical evaluation as an anti-cancer agent (Ferry *et al.*, 1996).

There is good evidence which supports its potential role as an anti-cancer agent. It has been shown to be anti-proliferative in breast (Scambia *et al.*, 1991), luekaemic (Larrocca *et al.*, 1990), ovarian (Scambia *et al.*, 1990a), gastro-intestinal (Yoshida *et al.*, 1990) and colonic (Agullo *et al.*, 1994) cancer cell lines at concentrations around 10 μM. Quercetin possesses a number of interesting biological activities. It binds to type II estrogen binding sites (Scambia *et al.*, 1991), and down regulates signal transduction in breast MDA-MB-435 cell lines via inhibition of phosphatidylinositol 4-kinase which parallels a concomitant decrease of 1-phosphatidylinositol-4-phosphate kinase (Singhal *et al.*, 1995). It has been shown to down-regulate mutant p53 in human breast cancer cell lines MDA-MB468 (Avila *et al.*, 1994). Furthermore, it inhibits skin carcinogenesis induced by benzo(a)pyrene and (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetra-benzo(α)pyrene (Chang *et al.*, 1985) and also inhibits the development of 7-12-0dimethylbenz(a)-anthracene (DMBA) and *N*-nitrosomethyl urea-induced rat mammary cancer (Verma *et al.*, 1988). Finally, quercetin enhances synergistically the anti-proliferative effect of *cis*-platin *in vitro* (Hofman *et al.*, 1988; Scambia *et al.*, 1990a, b, 1992) and increases the anti-tumour activity of *cis* platin in mice bearing human-derived carcinomas *in vivo* (Hofmann *et al.*, 1990).

While certain tests have suggested that quercetin is mutagenic *in vitro* (Brown, 1980) and may exacerbate the genotoxic effect of *cis* platin (Cross *et al.*, 1996). Zhu *et al.* (1994) hypothesized that the lack of its mutagenicity *in vivo* is due to deactivation by the enzyme catechol *O*-methyl transferase (COMT).

Several HPLC methods have been reported for the separation of quercetin and related flavonoids (Brown and Griffiths, 1983; Dondi *et al.*, 1989; Hertog *et al.*, 1993; Manach *et al.*, 1995; Sägesser and Deinzer, 1996). Few, however, described the analysis of quercetin in human plasma (Liu *et al.*, 1995; Paganda and Rice-Evans, 1997). The present paper describes a novel, sensitive, specific and simple HPLC procedure for the determination of quercetin in the plasma of patients who received quercetin in a phase I clinical study.

EXPERIMENTAL

Materials and reagents. Quercetin, fisetin, myricetin, morin, kaempferol and naringenin were from Sigma Chem. Co. (Poole, Dorset, UK). Ammonium acetate, disodium ethylenediaminetetraacetic acid (EDTA) and dimethyl sulphoxide (DMSO) all Analar grade were obtained from BDH (Poole, Dorset, UK), methanol (HPLC grade) from Fisher (Loughborough, Leics., UK).

Sample preparation. Plasma (200 μL) was vortex-mixed with 400 μL of DMSO–MeOH (1:4 v/v) for 30 s and then centrifuged (Hettich Zentrifugen, Tuttlingen, Germany) at 17060g 12,000 rpm for 12 min. The supernatant was thoroughly mixed with an equal volume of water before HPLC analysis.

High performance liquid chromatography (HPLC). A Varian model 9012 liquid chromatograph (Walton-on-Thames, Surrey, UK) with a Linear UVIS-204 detector set at 375 nm, or a Gynkotek model 300 solvent delivery system with an Antec electrochemical detector (Presearch, Letchworth Garden City, Herts., UK) set at oxidation mode (+0.69 V) was used. Injection of sample was via a rheodyne 7125 injector fitted with a 100 μL loop.

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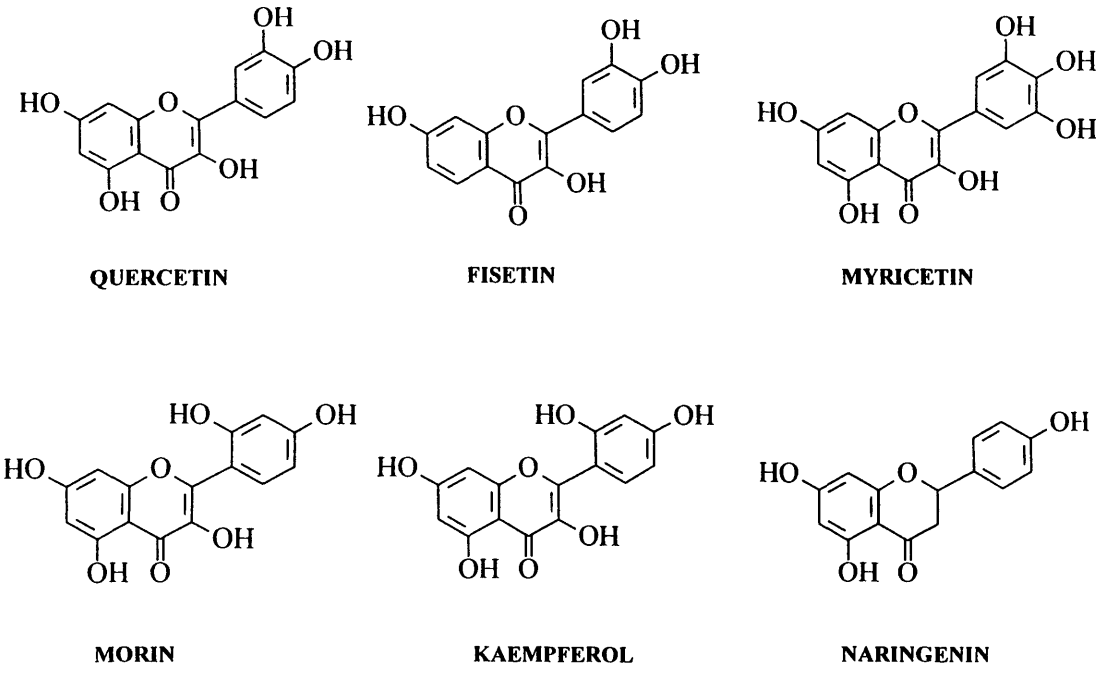

Figure 1. Structures of quercetin and related flavonoids.

The flavonoids were separated on a 250 × 5 mm I.D. BDS-Hypersil C_{18} column (5 μm particle size) from Hypersil, Runcorn, Cheshire, UK. The optimal mobile phase which was arrived at in preliminary experiments, was a mixture of 56% (v/v) 0.1 M ammonium acetate (pH 5.15) and 44% methanol. The flow-rate was 1.0 mL/min. For ECD, the mobile phase was continuously purged with a slow stream of helium during the separation.

Quantitation. The plasma concentration of quercetin was determined from calibration curves constructed by plotting the peak-area of quercetin against concentration from spiked plasma calibration standards following extraction and HPLC separation. The curve was linear within the range of 0–50 μg/mL ($y=9653.376x-7506.714$, $r^2=0.999$) when spectrophotometric detection at 375 nm was used, and within the range of 0–50 ng/mL ($y=1422.585x+733.500$, $r^2=0.996$) when ECD was employed.

RESULTS AND DISCUSSION

Extraction of quercetin from plasma

Quercetin in plasma is tightly bound to proteins (Manach *et al.*, 1995) and although it is soluble in methanol and acetonitrile, these organic solvents proved unsuitable for the extraction of quercetin from plasma yielding insufficient recovery because of co-precipitation of the drug with proteins. It has been demonstrated that a mixture of methanol – DMSO (4:1 v/v) extracted protein-bound drugs from plasma effectively (Wang *et al.*, 1993). We therefore used this mixture as the precipitation–extraction solvent. The recovery of quercetin (0.10, 7.5 and 15.0 μg/mL) added to drug-free control plasma after dissolution in DMSO (so that DMSO constituted less than 1% of final volume) was assesssed by comparison with suitable quercetin standards dissolved in methanol – DMSO (4:1 v/v) and an equal volume of water. The mean apparent recovery was 96.8±7.5, 96.0±5.9 and 92.9±10.3%, respectively (mean±SD, $n=6$ for each concentration).

HPLC

In most of the reversed-phase HPLC systems reported for the separation of flavonoids, quercetin was shown as a broad and tailing peak. This is most likely the result of the ability of the polyphenols to chelate with the metal ions bound to the residue geminal silanol of the stationary phase. Detailed studies by Euerby *et al.* (1995) have shown that compounds with catechol structure are particularly prone to chelation with metals and metal contamination varies according to the commercial source of the column. However, chelation with metals can be effectively eliminated by the addition of EDTA to the mobile phase. The effect of EDTA on peak shape is shown in Fig. 2.

Satisfactory separation of a mixture of the six polyphenols: quercetin, fisetin, myricetin, morin, kaempferol and naringenin (structures shown in Fig. 1) was achieved on a Hypersil-BDS column with 44% methanol in 0.1 M ammonium acetate (pH 5.15 adjusted with acetic acid) containing 0.27 mM EDTA as eluant (Fig. 3). All six compounds were separated with sharp symmetrical peak shape.

Application to the analysis of human plasma

Figures 4(a) and 4(b) show HPLC traces of plasma obtained from a patient prior to administration and 5 min after administration of quercetin (1400 mg/m^2). At the detection wavelength of (375 nm), the absorption maximum of quercetin in the mobile pase, interfering peaks were not observed in the retention time-window of quercetin when control plasma was analysed. The detection limit at 0.001 absorbance unit full scale (AUFS) was 80 ng/mL with a signal-to-noise ratio (S/N) of 3 for a 100 μL sample. This is adequate for pharmokinetic and metabolism studies. Several metabolites were detected in the plasma following quercetin administration (Fig. 4b). The identification of these metabolites by liquid chromatography-mass spectrometry is currently in progress.

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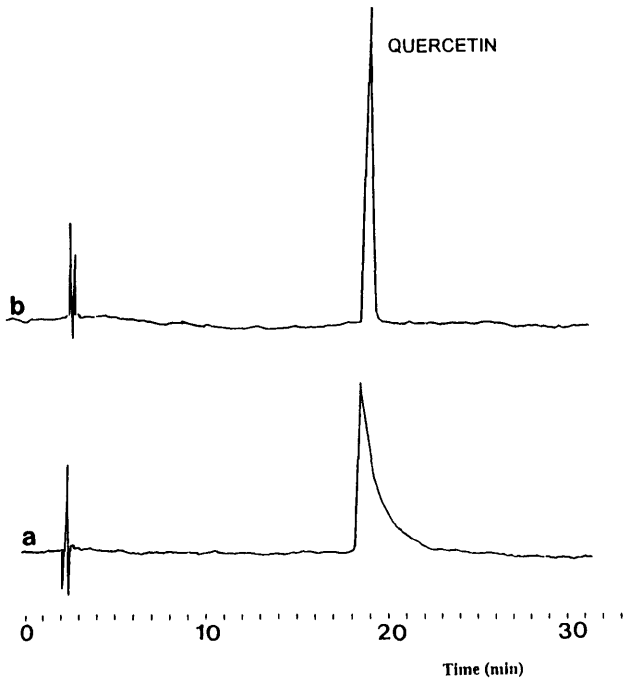

Figure 2. Effect of EDTA on the peak shape of quercetin. The separation was carried out on a C_{18}-Hypersil BDS column with (a) 44% (v/v) methanol in 0.1 M ammonium acetate (pH 5.15) and (b) 44% (v/v) methanol in 0.1 M ammonium acetate (pH 5.15) containing 0.27 mM EDTA.

The sensitivity of detection was improved considerably by employing ECD which is very suitable for the sensitive detection of polyphenolic flavonoids using the oxidation mode. The detection limit was reduced to 2 ng/mL (S/N=3) at an operation potential of +0.6 V with attenuation set at 1 nA/V. Figure 5 shows the separation and detection of quercetin in a plasma extract using ECD. An obvious advantage of ECD over spectrophotometric detection is that it allows a considerable reduction in sample size to be used for analysis.

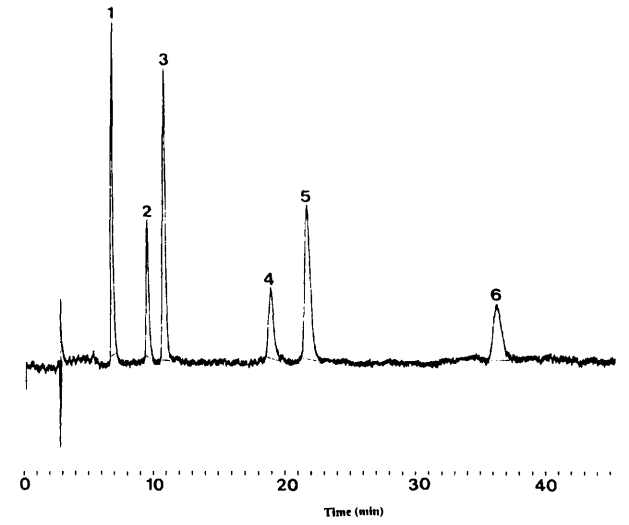

Figure 3. HPLC separation of a standard mixture of flavonoids. Column, C_{18}-Hypersil BDS (250×4.6 mm); eluant, 44% (v/v) methanol in 0.1 M ammonium acetate (pH 5.15) containing 0.27 mM EDTA; flow-rate, 1 mL/min; detection, 375 nm, 0.01 AUFS. Peaks correspond to 1=morin, 2=myricetin, 3=fisetin, 4=quercetin, 5=naringenin and 6=kaempferol.

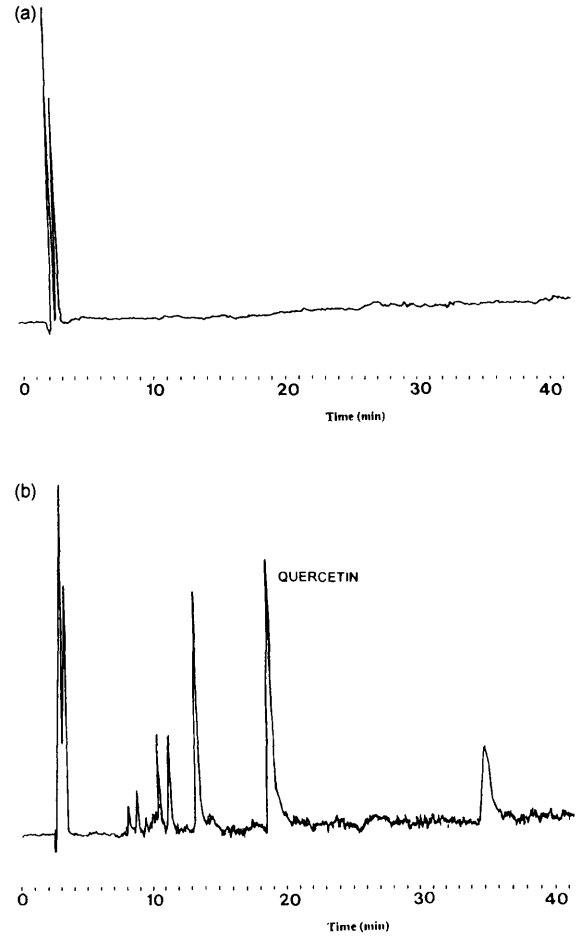

Figure 4. HPLC analysis of a plasma sample of a patient prior to (a) and 5 min after (b) administration of quercetin. HPLC conditions as described in Fig. 3.

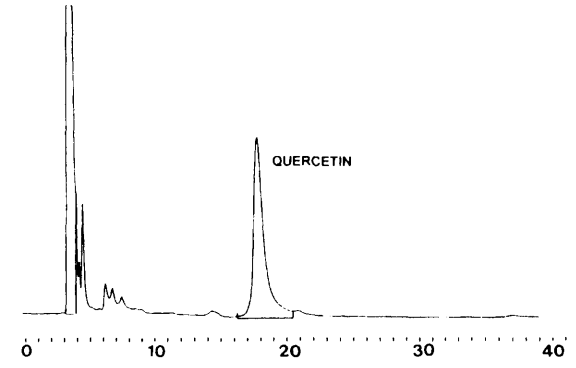

Figure 5. HPLC separation of quercetin in human plasma. HPLC conditions as in Fig. 3 except detection was by ECD using the oxidation mode at +0.6 V.

CONCLUSIONS

The method outlined here is sensitive and specific for the determination of quercetin in plasma. The assay is well suited for monitoring drug levels in patient plasma. It is expected, with suitable modification of the mobile phase, to be applicable to the analysis of other flavonoids.